Uncovering a MYC-driven Tumor-suppressive Program in Proliferating Lymphocytes

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Uncovering a MYC-driven Tumor-suppressive Program in Proliferating Lymphocytes

by

Elena Tonc

A dissertation presented to
The Graduate School
of Washington University in
partial fulfillment of the
requirements for the degree
of Doctor of Philosophy

August 2021
St. Louis, Missouri
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Acknowledgments

This work would not have been possible without the continuous support of faculty, friends, and family. First and foremost, I would like to thank my mentor, Dr. Takeshi Egawa, for his support, guidance, and faith in me. Thank you for encouraging me to keep learning and asking questions, and fostering my development as an immunologist by always challenging and motivating me to do my best. I can confidently say that a lot of what I know, I have learned from you. Also, thank you for being an understanding mentor who supported my professional development outside of the lab. Thank you to my committee members, previous and current, Dr. Kenneth Murphy, Dr. Jeffrey Bednarski, Dr. Chyi-Song Hsieh, Dr. Jacqueline Payton, Dr. Todd Fehniger, and Dr. Eugene Oltz for taking the time to serve on my committee and provide constructive criticism, advice, and encouragement throughout my thesis work. I would also like to thank Dr. Gaya Amarasinghe and Dr. Daisy Leung for their instrumental career guidance.

Importantly, I would like to thank the previous and current members of the Egawa lab. Chun "Jim" Chou for his work generated the basis for the work presented in this thesis. I would also like to especially thank Saravanan Raju and Daniel Verbaro for their support and advice as I started working on this project and for all their patience when teaching me the necessary techniques. Thank you also to Yu Xia, Sunnie Hsiung, Maegan Murphy, Tenzin Yangdon, Melanie Holmgren, Chika Fujii, and Elliot Bradshaw for the experimental and technical support and for being wonderful lab mates who made the long days in the lab more enjoyable.

The many resources and people at Washington University in St. Louis that helped me in this work include, but are not limited to, the members of Dr. Kenneth Murphy's Lab, Dr. Eugene Oltz's lab, Dr. Deepta Bhattacharya's lab, the Flow Cytometry & Fluorescence-Activated Cell Sorting Core, and the Genome Technology Access Center. My research would also not be
possible without the generous funding from the government and private sources. The following work has been directly supported by the US National Institutes of Health (NIH) grants R56AI114593-01A1, R01AI130152-01A1, the Leukemia and Lymphoma Society Scholar Award #1349-18, and the Siteman Investment Program Research Development Award to Dr. Takeshi Egawa and Hu and Zeng Predoctoral Scholarship that has funded a part of my doctoral studies.

Thank you to my loving friends, who are too many to be listed here for their individual contributions, for being supportive and kind during this process, and for putting up with my schedule, feeding me, making me laugh, and making sure I leave the lab every once in a while and for joining me at the barre. Thank you to the extensive Winegar Clan for being my American family, for your encouragement and understanding, and for creating my home away from home.

Finally, thank you to my family, parents, siblings, and grandparents, for your willingness and sacrifices to support me in following my dreams even though you do not always understand what I am doing. It has never been easy to be away from you, but your trust and love make my endeavors worthwhile. Without you, I would have never achieved as much as I have, and I hope I can always make you proud. Hvala.

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August 2021
ABSTRACT OF THE DISSERTATION

Uncovering a MYC-driven Tumor-suppressive Program in Proliferating Lymphocytes

by

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Doctor of Philosophy in Biology and Biomedical Sciences
Immunology
Washington University in St. Louis, 2021
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Rapid cell proliferation is a hallmark feature of adaptive immune cells lymphocytes. It is essential for the establishment of diverse antigen receptor repertoires and amplification of antigen-specific immune responses. While such proliferation is beneficial for host protection from infections and cancers, it inevitably elevates the risk of oncogenic transformation. In developing and germinal center B lymphocytes, the risk is further increased by endogenous, genomic insults due to antigen receptor rearrangements and somatic mutations, with which expression of the proto-oncogene c-MYC is closely associated. Nonetheless, frequencies of cancers originated from B lymphocytes are relatively low, suggesting that they are protected from transformation through a putative tumor-suppressive program coupled with their cell proliferation. In this work, we found that the proliferative driver c-MYC not only facilitates rapid cell proliferation but also unexpectedly engages a counteracting tumor-suppressive program through its downstream protein TFAP4. Missense mutations of TFAP4, including loss of function variants, were detected in human lymphoid malignancies, particularly in 12% of Burkitt lymphomas driven by overexpression of c-MYC. Furthermore, B-ALL patients with c-MYC
overexpression and reduced expression of TFAP4 had worse survival compared to patients with high TFAP4 expressing B-ALL. In mice, haploinsufficiency for AP4 drastically accelerated tumor development in MYC-driven B cell lymphoid tumor models in a cell-intrinsic manner. Mechanistically, TFAP4 restricted the expression of protooncogene Erg, which, together with c-MYC, is required for B cell development. The risk of transformation was thus limited by blocking inappropriate co-expression of the two oncogenic proteins. TFAP4-mediated restriction of Erg also ensured appropriate coupling of c-MYC-mediated proliferation and the loss of self-renewing capacity in developing B cells. Thus, c-MYC suppresses the stemness of B cell progenitors by inducing TFAP4 while driving proliferation, and this transcription factor cascade functions as a tumor suppressor module that safeguards against the transformation of developing B cells. Thus, c-MYC concurrently engages proliferative and tumor-suppressive programs in B lymphocytes.
Chapter 1:

Introduction
1.1 Immune system

The immune system is comprised of a variety of effector cells and molecules that protect against infectious microorganisms or parasites. Generally, aspects of the immune system response can be categorized as “innate” and “adaptive”, with the major differences being the speed and the specificity of the elicited reaction.

The innate responses tend to occur on the scale of minutes to hours and involve physical barriers such as the skin and the mucous surfaces of the body, the proteins of the complement pathway and innate immune cells; macrophages, dendritic cells, granulocytes, mast cells, natural killer and innate lymphoid cells. The innate immune cells recognize common pathogen or damage-associated molecular patterns and induce effector molecules that directly control the pathogen but can also activate and recruit other cells, including adaptive immune cells. As the innate immune cells recognize common patterns across pathogens, their receptors are germline-encoded and broadly shared within the species.

In contrast, the slower adaptive immune response is highly specific to the invading pathogen due to germline rearrangements of the antigen receptor loci in individual lymphocytes and thus unique to the host. The expression of these uniquely derived antigen receptors on B and T lymphocytes generates a repertoire of B cell receptors (BCRs) and T cell receptors (TCRs) that can recognize virtually any pathogen a host might encounter. Following the clearance of an infection, the adaptive immune response, unlike the innate, persists in the form of immunological “memory” that helps in the prevention of reinfection for years to come through a faster and more robust immune response upon secondary exposure to the pathogen (1).
1.2 Hematopoiesis

Despite the significant differences in innate and adaptive immune cells, all immune cells originate in the bone marrow from a shared precursor, hematopoietic stem cell (HSC). In fact, all mature blood cells are derived through hematopoiesis; a process by which HSCs give rise to a larger number of differentiated progeny cells. In this process self-renewing, pluripotent HSCs differentiate into multipotent and lineage-committed progenitors. Unlike HSCs, and as the name implies, the lineage-committed progenitors have a limited lineage differentiation capacity. Thus, this stepwise differentiation process is associated with the loss of self-renewal potential and increased frequency and specificity of each following progeny cell type (1-5).

In adults, hematopoiesis primarily occurs in the bone marrow, and this is where the progenitors of the two main lineages of immune cells, myeloid and lymphoid, bifurcate (1-3). The common myeloid progenitor is the precursor for most of the cells of the innate immune response: macrophages, dendritic cells, granulocytes, and mast cells, as well as red blood cells and megakaryocytes. The common lymphoid progenitor, on the other hand, gives rise to both innate immune cells, natural killer, and innate lymphoid cells, as well as the effector cells of the adaptive immune system, B lymphocytes (B cells) and T lymphocytes (T cells) (1, 3, 6). T cell progenitors do not stay in the bone marrow and instead migrate into the thymus where they continue their maturation, undergoing antigen receptor loci rearrangements, and differentiate into either CD4 or CD8 T cells (1, 7). B cell progenitors, on the other hand, complete the majority of their development in the bone marrow. Lymphocytes and their receptors will be introduced in the following section.
1.3 Adaptive lymphocytes and their receptors

B lymphocytes, together with T lymphocytes, are the central effector cells of the adaptive immune response. Whilst both can recognize virtually any antigen in the environment through their rearranged antigen receptors, a key distinction between these receptors is their structure. B cell receptor is formed by immunoglobulin (Ig) proteins and can be either membrane-bound as BCR or secreted in the form of an antibody. On the other hand, while the TCR is related to Ig in structure, T cells always retain it on the cell surface. Both B and T cell receptors are made from constant and variable regions that are important for their effector functions. BCR, and antibody molecule, are composed of two identical heavy (IgH) and two identical light (IgL) chains, with each having a variable and constant region. TCR is composed of two chains, TCRα and TCRβ, that also have a variable and a constant region. In both T cells and B cells the variable region of their TCRβ and IgH chains, respectively, is derived through somatic gene rearrangements of a number of variable (V), diversity (D) and joining (J) gene segments, while the TCRα and IgL chain are generated by V to J rearrangement through a process known as V(D)J recombination. This process is the key to generating the vast diversity of lymphocyte receptors and occurs at strictly defined stages of B and T lymphocyte differentiation. B lymphocytes and their differentiation will be the focus of the remaining sections, as they are the primary focus of the work presented herein, but occasional parallels might be drawn to T cell lymphocytes throughout.
1.4 B lymphocyte differentiation

The development of B lymphocytes from HSC is a well-studied process and has been defined by several stages marked by distinct surface marker expression and associated gene expression programs. While there is evidence of minor pathways through which B lymphocytes can be generated, the generally agreed upon, major pathway of B cell development will be described here. The first steps of the differentiation include HSC-derived multipotent progenitor (MPP) and MPP-derived common lymphoid progenitor (CLP). CLP, in turn, gives rise to the pre-pro-B cells, followed sequentially by differentiation into the early pro-B cells, late pro-B cells, large pre-B cells, small pre-B cells, immature B cells, and finally mature B cells. The cell surface markers often used to define these developmental stages in terms of commonly known “Hardy fractions” are B220, CD43, CD24(HSA), BP-1, IgM, and IgD (8), but additional lineage defining markers, including CD117 (c-Kit), CD127 (IL7Rα) and CD25 (IL2Rα), have been used (9). These developmental stages and commonly used markers are shown in Figure 1.1.

While committed to the lymphoid lineage, the CLP in the bone marrow retains the ability to differentiate into B lymphocytes, T lymphocytes, or NK cells. In a strictly regulated process, the CLP goes down the B cell lineage path in response to specific cues from the bone marrow niche, including signaling from cytokines, chemokines, and cell to cell contacts. For example, chemokine CXCL12 is thought to be important to retain the B cell precursors in the bone marrow, and IL-7 is responsible for the survival and proliferation of the CLP and pro-B cells whilst SCF supports their growth and proliferation (10-14).

These signals, especially IL-7 availability, are critical for the specification and commitment to the B cell lineage and play different roles in B cell development depending on the stage (10, 15). The B cell lineage specification of the pre-pro-B cell is achieved through the
induction of a B-lineage specific transcription factor E2A which prompts the induction of early B cell factor (EBF) (16-20). Decisive fate commitment to the B cell lineage is in turn sealed by EBF mediated induction of Pax5 in early pro-B cells, which represses non-B lineage gene expression (10, 19-22). These transcription factors act in a hierarchical as well as combinatorial manner. E2A and EBF1 promote the initial antigen receptor gene rearrangements between the D and J gene segments of the IgH heavy chain, while PAX5 promotes V to the DhJH recombination by recombination-activating gene (RAG) proteins. These transcription factors also induce the expression of the additional signaling components of the BCR, CD79α and CD79β. These molecules, together with the rearranged heavy chain complexed with VpreB and λ5 are expressed on developing B cells as pre-BCR. The signaling through the pre-BCR induces proliferation and differentiation into large pre-B cells, selecting only cells with a productively rearranged BCR in a process known as positive selection, while others undergo apoptosis due to lack of pre-BCR signaling (23). Proliferation at this step is crucial for increasing the diversity of the final BCR repertoire by increasing the number of cells that will undergo light chain rearrangements and thus increasing the possible combinations of rearranged heavy and light chains. Following 2-5 rounds of divisions (24), the positively selected cells lose surface pre-BCR, discontinue proliferation and differentiate into small pre-B cells, which start to rearrange the V_{L} to J_{L} chains. To maintain genomic integrity of the developing B cells, these processes, proliferation and immunoglobulin light chain rearrangement, need to be mutually exclusive to avoid potentially oncogenic chromosomal translocations from occurring. Following successful rearrangement of the light chain, the complete BCR is expressed on the surface of immature B cells in the form of IgM receptor. The immature B cells continue their maturation in the
periphery, usually in the spleen, where they acquire the expression of IgD and differentiate into either follicular or marginal zone B cells.

Follicular B cells are the main players of the T cell-dependent adaptive B cell responses that give rise to antibody-secreting plasma cells and memory B cells through the germinal center reaction. Mature B cells in the B cell follicles of the spleen and lymph nodes become activated following BCR-mediated recognition of their antigen. Activated B cells then migrate towards the T cell zone and effectively form germinal centers composed of two anatomically distinct regions, the light and dark zone. The dark zone is the site of actively proliferating B cells, while the light zone contains mostly nondividing B cells, antigen-presenting follicular dendritic cells, and antigen-specific T follicular CD4 T cells. It is in the light zone that B cells receive additional activation signals, or “help”, from follicular CD4 T cells and are selected for expansion based on antigen affinity. They also undergo genome editing by activation-induced cytidine deaminase (AID) protein resulting in somatic hypermutation (SHM) and class switch recombination (CSR) of their BCR that allows for selective expansion of B cells with increased antigen affinity and specialized effector functions. Ultimately after rounds of proliferation in the dark zone and positive selection in the light zone, B cells exit the germinal center and differentiate into either memory B cells or long-lived plasma cells that are indispensable for forming long-term immunity against the invading pathogen (25-28).

Unlike B cells, T cells undergo gene rearrangements of their TCR loci only during development in the thymus but similarly to B cells, this process increases the diversity of the TCR repertoire (7, 29, 30). In both cases, the broad diversity of the repertoire results in a low frequency of individual antigen-specific B cell and T cell clones. These antigen-specific clones need to proliferate in a timely manner for efficient pathogen clearance. Antigen-specific T cells
rapidly divide to increase their numbers to eliminate infected cells, and B cells participate in the germinal center reaction, as discussed in the previous paragraph.

Therefore, successful adaptive immune responses are dependent on the initial establishment of a diverse lymphocyte repertoire and subsequent activation-induced clonal expansion of antigen-specific lymphocytes. Both processes require rapid proliferation of the lymphocyte progenitors and their mature progenies, with cells dividing multiple times a day. In normal B and T cells, and their precursors, such clonal expansion is supported by metabolic reprogramming triggered by signals through the antigen receptors (31, 32).

1.5 c-MYC in hematopoiesis and B cell differentiation

In developing and mature B cells, pre-BCR- and BCR-triggered proliferation absolutely requires the expression of transcription factor c-MYC (31, 33-37). c-MYC functions as a sequence-specific transcription factor and a central regulator of cell growth and biogenesis. It establishes active cell cycle, anabolic states, and amplifies global gene transcription in proliferating cells (30, 31, 38-43). In addition to its well-studied role in cell proliferation, c-MYC has been implicated in many biological functions, including hematopoiesis and differentiation, reducing cell adhesion, and promoting genomic instability (44-47). However, its cell differentiation and proliferation roles are the most relevant to the current study and will be described below.

c-MYC belongs to the Myc family of proteins that also includes N-MYC, and L-MYC in humans. All members of the family are basic region/helix-loop-helix/leucine zipper (bHLHZip) transcription factors that heterodimerize with Myc associated factor X (MAX) and bind E-box element CACGTG on DNA to drive target gene expression (41, 42, 48). However, there are
functional and tissue expression pattern differences amongst the family members. Most strikingly, while L-MYC-deficient mice are viable, both c-MYC and N-MYC-deficient mice are embryonic lethal (49-53) with strong indications that defective hematopoiesis in these animals is a major contributor to the lethality (54). Consistently, c-Myc and N-Myc are co-expressed in long-term HSCs and hematopoietic progenitor cells, while there is no detectable expression of L-Myc in these cells or the vast majority of haematopoietically derived cells (28, 45, 54). Myc expression throughout most of hematopoiesis is controlled by a “blood enhancer cluster” located 1.7 megabases downstream from the Myc gene except in megakaryocyte-erythroid and T cell lineages (55).

Overexpression of c-MYC in HSCs results in depletion of HSC numbers, presumably due to premature differentiation. Consistent with these findings, conditional deletion of c-MYC in HSCs results in their accumulation and loss of differentiation. Both phenotypes are believed to arise due to disruption of HSC localization to its bone marrow niche. Nonetheless, c-MYC-deficient HSCs could still survive and proliferate, suggesting a role for N-MYC in these processes (44). Surprisingly, however, N-MYC was not necessary for steady-state hematopoiesis, but a double deletion of both c-Myc and N-Myc resulted in HSC depletion (45). These results suggest that while N-MYC has an important role in the survival and proliferation of c-Myc-deficient HSCs, it is dispensable in the presence of c-MYC. Coincidentally, the expression of N-Myc decreases with HSC differentiation, and no hematopoietic cells express N-Myc without c-Myc (45). Additional studies have given support to the idea that c-MYC loss in the HSCs results in their accumulation in the bone marrow and impaired differentiation ability (56) and that increased expression of c-Myc correlates with loss of self-renewal and differentiation of HSCs into MPPs, marked by higher proliferative capacity (57, 58). However,
there have been reports that c-Myc overexpression enhances self-renewal but does not ensure the long-term repopulation capacity of these HSCs (59). Moreover, a recent study found that c-Myc haploinsufficiency inhibited HSC self-renewal (60), and another found that deletion of an acetyltransferase Tip60 suppressed expression of c-MYC target genes and impaired HSC maintenance by inducing apoptosis and blocking cell cycle progression (61). Taken together, these studies show that MYC needs to be tightly regulated in HSCs, with low level expression necessary for the maintenance of self-renewal and higher expression driving differentiation towards multipotent and lineage-committed progenitors.

Similar parallels have been drawn to the role of MYC in lymphocyte differentiation. N-MYC and c-MYC are co-expressed in the earliest lymphocyte progenitors, pro-B cells and pro-T cells, but only c-MYC is induced in later stages of differentiation and in activated mature lymphocytes (32, 33, 62-64). Conditional deletion of c-Myc in developing B cells resulted in impaired B cell differentiation with a block in pre-B cell formation, while single deletion of N-Myc did not have a detectable effect on differentiation (31). c-Myc-deficient progenitors were also unable to divide in response to IL-7 (31), a signal known to induce Myc expression and proliferation of pro-B cells (62). B cell progenitors also induce c-MYC in response to pre-BCR signaling (65). Therefore, c-MYC expression peaks at the height of cellular expansion as developing B cells transit from pro-B to pre-B cell stages while testing their newly rearranged IgH chain (58). In addition, in its sequence-specific canonical transcription factor role, c-Myc induces transcription factor E2A and promotes activation of other B cells identity genes, namely EBF and Pax5 (48). Therefore, c-Myc is indispensable for the expansion of pro-B cells and their transition to the pre-B cell stage, with proliferation and differentiation being intertwined during B cell development in the bone marrow.
In response to BCR stimulation *in vitro*, mature B cells induce *c-Myc* (66). Furthermore, *in vivo* BCR activation of mature B cells and CD4 T cell help together stimulate *c-Myc* expression, which is necessary for the germinal center formation and clonal expansion of selected B cells in the germinal centers (36, 37, 67). However, surprisingly, *c-MYC* expression is limited to the light zone B cells while proliferation predominantly occurs in the dark zone (26, 36, 37). *c-MYC* maintains cell proliferation in the dark zone by inducing transcription factor AP4 (TFAP4) in positively selected light zone B cells. TFAP4 persists in dark zone B cells and supports their proliferation until the next round of selection in the light zone and is required for the production of high-affinity antibodies (68). After multiple rounds of cyclic re-entry of cells from dark zone to the light zone, B cells with somatically mutated BCR exit the germinal center and differentiate into memory or plasma B cells. Terminal differentiation into plasma cells is driven by B-lymphocyte-induced maturation protein 1 (BLIMP1), which suppresses *c-Myc* transcription, while *c-Myc* overexpression blocks this differentiation (69, 70). Therefore *c-Myc* has specific roles in the differentiation of B cells depending on the cellular context and its interactions with other transcription factors. This also suggests that just like in HSCs, *c-Myc* expression needs to be tightly regulated at different stages of B lymphocyte differentiation. The need for tight regulation of *c-Myc* in cells is also stressed by the fact that it is commonly dysregulated in hematopoietic and other malignancies (28, 54, 71).

### 1.6 c-MYC in hematopoietic oncogenesis

The *Myc* gene was first discovered as an oncogene (v-myc) in avian leukemogenic retroviruses that could transform fibroblasts and hematopoietic cells (72, 73). Consequent research has revealed that MYC family members have critical physiological functions in cell
proliferation and differentiation, as discussed earlier. Nonetheless, MYC activation is regarded as a hallmark of cancer, with over 28% of cancers, including hematopoietic and solid tumors, exhibiting some form of MYC alterations. These most often include genetic aberrations such as chromosomal translocations and gene amplifications or aberrant regulation of its expression, and rarely oncogenic mutations in its coding sequence (74). These alterations result in the loss of the tightly regulated control of Myc expression, often leading to constitutive overexpression of MYC that no longer responds to its normal regulatory signals. In turn, many biological programs usually regulated by MYC also become dysregulated, including cell proliferation and growth, DNA replication, protein synthesis, cell metabolism, and others (41, 75).

The first hematopoietic human cancer where Myc deregulation was identified is Burkitt Lymphoma (BL), a germinal center B lymphocyte malignancy (76). Overall, genetic abnormalities involving MYC are more commonly found in B cell lymphomas vs. T cell lymphomas. However, both B cell and T cell lymphomas and leukemias frequently involve upregulation of c-MYC, and these include diffuse large B cell lymphoma (DLBCL), chronic lymphocytic leukemia (CLL), plasma cell myeloma (multiple myeloma), B and T cell lymphoblastic leukemia (B-ALL, T-ALL) and others (28, 54, 77-80). Myc is also involved in some myeloid neoplasms (54). Lymphoid cells are thought to be more sensitive to dangerous genomic aberrations involving c-Myc due to endogenous genomic rearrangements at the time when they are also highly dependent on MYC for progression through differentiation.

Despite being one of the most recognized oncogenes, Myc alone is not sufficient to cause the transformation of normal cells. Malignant transformation often requires at least one additional mutation as c-MYC overexpression alone can lead to proliferative arrest, senescence, or apoptosis, thus limiting tumorigenesis (75, 81-85). A frequently utilized model to study how
MYC drives tumorigenesis is the Eμ-Myc mouse model that mimics the t(8:14) translocation of c-Myc to IgH regulatory elements, representative of human BL, where all mice develop B cell malignancy and succumb to the disease (86). The first hint that additional mutations are necessary for transformation was the clonal nature of the B cell leukemias (immature B cell phenotype) or lymphomas (mature B cell phenotype) that develop in these animals (87-89).

Additional evidence that a second, or a third, mutation is necessary for malignant transformation was revealed by discovering cooperating mutations in MYC-driven tumors (75, 90-93). Furthermore, mechanisms that inhibit apoptosis and cellular senescence, mediate DNA damage response, and ultimately promote proliferation were also implicated in c-MYC-driven lymphomagenesis in the Eμ-Myc mouse model. Most of these involve genes with known roles in these inherently intertwined processes and some of the most prominent players will be briefly described below.

Overexpression of Bcl2, an anti-apoptotic protein, cooperated with c-Myc in the induction of lymphoid progenitor-derived cancers (94, 95). Bmi-1 cooperated with c-MYC in tumorigenesis by blocking apoptosis through downregulation of ink4a-ARF, which is typically induced by Myc and leads to p53-dependent apoptosis (96, 97). Deletion of pro-apoptotic regulators, Bim, Bmf, Bad, and Puma, accelerated tumor development (87, 98, 99). However, mice overexpressing c-MYC in the absence of P53 developed malignant lymphomas faster due to enhanced proliferation and not reduced apoptosis (100).

MYC overexpression leads to rapid proliferation and accelerates S-phase predisposing the cells towards replication stress and genomic instability, which could be contributing to cell death or tumorigenesis (47, 101, 102). In agreement with this, deletion of DNA damage mediator Atm accelerated cancer formation due to reduced apoptosis and increased proliferation (103). In
contrast, deletion of histone H2AX, which mediates recruitment of ATM to the sites of double-strand break, did not impact latency to transformation (104). Furthermore, deletion of Wrn, a DNA helicase involved in DNA repair following DNA-replication associated damage, delayed tumorigenesis due to increased accumulation of DNA damage and accompanying induction of senescence (105). On the other hand, deletion of acetyltransferase Tip60 accelerated tumor formation due to impaired MYC-driven DNA damage response (106). However, main inducers of DNA damage in B lymphocytes, RAG and AID proteins, did not impact the formation of immature or mature lymphomas in one study, while AID-insufficiency in another slightly delayed formation of B cells cancers but skewed them towards immature phenotype. However, these findings are difficult to interpret due to extrinsic, anti-cancer contributions by other immune cells (88, 89).

Deletion of Cks1, which normally targets cyclin-dependent kinase inhibitor p27Kip1 for degradation and thus permits proliferation, slowed down lymphoma formation (107). Cell cycle progression requires cells to accumulate sufficient biomass to safely divide into two daughter cells. In addition to its role in cell cycle progression, c-MYC has also been shown to enhance protein synthesis and cell size, suggesting that its deregulation might decouple cell growth from cell division in a way that supports uncontrolled cell proliferation (30).

Taken together, these and many other studies show that there is not a single “oncogenic” pathway by which c-MYC-overexpression leads to tumorigenesis. Instead, it appears that c-MYC subverts specific pathways that normally function to regulate cell proliferation, survival, and differentiation, thus eventually facilitating the emergence and preferential survival of cells with additional oncogenic mutations that allow unrestrained proliferation, a hallmark of cancer.
The critical question in the field of cancer, then, is how the latency to transformation can be extended or transformation be prevented.

Transcription factors, such as c-MYC, that have a broad spectrum of functions are typically considered “undruggable” targets. However, the above discussed secondary pathways that could indirectly suppress MYC-driven oncogenesis can be targeted instead. Thus, our continued efforts to understand the mechanisms that drive and block cancer formation are crucial for creation of effective therapies.

Numerous studies have shown that tumor-suppressive pathways allow normal B cell development by counteracting deleterious aspects of c-MYC function. However, because c-Myc activation in lymphocytes is absolutely necessary for their function and ultimately host defense against pathogens, there is a possibility that c-MYC directly activates an intrinsic tumor-suppressive pathway linked with the proliferative program. There are two distinct, but not mutually exclusive, possibilities that could account for such a protective mechanism: 1) lymphocytes temporally restrict their dependency on c-MYC during proliferation by utilizing an alternative program for continued proliferation ("passive" tumor suppression), and 2) they concurrently engage an active, MYC-driven tumor-suppressive program as they proliferate.

1.7 AP4 as a MYC-induced regulator of oncogenesis

As a sequence-specific transcription factor, c-MYC activates and represses transcription of discrete genes leading to global cellular changes (43). Activating enhancer-binding protein 4/transcription factor 4 (TFAP4) is a bHLHZip protein and one of the many c-MYC targets. Similar to c-Myc, Tfap4 was first identified as an activator in the Simian virus 40 and binds to the E-box motif CAGCTG on the DNA (108). Functionally, TFAP4 has been reported to act
similarly to c-MYC in many ways, regulating genes involved in cell proliferation, metabolism, and cell cycle (109).

In agreement with this and with regards to the first possibility of MYC-induced tumor-suppression mentioned above, previous work has demonstrated that antigen-specific CD8 T cells and GC B cells require only transient expression of c-MYC for their clonal expansion by utilizing TFAP4. TFAP4 maintains the expression of many c-MYC target genes involved in cell metabolism and protein translation, thus supporting continued cell proliferation even after c-MYC decays (68, 110). This c-MYC to TFAP4 cascade allows activated lymphocytes to temporally restrict their dependency on c-MYC to achieve requisite clonal expansion during the immune response. Thus, these findings may support the possibility of passive tumor suppression, although it is yet to be determined whether TFAP4 is not oncogenic, as discussed below.

There has been some evidence of an oncogenic role for TFAP4 in a colon cancer model in vivo, as well as epithelial-mesenchymal transition involved in metastasis of breast cancer and colorectal cancer cell lines (111-113). More frequently, TFAP4 has been demonstrated to play a role in cell cycle progression in various cell lines. TFAP4 was required for cell cycle entry and proliferation of MEFs in response to mitogenic stimuli following initial c-MYC activation (114) as well as proliferation and migration of neuroblastoma cells (115). Moreover, TFAP4 was shown to be involved in suppressing cellular senescence through its ability to downregulate p16 and p21 in mouse embryo fibroblasts (116, 117). On the other hand, in retinal pigment epithelium immortal cells, TFAP4 stimulated senescence through induction of p53 in low c-Myc but lead to apoptosis in high c-Myc expressing cells suggesting relative levels of c-MYC and TFAP4 might play an important role in determining cell fate (118, 119). However, the majority of these and other findings suggesting TFAP4 plays a detrimental role in cancer come from the
study of immortalized and tumor cell lines that may have altered biology in many ways. TFAP4 function in normal cells might differ significantly, and additional studies in primary cancer models are needed for more straightforward interpretations.

Nevertheless, there is evidence of TFAP4 being upregulated in various human cancers, including colorectal cancer \((120, 121)\), pancreatic cancer \((122)\), prostate cancer \((123)\), hepatocellular carcinoma \((124)\), non-small cell lung cancer \((125)\), and neuroblastoma \((115, 126)\). Moreover, poor prognosis in patients with higher \(Tfap4\) expression was found for some of these, notably colorectal cancer, hepatocellular carcinoma, prostate cancer, and neuroblastoma \((115, 120, 121, 123, 124)\). However, \(TFAP4\) expression was much higher in tumors with high \(MYC\) and was not an independent prognostic marker of survival in primary neuroblastoma. The authors suggested that its upregulation could thus be secondary to overexpression of \(MYC\) in these tumors \((115)\). It is quite possible this could be the case in other tumor types as well, so close attention should be given to how the relative levels of \(MYC\) and \(TFAP4\) contribute to patient survival.

Interestingly, however, somatic mutations of TFAP4 in hematopoietic malignancies driven by high \(MYC\) have been reported across various cancer databases (Duke, Catalogue of Somatic Mutations in Cancer, Pediatric Cancer Genomic Data Portal, and The Cancer Genome Atlas) \((Figure\ 2.1G-H)\). This suggests that TFAP4 might play an important protective role in blood cell cancers, primarily lymphoid malignancies.

Taken together, the aforementioned findings suggest that the role of TFAP4 in cancer might be highly cell-type and context-dependent, as well as dependent on its relative levels in regard to c-MYC.
1.8 Erg in hematopoiesis and hematopoietic oncogenesis

Transcriptional regulators of HSC differentiation are common targets of mutations, translocations, or aberrant expression in leukemias and lymphomas, and c-Myc by far is not the only example of such proto-oncogenes (127-129). ETS family transcription factors are known to regulate HSC homeostasis and differentiation and have been linked to leukemic development (127, 130). One member of this family, proto-oncogene Erg, will be briefly introduced in the following sections due to the multiplicity of its function in HSC and B lymphocyte differentiation and relevance to this study.

The role of Erg in adult HSCs has been first shown using a sensitized genetic screen where a missense mutation in the DNA binding Ets domain of ERG compromised its transactivating ability. While homozygous mutation was embryonic lethal due to hematopoiesis defects, no gross abnormalities, including frequency and number of hematopoietic cell subsets, were observed for heterozygous mice. However, the heterozygous mutation in the HSCs was sufficient for their lower colony-forming capacity, impaired bone marrow engraftment, and reconstitution impairment in competition with wild-type HSCs, all defects shown to be cell-intrinsic. Mechanistically, the authors suggested that Erg regulates the expression of genes essential for stem cell function (131). Additional studies confirmed these findings showing that mutant Erg was insufficient to support hematopoiesis during requirements for high HSC cycling due to its role in HSC maintenance (132, 133).

A conditional deletion of Erg in HSCs using Mx1Cre similarly found that loss of Erg leads to a decrease in HSC numbers and their reconstitution ability. These defects were shown to be a result of ERG role in the maintenance of HSC quiescence and prevention of differentiation and not due to defects in HSC migration, homing, adhesion, or proliferation. ERG-deficient cells
also had lower expression of signature HSC genes that correlated with upregulation of MYC-target genes. The authors thus proposed a model where ERG functions to maintain HSC stemness through repression of MYC-downstream pathways involved in differentiation. In agreement with this, MYC inhibition partially restored HSC function (134).

More recently, ERG has been shown to have an important role in B lymphocyte development. Two independent studies showed that the conditional deletion of Erg in common lymphoid progenitors impairs B cell differentiation at the pro-B cell stage. Block at this stage of differentiation is due to ERG requirement for expression of IgH chain on developing B cells via its role in promoting \( V_H \) to \( D_HJ_H \) recombination (135, 136). Differentiation of ERG-deficient B cell progenitors was thus rescued by the expression of a functional \( IgH \) (136). Ng et al. determined that ERG initiates a co-dependent transcriptional network with EBF1 and PAX5, thus directly and indirectly promoting the expression of genes for V(D)J recombination and pre-BCR. On the other hand, Sondergard et al. proposed that ERG functions via its transcriptional regulation of regulatory PAX5-activated intergenic repeat (PAIR) elements whose expression is associated with \( V_H \) to \( D_HJ_H \) recombination. Unlike in HSCs, loss of Erg was accompanied by a decrease in the expression of MYC target genes (135), and \( c-Myc \) expression itself (136), suggesting the lack of \( c-Myc \) expression could also be contributing to the differentiation block observed. However, overexpression of ERG in fetal pro-B cells inhibits their differentiation to pre-B cells, as evidenced by the retention of c-Kit expression on these progenitors (137). Together, these findings suggest that the interplay of MYC and ERG on B cell development might be highly regulated and context-specific.

ERG overexpression has long been implicated in malignant transformation in hematopoietic and solid tumors (138-141). Indeed, ectopic expression of ERG in fetal liver
HPCs lead to the development of mostly megakaryoblastic leukemia in mice (142, 143), and its overexpression in 5-FU treated bone marrow cells leads to the development of both erythroid and T-ALL-like leukemia (137, 143). Moreover, ERG overexpression in hematopoietic cells using vav caused T-ALL in recipient mice (144). The ERG-driven T-ALL-like disease cooperated with Notch1 mutations, and its deletion in a NOTCH-intercellular domain (NOTCH-ICD)-driven T-ALL model slightly delayed tumor development (134, 137, 144).

High Erg transcripts have been reported across B-cell leukemias when compared to T-ALL and normal bone marrow B cells, suggesting it could have an oncogenic role in B cell malignancies as well (145-148). However, while ERG-overexpressing bone marrow pro-B cells formed more colonies in the presence of cytokine in a feeder cell culture, ERG alone was not sufficient for the formation of B cell leukemia in recipient mice (137). Interestingly, however, another ETS family member, TEL2, accelerated B cell lymphoma formation in the Eμ-Myc mouse model (149) suggesting c-Myc could be cooperating with ETS family transcription factors in lymphoid oncogenesis.

Taken together, ERG and MYC both have essential roles in HSC and B lymphocyte differentiation, and their interplay in the balance of self-renewal and differentiation might be highly dependent on their relative amounts, differentiation stage of the cells and possibly even interactions with other genes.

### 1.9 Concluding remarks

Since its discovery forty years ago, Myc has been the focus of studying many biological pathways. Due to its highly oncogenic nature, particular emphasis has been placed on
understanding its function in cancer formation. Despite the large body of research on Myc, however, adequate therapeutic targeting is mainly lacking, and Myc has often been described as “undruggable”. Clarification of the cooperative and antagonistic networks involved in MYC-driven tumorigenesis will thus lead to the development of therapeutics that target “inappropriate” MYC function and minimize the risk of malignant transformation. Thus, identification of an MYC-inducible tumor-suppressive program in lymphocytes and elucidating its anti-transformation strategies will provide significant conceptual advances to the field.
1.6 References


Figure 1.1. Developmental stages of B lymphocyte differentiation.

B cell differentiation from hematopoietic stem cell to mature B cell through distinct stages marked by expression of surface proteins and rearrangement and expression of immunoglobulin (Ig). Hardy fraction nomenclature is included below each stage name. The HSC, MPP, and CLP maintain germline Ig configuration. CLP, while expressing IL-7R and c-Kit that support survival and B lineage specification do not yet express earliest B lineage surface markers B220 and CD19. B220 and CD19 expressed at pre-pro and pro-B stage following B cell lineage commitment remain expressed throughout. At the early pro-B cell stage, developing B lymphocytes start to rearrange D_H to their J_H gene segments, giving rise to late pro-B cells that continue rearranging their Ig, marked by V_H to D_HJ_H rearrangements. Successfully rearranged IgH chain is expressed on the surface of large pre-B cells as pre-BCR that following positive selection leads to proliferation and differentiation into small pre-B cells. Small pre-B cells rearrange their IgL chain and upon pairing with the rearranged heavy chain express a complete IgM molecule (BCR) on its surface. Following maturation in the peripheral secondary organ spleen, mature B cells express IgM and IgD on their surface. Created with BioRender.com.
Chapter 2:

Unexpected suppression of tumorigenesis by c-MYC via TFAP4-dependent restriction of stemness in B lymphocytes

This research was originally published in Blood. Elena Tonc, Yoshiko Takeuchi, Chun Chou, Yu Xia, Melanie Holmgren, Chika Fujii, Saravanan Raju, Gue Su Chang, Masahiro Iwamoto, Takeshi Egawa. Unexpected suppression of tumorigenesis by c-MYC via TFAP4-dependent restriction of stemness in B lymphocytes. Blood. 2021;doi:10.1182/blood.2021011711. © by the American Society of Hematology.
2.1 Abstract

The proliferative burst of B lymphocytes is essential for antigen receptor repertoire diversification during the development and selective expansion of antigen-specific clones during immune responses. High proliferative activity inevitably promotes oncogenesis, the risk of which is further elevated in B lymphocytes by endogenous gene rearrangement and somatic mutations. However, B cell-derived cancers are rare, perhaps owing to putative intrinsic tumor-suppressive mechanisms. We show that c-MYC not only facilitates B cell proliferation as a pro-tumorigenic driver but unexpectedly also co-engages counteracting tumor suppression through its downstream factor TFAP4. TFAP4 is mutated in human lymphoid malignancies, particularly in >10% of Burkitt lymphomas, and reduced TFAP4 expression was associated with poor survival in MYC-high B-ALL cases. In mice, insufficient TFAP4 expression accelerated c-MYC-driven transformation of B cells. Mechanistically, c-MYC suppresses the stemness of developing B cells by inducing TFAP4 and restricting self-renewal of proliferating B cells. The pursuant transcription factor cascade thus functions as a tumor suppressor module that safeguards against the transformation of developing B cells.
2.2 Introduction

Lymphocytes are one of the most rapidly proliferating cell types in the postnatal organism (1-6). Extensive proliferative burst is essential to form diverse antigen-receptor repertoires and facilitate requisite antigen-specific immune responses. Besides demands for accurate DNA replication during extensive cell division, B lymphocytes are vulnerable to genomic instability due to DNA editing by recombination-activating gene (RAG) or activation-induced cytidine deaminase (AID) that primarily target antigen receptor loci but and also attack other sites throughout the genome (7), potentially rendering lymphocytes highly cancer-prone. However, cancers derived from developing lymphocytes are rarer than would be expected from their inherent risk, and their incidence is lower than that of common epithelial cancers (e.g., 1.7 for ALL versus 38-127 cases per 100,000 population for colon, lung, and breast cancers per year, https://seer.cancer.gov/statfacts/). Accordingly, we hypothesized that lymphocytes are protected from transformation by putative tumor-suppressive programs tightly linked to their proliferative program. Such programs may preserve the genome integrity or restrict specific gene expression programs that would enhance transformation.

The transcription factor c-MYC is a strong oncogenic driver as activating mutations and translocations that deregulate its expression are hallmark features of numerous tumors in humans and mice (8-10). c-MYC is also essential for proliferation of normal lymphocytes through the control of cell cycle and metabolism (11-14). If lymphocytes activate putative tumor suppressors tied to their proliferation, c-MYC itself may directly induce such tumor suppressor genes as a feedback pathway. Therefore, it is conceivable that perturbation of any such pathways might
accelerate development of lymphoid malignancies induced by aberrant c-MYC expression, although it remains unknown if such a c-MYC-induced tumor-suppressive mechanism exists.

In this work, we profiled direct c-MYC-target genes that are mutated in primary human lymphoid cancers and identified the transcription factor TFAP4 as a c-MYC-inducible tumor suppressor in B lymphocytes. Recurrent mutations of TFAP4 are found predominantly in its DNA binding domain in human cancers, including ALL and a notable proportion of Burkitt Lymphomas (BL). In c-MYC-driven mouse B cell tumor models, TFAP4 functions as a c-MYC-induced, haploinsufficient tumor suppressor. Single allele deletion of Tfap4 or a specific deletion of a c-MYC binding site in the locus shortened the latency of the MYC transgene-driven tumorigenesis, with frequent loss of heterozygosity (LOH) occurring. Mechanistically, c-MYC not only drives proliferation of B cell progenitors but also promotes their differentiation, thus restricting the self-renewing capacity of B cells in undifferentiated states through TFAP4-dependent repression of the stemness factor Erg. Finally, reduced expression of TFAP4 despite high c-MYC expression is associated with poor prognosis of a subset of B-ALL patients. These results collectively show that the c-MYC-TFAP4 axis has dual roles in supporting the clonal expansion of normal lymphocytes (15, 16) and paradoxically suppresses transformation of proliferating B cells.
2.3 Materials and Methods

Mice

*Tfap4*+/− (17), *Tfap4*F/F (15), *Erg*F/F (18), *Cd79a*-icre(19), and *Myc*-GFP (20) mice were described previously and were backcrossed to the C57BL/6N background for >10 generations. *Eμ*-Myc(21), *Ighg1*-cre(22), *Rosα26CAG-MYC*(23), and *Rosα26Pik3ca* (24) mice were from JAX. C57BL/6N and B6-Ly5.1 mice were purchased from Charles River. All mice were maintained in a specific pathogen-free environment, and all experiments were conducted according to the protocol approved by the Washington University Animal Studies Committee.

To generate mutant *Tfap4* alleles lacking a 1-kb enhancer region bound by c-MYC or each of the MYC-binding sites, *in vitro* transcribed RNA containing a 21-bp complementary sequence corresponding to and PAM following short guide RNA sequence complementary to MYCBS1 or MYCBS2 was generated using MEGAshortscript T7 Transcription Kit (Thermo Fisher) from DNA templates which were amplified using oligonucleotide primers, TTA ATA CGA CTC ACT ATA GGG GAC TGC TGC AGC ACC ACG TGT TTT AGA GCT AGA AAT AGC AAG or TTA ATA CGA CTC ACT ATA GGG GGT GTG TCG GAG CAC GGT TTT AGA GCT AGA AAT AGC AAG, and AAA AGC ACC GAC TCG GTG CC, the pX-330 plasmid (Addgene) and Phusion PCR polymerase (Thermo Fisher). A mixture of sgRNAs and Cas9 mRNA was microinjected into C57BL/6N zygotes and founders were bred to C57BL/6NCr mice at the Transgenic, Knockout and Micro-Injection Core affiliated by the department of Pathology and Immunology and Rheumatic Diseases Research Resource-based Center of Washington University.
**Tumorigenesis studies**

Eμ-Myc mice were monitored weekly for palpable tumor development and body weight loss. Mice were euthanized when tumor sizes exceeded 20 mm in diameter or mice showed >20% body weight loss or paralysis. The immunophenotypes of tumor cells were determined by flow cytometry and qRT-PCR. Since we did not observe differences in survival of Eμ-Myc Tfap4+/– mice between males and females, we pooled both genders for the analysis presented in this work, without randomization or blinding. To generate mixed bone marrow chimeras, CD45.1 mice were lethally irradiated (9.5 Gy) and reconstituted with a 1:1 mixture of BM cells from congenic Eμ-Myc mice (total cell numbers, 5x10^6 cells). For transplantation of retrovirally transduced B cells, CD19+ BM cells isolated using anti-CD19 microbeads (Miltenyi) from 6-10-week-old mice were infected with viral supernatants, followed by intravenous transfer of 1x10^6 cells into sublethally irradiated (5.5 Gy) mice. For *in vivo* persistence analysis, transduced cells were transferred into congenic mice at a ~1:1 ratio of Tfap4+/–, Tfap4+/–Erg+/– or Erg+/– to wild-type (WT) cells.

**Retroviral infection**

The retroviral plasmids encoding mouse MYC and mouse ERG were generated by cloning PCR-amplified fragment into pMIGR or pMSCV-Thy1.1. A retroviral plasmid encoding human BCR-ABL P190 was purchased from Addgene (Plasmid #27483). Viral supernatants were prepared by transiently transfecting PlatE cells (25) with a plasmid encoding either mouse MYC (T58A), mouse ERG, or human BCR-ABL P190, as indicated in the figures, together with a helper plasmid pCL-10A1 using TransIT-293 transfection reagent (Mirus Bio). Viral
supernatant was harvested 48 hrs after transfection and used to infect freshly isolated CD19+ BM cells at 1000 x g, 30°C for 1.5-2 hrs in the presence of 10 mg/ml of polybrene (Sigma).

**Human BL and TARGET data analysis**

Gene expression data from BL samples (26) were downloaded from European Genome-Phenome Archive (EGAS00001003778). Data were analyzed using Galaxy as follows: counts per gene for each sample were obtained by running featureCounts on GRCh38 aligned BAM files and normalized using DESeq2. The normalized data were imported into the Phantasus application (27) for further analysis. After log2 transformation and quantile normalization, ENTREZ-annotated genes were filtered for mean normalized counts >2 across samples. Data for B-ALL samples were downloaded from TARGET Phase II and gene expression analyzed in Phantasus Differentially expressed genes in both patient cohorts were analyzed using a limma tool followed by GSEA analysis.

**Flow cytometry**

Single cell suspension was prepared by mechanical dissociation of bones, spleen and lymph nodes in phosphate buffered saline supplemented with 2% fetal calf serum (Thermo Fisher) and 2 mM EDTA, stained with monoclonal antibodies listed below and analyzed using LSR Fortessa, LSR-II or X-20 flow cytometer (BD Biosciences), or sorted using a FACS Aria II (BD Biosciences). After cell sorting, the purity of sorted cells was confirmed to be 95% or higher.

**Antibodies**
The following monoclonal antibodies were purchased from Biolegend unless specified otherwise:

Fluorescein isothiocyanate-conjugated anti-CD8α (53-6.7), anti-CD24 (M1/69, Biolegend), anti-IgM (II/41), anti-Ly-51 (BP-1); Alexa Fluor (AF) 488-conjugated anti-CD45.1 (A20); phycoerythrin (PE)-conjugated anti-human CD2 (RPA-2.10), anti-CD25 (PC61), anti-CD117 (2B8), anti-IgM (RMM-1); peridinin chlorophyll protein–cyanin 5.5-conjugated anti-CD4 (GK1.5), anti-CD11b (M1/70), anti-CD19 (6D5), anti-CD43 (S7, Thermo Fisher), anti-CD45R/B220 (RA3-6B2), anti-CD90.1 (OX-7); PE-indotricarbocyanine-conjugated anti-CD8α (53-6.7), anti-CD11b (M1/70), anti-CD45.1 (A20), anti-CD45R/B220 (RA3-6B2), anti-CD95 (Jo2, Thermo Fisher), anti-Ly-51 (BP-1); allophycocyanin (APC)-conjugated anti-CD93 (AA4.1), anti-CD117 (2B8), anti-Ly6A/E (E13-161.7), anti-TCRβ (H57-597, Thermo Fisher); AF647-conjugated anti-CD11b (M1/70), anti-GL-7 (GL-7); APC-indotricarbocyanine conjugated anti-CD45.2 (104), anti-CD45R/B220 (RA3-6B2), anti-CD117 (2B8), anti-Igκ (187.1, BD Pharmingen); AF700-conjugated anti-CD11b (M1/70); Pacific Blue-conjugated anti-CD11b (M1/70), anti-CD44 (IM7), anti-CD45R/B220 (RA3-6B2), anti-IgD (11-26c.2a), anti-TER119 (TER-119); Brilliant Violet (BV) 510-conjugated anti-CD4 (RM4-5), anti-CD45R/B220 (RA3-6B2); BV605-conjugated anti-CD11b (M1/70), anti-CD45.1 (A20), anti-IgD (11-26c.2a); BV650-conjugated streptavidin; biotin-conjugated anti-IgM (AF6-78).

**RNA-seq**

Total RNA was extracted from sorted cells by Trizol and treated with recombinant DNase using Nucleospin RNA XS extraction kit (Macherey-Nagel). The integrity of the extracted RNA was determined using an Agilent Bioanalyzer or Tapestation. mRNA was
enriched by depleting ribosomal RNA using a Ribo-ZERO kit (Illumina), and was then fragmented in buffer containing 40 mM Tris-Acetate (pH 8.2), 100 mM Potassium Acetate and 30 mM Magnesium Acetate at 94 °C for 150 seconds. mRNA was reverse transcribed to yield cDNA using SuperScript III RT enzyme (Thermo Fisher) and random hexamers followed by synthesis of the second strand to yield ds-cDNA. cDNA was blunt ended, had an A base added to the 3’ ends, and then had Illumina sequencing adapters ligated to the ends. Ligated fragments were then amplified for 15 cycles using primers incorporating the p5 and p7 sequences and unique index tags. Fragments were sequenced on an Illumina HiSeq2500 or HiSeq3000 using single reads extending 50 bases. Sequencing reads were mapped to the mouse genome mm10 using STAR with default options. Gene counts were derived from the number of uniquely aligned reads by Subread:featureCount version 1.4.5. Sequencing performance was assessed with RSeQC version 2.3. Genes with RPKM>2 in at least one sample were initially filtered, resulting in a list of 11 637 genes further analyzed using the Phantasus web application (https://fgenome.ifmo.ru/phantasus/) for PCA. Reads were log₂ transformed and differential expression analysis was conducted using a limma tool Release 3.9 (https://bioconductor.org/packages/release/bioc/html/limma.html). This output was further analyzed for gene set enrichment using the Molecular Signatures Database (http://software.broadinstitute.org/gsea/msigdb/annotate.jsp) using Phantasus. Additionally, for transformed pre-B cells unsupervised clustering of expression data was performed, with indicated clusters being used for additional GSEA analysis. For Figure 4B and Supplemental Figure 4C and F, only annotated genes are shown. Human data was analyzed using limma tool Release 3.9 and GSEA was performed using the human Hallmark Molecular Signatures Database in Phantasus following processing described in the main methods.
Genomic PCR and variant validation

Genomic DNA was purified from tumor cells and a tail-tip using QIAmp Fast DNA Tissue Kit (Qiagen). Genomic sequences of wild-type and targeted Tfap4 alleles as well as Runx1, as a control positioned on the same chromosome, were amplified using Taq polymerase (MidSci) and primers listed below. To confirm mutations identified by whole exome sequencing, cDNA from sorted pro/pre-B tumor cells was used to PCR amplify Kras using Phusion high-fidelity PCR master-mix (Thermo Fisher) and the fragments were sequenced following TA-cloning into pCR2.1-TOPO (Thermo Fisher). Five to seven clones from each sample were sequenced using the T7 primer. Obtained sequencing data were manually inspected for Kras point mutations. The following primers were used:

*Tfap4* (WT), CAG ACC CAG GGT ATG TGC TC and GCA GCT CAC TCT GTG TAA AGA ACT G;

*Tfap4* (targeted), CAG ACC CAG GGT ATG TGC TC and GTA ACA AGA TCT GAT GCC CTC TTC;

*Runx1*, GCG TTC CAA GTC AGT TGT AAG CC and CTG CAT TTG TCC CTT GGT TGA CG;

*Kras*, CGC GGA GAG AGG CCT GCT GAA A and GTG TGC CTT AAG AAA GAG TAC A.

Real-time quantitative RT-PCR

RNA was extracted with Trizol (Thermo Fisher) and was reverse-transcribed with qScript Supermix (Quanta Bio). Luminaris SYBR green qPCR mix (Thermo Fisher) and a LightCycler 480 (Roche) were used for real-time quantitative RT-PCR. Quantities of transcripts were normalized to that of *Hprt1* RNA unless specified otherwise. For quantification of gene
expression normalized to that of 'spiked-in' RNA, 5 µl of ERCC (External RNA Controls Consortium) RNA Spike-In Control Mixes (Thermo Fisher) at a dilution of 1:1000 was added to cell lysates in Trizol per 0.5 × 10^6 cells prior to RNA extraction. The following oligonucleotide primers (Sigma) were used:

\textit{Tfap4}, GGA GAA GCT AGA GCG GGA AC and TTT TGC CGG GAT GTA GAG AC;
\textit{Myc}, AGT GCT GCA TGA GGA GAC AC and GGT TTG CCT CTT CTC CAC AG;
\textit{Erg}, GGA GTG CAA CCC TAG TCA GG and GTA GCT GCC GTA GCT CAT CC;
\textit{Rag1}, CAA CCA AGC TGC AGA CAT TC and AAT TTC ATC GGG TGC AGA AC;
\textit{Vpreb1}, ATG CTG CTG GCC TAT CTC AC and GAT GCT AAT GGT GGC TGA TG;
\textit{Hprt1}, AGG TTG CAA GCT TGC TGG T and TGA AGT ACT CAT TAT AGT CAA GGG CA;
\textit{ERCC-00108}, GCT ATC AGC TTG CGC CTA TTA T and GTT GAG TCC ACG GGA TAG AGT C.

**Immunoblot analysis**

Whole cell lysates were prepared in Laemmli buffer containing 1% SDS and 2% 2-mercaptoethanol. Lysates from 5 x 10^5 B220^+ IgM^- cells or 1 x 10^5 activated T cells were separated on 10% SDS-PAGE and transferred to nitrocellulose membranes (GE Healthcare). The blots were incubated with primary antibodies (identified below), followed by detection with horseradish peroxidase–conjugated antibody to rabbit immunoglobulin light chain (211-032-171; Jackson ImmunoResearch) and a Luminata HRP substrate (Millipore). Anti-AP4 was described previously (15) and anti-c-MYC was purchased from Santa Cruz Biotechnology (sc-764). Anti-Histone H3 (ab1791; Abcam) or anti-HDAC1 (ab7028; Abcam) were used as loading
controls. For inhibition of translation in an assay for AP4 protein stability assay, 10 μM cycloheximide (Sigma) was added to the activated T cell culture as described.(15)

**In vitro stimulation of T cells**

Naive CD8+ T cells were purified using Dynabeads CD8 positive isolation kit (Thermo Fisher) and cultured in RPMI medium supplemented with 10% FBS (Thermo Fisher) in the presence of soluble anti-CD3 (145-2C11; Biolegend) and anti-CD28 (37.51; Bio X Cell) at concentrations of 0.1 μg/ml and 1 μg/ml, respectively, in multiwell tissue culture plates coated with goat anti-hamster IgG (55397; MP Biomedicals). For retroviral transduction of activated T cells, T cells were activated overnight and then 'spin-inoculated' as described.(15) Following retroviral infection T cells were cultured in original media with anti-CD3 and anti-CD28 stimulation for additional 24 hrs. Subsequently, infected T cells were cultured in the presence of 100 U/mL of recombinant IL-2 (Thermo Fisher) for 24 hrs and were transferred to culture with 1 U/ml of IL-2 for analysis of CD25 expression 12-16 hrs later.

**Chromatin Immunoprecipitation and sequencing (ChIP-seq)**

ChIP-seq was performed essentially as described previously (15, 16). Briefly, CD19+ cells from 3-4-week-old Eμ-Myc Tαp4+ and Eμ-Myc Tαp4− mice were purified from BM using anti-CD19 microbeads and LS columns (Miltenyi), and fixed with 1% paraformaldehyde for 10 min at room temperature. Sonicated chromatin was immunoprecipitated using 5 μg of anti-AP4, 1 μg of anti-H3K27ac (ab4729, Abcam) and Dynabeads protein-G magnetic beads (Thermo Fisher). After reverse crosslinking, precipitated DNA was purified using a GenElute PCR Clean Up Kit (Sigma) and quantitated using a Qubit DNA quantitation kit (Thermo Fisher).
DNA size range was assayed on Agilent Bioanalyzer High Sensitivity DNA chips. The purified DNA was blunt ended, had addition of "A" base to 3' end, and had sequencing adapters ligated to the ends. The fragments were size-selected to 200-600 base pairs, and underwent amplification for 15 cycles with primers incorporating p5 and p7 sequences and a unique index tag for multiplexing. The resulting libraries were sequenced using the Illumina HiSeq3000 as single reads extending 50 bases. Sequenced reads were mapped to the mouse genome mm9 using bowtie-2 with default parameters and the Homer software package (28) was used for peak calling, motif analysis and visualization on the UCSC genome browser. Homer was used for peak 'calling' with a --style factor option and default False Discovery Rate of 0.001. Previously published ChIP-seq and ATAC-seq data were obtained from the NCBI-GEO database with the following accession IDs. GEO: Eμ-Myc anti-MYC ChIP-seq: GSE51011; ATAC-seq GSE100738; activated T cell ChIP-seq GSE58081; activated B cell ChIP-seq GSE80669, T cell ATAC-seq GSE87646.

**Whole Exome Sequencing and somatic variant identification**

Library preparation was performed with 1 μg genomic DNA from sorted tumor cells and a tail-tip of each corresponding tumor bearing mouse. The integrity of genomic DNA was determined using Agilent Tape station. Genomic DNA was sonicated to an average size of 175bp. The fragments were blunt ended, had addition of "A" base to 3' end, and had Illumina's sequencing adapters ligated to the ends. The ligated fragments underwent amplification for 8 cycles. Fragments were hybridized to biotinylated RNA oligos (Agilent Sure Select Mouse Exome) specific to regions of interest, and selected from remaining fragments using streptavidin beads. Enriched fragments were amplified for 11 cycles with primers that incorporate a unique
indexing sequence tag. The resulting libraries were sequenced using the Illumina HiSeq-3000 as paired end reads extending 150 bases from both ends of the fragments. to achieve at least 25-fold coverage in >90% of exome for tumor samples and at least 10-fold coverage in >95% of exomes for tail samples.

Whole exome sequencing data were aligned to the mouse reference sequence mm9 using BWA (29) version 0.7.10. BWA-MEM algorithm was used with the parameter, -t 8. BAM files were de-duplicated by using picard version 1.113 (https://broadinstitute.github.io/picard/). Somatic variants in tumor samples were called against matched normal tail tip samples. In detail, single nucleotide variants (SNVs) were detected using the union of three callers: 1) Samtools (30) version r982 (params: mpileup -BuDS) intersected with Somatic Sniper (31) version 1.0.4 (params: -F vcf -G -L -q 1 -Q 15) and processed through false-positive filter v1 (params: --bam-readcount-version 0.4 --bam-readcount-min-base-quality 15 --min-mapping-quality 40 --min-somatic-score 40) 2) VarScan (32) version 2.3.6 (params: --nobaq --version r982:) filtered by varscan-high-confidence filter v1 and processed through false-positive filter v1 (params: --bam-readcount-version 0.4 --bam-readcount-min-base-quality 15), and 3) Strelka (33) version 1.0.11 (params: isSkipDepthFilters = 1). INDELs were detected using the union of 4 callers: 1) GATK somatic-indel (34) version 5336, 2) Pindel (35) version 0.5 filtered with pindel somatic-calls v1 and vaf-filter v1 (params: --variant-freq-cutoff=0.08), 3) VarScan version 2.3.6 (params: --nobaq --version r982:) filtered by varscan-high-confidence-indel v1, and 4) Strelka version 1.0.11 (params: isSkipDepthFilters = 1). Furthermore, low confidence SNVs were filtered out given known tumor and normal contamination rates, by using “gmt validation identify-outliers” tools (params: -llr-cutoff 3 -tumor-purity 0.95) (Genome Modeling System (36): (https://github.com/genome/gms). Finally, at least 20x coverage was required at each variant site,
then variant calls by read alignment in paralogous sequence regions or unstable contigs were filtered out using the mapping quality score.

**EdU labeling**

Three week-old mice received 1.5 mg EdU (5-ethynyl-2′-deoxyuridine) by intraperitoneal injection 1 hr before euthanasia. EdU incorporation was determined by a Click-iT Plus EdU Alexa Fluor 647 Flow Cytometry Assay Kit according to the manufacturer's instructions (Thermo Fisher).

**Annexin V staining**

Developing B cells from the bone marrow of 3-week-old mice were stained for Annexin V-APC (Biolegend) and Live/Dead Fixable Aqua (Thermo Fisher) according to the manufacturer's instructions.

**Ex vivo B cell survival assay**

CD19+ pro/pre-B cells of 3–4-week-old Eμ-Myc mice were cultured 10% FBS-containing medium without or with 10 ng/μl of recombinant IL-7 (Peprotech). Cell viability was determined by Live/Dead Fixable Aqua staining at 24, 48 and 72 hrs post isolation.

**Statistical analysis**

Survival data were compared using the log-rank test with adjustment for multiple comparisons when necessary. *P*-values were calculated with an unpaired two-tailed Student's *t*-
test for two-group comparisons and one-way ANOVA for multi-group comparisons with the Tukey post-hoc test or Kruskal-Wallis test with Dunn's multiple comparison test. Human microarray data from previously published studies were compared using Mann-Whitney's U-test for two-group comparisons and by Kruskal-Wallis test for multi-group comparisons. All statistical analyses were done using Prism. Adjusted $P$-values of $<0.05$ were considered significant; *$P<0.05$; **$P<0.01$; ***$P<0.001$; ****$P<0.0001$.

Data Availability and Sharing

Sequencing data have been deposited to the NCBI-GEO and SRA with accession numbers GSE133514 and PRJNA551263.
2.4 Results

The c-MYC downstream transcription factor TFAP4 is mutated in primary human cancers

To determine whether c-MYC engages tumor-suppressive programs in B lymphocytes, we initially defined c-MYC-dependent genes in developing B cells between Eμ-Myc and littermate wild-type B220+ IgM− pro/pre-B cells. We combined this analysis with gene expression profiles of mouse germinal center B (GCB) cells (12) and identified 107 genes commonly dependent on c-MYC in pro/pre-B and GCB cells (Figure 2.1A-C). The candidate genes were prioritized based on known roles in gene regulation, direct c-MYC targets (15, 16, 37), and mutation frequencies in human hematopoietic malignancies using the COSMIC (Catalogue Of Somatic Mutations In Cancer) database. Tfap4, encoding a bHLH transcription factor, was identified as a gene meeting all the criteria (Figure 2.1D). We confirmed that TFAP4 expression was increased in B220+ IgM− bone marrow (BM) cells from Eμ-Myc compared to those from littermate WT mice (Figure 2.1E).

Somatic mutations of TFAP4 were found in primary lymphoid and other cancers registered in COSMIC, TCGA (The Cancer Genome Atlas), and PeCan (Pediatric Cancer Genomic Data Portal). Notably, five out of six PeCan cases with TFAP4 mutations were found in lymphoid tumors (3 BL, 1 B-ALL, and 1 T-ALL cases). While TFAP4 mutations in these databases were rare (288/37,420 unique samples in COSMIC, 65/10,202 in TCGA, and 6/2,578 in PeCan), analysis of cases in the BL dataset(26) identified missense TFAP4 mutations in 12 out of 101 cases, with a slightly higher frequency in endemic BLs (6/32) compared to sporadic and HIV+ cases (6/69) (Figure 2.1F; Figure 2.2A-B). Across these datasets, the majority of recurrent somatic mutations (≥4 independent mutations), including 4 out of 6 cases in the PeCan lymphoid
tumors and three additional cases in the BL data, were mapped to the DNA-binding domain (basic region) (38) (Figure 2.1G-H). A single amino acid substitution in the DNA-interacting domain was sufficient to compromise its transactivating potential, as we observed reduced expression of CD25/Il2ra, a direct TFAP4-target gene, in Tfap4+/− CD8 T cells retrovirally complemented with the R50W or R60H variant compared to those expressing WT-TFAP4 or the R129W variant harboring a mutation outside of the basic region (Figure 2.1I; Figure 2.2C-F). These results suggest that the somatic mutations found in the basic region impair TFAP4 function and that the dysfunction of TFAP4 contributes to oncogenesis in lymphoid malignancies.

**TFAP4 is a cell-intrinsic tumor suppressor**

Since Tfap4 is a c-MYC-inducible gene (15, 16, 39) and many tumors associated with TFAP4 mutations express high levels of c-MYC, we next determined whether TFAP4 modulates c-MYC-induced tumorigenesis in B cells. We first employed the Eμ-Myc mouse model, in which Myc is expressed under the control of the Igh μ enhancer, mimicking the t(8;14) IGH-MYC translocation (21). In this model, c-MYC is overexpressed in developing B cells starting at the pro-B cell stage, leading to B cell malignancy and almost all mice dying within a year (Figure 2.3A). In striking contrast, haploinsufficiency of Tfap4 in Eμ-Myc mice accelerated tumorigenesis, with all mice dying by day 92, while homozygous Tfap4 deletion further accelerated tumorigenesis (Figure 2.3A). In 40% of tumors in Eμ-Myc mice, B220+ cells exhibited an immature phenotype, expressing Rag1 and VpreB1 (Figure 2.3B; Figure 2.4A-B), consistent with previous studies (40, 41). By contrast, in the Eμ-Myc Tfap4+/− group, 93% of mice formed tumors with the immature phenotype (Figure 2.3B). The skewing of tumors
towards the immature phenotype suggests that pro/pre-B cells with elevated c-MYC but reduced TFAP4 become transformed with shorter latency after c-MYC is turned-on in pro-B cells. Numbers of developing B cells, their short-term proliferation, and Annexin-V binding at the population level were comparable between Tfap4-haploinsufficient and -proficient Eμ-Myc mice before tumor formation (Figure 2.4C-F). No survival difference was observed between Tfap4+/+ and Tfap4+/– Eμ-Myc pro/pre-B cells ex vivo (Figure 2.4G-H).

To determine whether TFAP4 is cell-intrinsically required for suppression of Eμ-Myc tumors, we deleted one or two Tfap4 alleles in pro-B cells using Cd79aicre, recapitulating the accelerated tumorigenesis and immunophenotype observed in germline Tfap4-deficient mice (Figure 2.3C, Figure 2.4I). The cell-intrinsic requirement for AP4 was further confirmed by mixed BM chimeras, in which lethally irradiated CD45.1 mice were reconstituted with a mixture of 3-4-week-old Eμ-Myc Tfap4+/– or Eμ-Myc Tfap4+/+ (both CD45.2, tester) and CD45.1/2 Eμ-Myc (competitor) BM cells (Figure 2.5A). Recipients of Eμ-Myc Tfap4+/– tester BM cells developed tumors significantly faster than those receiving only Eμ-Myc Tfap4+/+ tester BM cells. The tumors in recipients of Eμ-Myc Tfap4+/– BM cells were predominantly derived from CD45.2/2 cells (Figure 2.5B-D). These results indicate that Eμ-Myc Tfap4+/– BM cells are intrinsically more tumor-prone.

We also tested whether TFAP4 functions as a suppressor of c-MYC-induced B cell malignancies in additional mouse models since almost all B cells express aberrant MYC throughout their development in Eμ-Myc mice. First, we retrovirally expressed c-MYC in CD19+ BM cells from Tfap4+/– or Tfap4+/+ mice, followed by transfer to sublethally irradiated mice. In this model, we typically achieve transduction of ~10% of pro/pre-B cells (data not shown), and tumor formation by c-MYC-transduced pre-B cells is dependent on a defect of a tumor
suppressor (42, 43). In contrast to control mice receiving c-MYC-transduced \( Tfap4^{+/+} \) CD19+ cells, the majority of mice receiving c-MYC-transduced \( Tfap4^{-/-} \) CD19+ BM cells died of pre-B cell tumors (Figure 2.3D, Figure 2.4J). We also observed accelerated B-ALL disease progression induced by ectopic expression of p190 \( bcr-abl \), which indirectly upregulates Myc, in CD19+ BM cells from \( Tfap4^{-/-} \) mice compared to those from \( Tfap4^{+/+} \) mice (Figure 2.3E, Figure 2.4K). In addition to these immature B cell malignancies, we observed accelerated death in the absence of TFAP4 in a mature B cell lymphoma model induced by c-MYC and a constitutively active PI3K(23) (Figure 2.3F). These data establish a cell-intrinsic requirement for TFAP4 in the suppression of MYC-mediated B cell cancers in multiple mouse models.

C-MYC bound two consensus sites within the H3K27ac-marked intronic region at the \( Tfap4 \) locus in E\( \mu \)-Myc B220+ BM cells (Figure 2.6A). To determine whether direct activation of \( Tfap4 \) by c-MYC binding to this enhancer is necessary for tumor suppression, we deleted a 1-kb intronic region containing the two consensus sites using CRISPR/Cas9 (Figure 2.6A). Heterozygous deletion of the 1-kb region in E\( \mu \)-Myc mice recapitulated accelerated tumorigenesis (Figure 2.6B). This might be caused by the loss of other transcription factors binding at the enhancer or indirectly by altered expression of the neighboring genes. However, a more specific single-allele deletion of one MYC binding site also shortened survival of these mice compared to E\( \mu \)-Myc mice harboring wild-type \( Tfap4 \) loci (Figure 2.6B), while expression of two genes around the \( Tfap4 \) locus, or \( Runx1 \) located on the same chromosome, was unaffected (Figure 2.6C). Thus, direct activation of \( Tfap4 \) by c-MYC is essential for tumor suppression.
LOH of TFAP4 confers interclonal competitiveness for tumor outgrowth

*Tfap4* mRNA expression was comparable between pro/pre-B cells before transformation and pro/pre-B tumor cells in *Eμ-Myc Tfap4*+/+ mice (**Figure 2.7A**). However, approximately one-third of pro/pre-B tumors from *Eμ-Myc Tfap4*+/− mice expressed substantially lower *Tfap4* mRNA than pre-transformed pro/pre-B cells (**Figure 2.7A**, dashed rectangle). This reduced *Tfap4* expression was caused by LOH in these tumors (**Figure 2.7B**). A genomic region encompassing exons 2-4, deleted in the *Tfap4*− allele, was detectable in all tumors from *Eμ-Myc Tfap4*+/+ mice and a subset of tumors from *Eμ-Myc Tfap4*+/− mice. By contrast, this genomic region was lost in the tumor cells with low *Tfap4* expression from *Eμ-Myc Tfap4*+/− mice (**Figure 2.7B**, dashed rectangle). LOH of *Tfap4* was also confirmed by loss of mCherry fluorescence in *Eμ-Myc* tumors harboring one null and one functional TFAP4-mCherry fusion knock-in allele(16) (*Tfap4*<sup>moCl−</sup>) (**Figure 2.7C-D**). These results indicate that although TFAP4 downregulation is not necessary for transformation, rare clones undergoing LOH at the *Tfap4* locus predominantly contribute to tumorigenesis.

Furthermore, to determine whether continuous AP4 expression is sufficient to suppress transformation of *Eμ-Myc* B cells, we generated an allele conditionally overexpressing AP4 fused with a self-cleaving 2A peptide and mCherry reporter, which were inserted into the *ROSA26* locus (*ROSA26<sup>LSL-AP4</sup>*) (**Figure 2.8A**). While AP4 rapidly turns over due to constitutive degradation by the proteasome23, we ensured sustained AP4 expression by generating a degradation resistant variant of AP4 (sAP4) by a serine to alanine substitution at position 139 (S139A) (**Figure 2.8B**). This allele was bred with *Eμ-Myc Tfap4*i/F+ or *Tfap4*F/F mice with a *Cd79a<sup>ics</sup>* allele to determine whether overexpression of AP4 is sufficient to suppress tumorigenesis. Survival of *Eμ-Myc Tfap4*i/F+ mice was significantly extended due to delayed
tumor onset by B cell-specific overexpression of AP4 (Figure 2.8A, red versus orange), while we observed only marginal extension of survival in Eμ-Myc Tfpap4F/F mice (Figure 2.8A, dark blue versus light blue). Importantly, however, all tumor samples harvested from the Eμ-Myc Tfpap4F/F mice lacked expression of the mCherry reporter, despite its expression in the vast majority of B cells prior to tumor onset (Figure 2.8C). These results indicate that tumors were derived predominantly from the minority of B cell precursors that escaped from Cd79aicre-mediated activation of AP4 overexpression or silenced the transgene expression. Together with the observed LOH in Eμ-Myc Tfpap4+/− mice, our data indicate that a minority of transforming pro/pre-B cells with defective or low AP4 expression are endowed with a strong selective advantage over AP4-proficient cells during c-MYC-driven transformation.

A c-MYC target gene signature is commonly enriched both in mouse Eμ-Myc Tfpap4+/− tumors and TFAP4-mutated BL in humans

To gain insights into the molecular mechanisms of c-MYC-TFAP4-dependent tumor suppression, we profiled gene expression of pre-transformed B220+ IgM− pro/pre-B cells, validated by unbiased expression of Ighv transcripts (Figure 2.9A), from Eμ-Myc Tfpap4+/− and control Eμ-Myc Tfpap4+/+ mice at 3-4 weeks-old. Due to rapid tumor development, analysis of pre-transformed Eμ-Myc Tfpap4+/− pro/pre-B cells was not included. Neither gross transcriptomic differences determined by a principal component analysis (PCA) nor the expression of genes related to apoptosis, proliferation, and senescence were detected between Eμ-Myc Tfpap4+/− and Eμ-Myc Tfpap4+/+ cells (Figure 2.9B-D). At the gene level, 29 genes were differentially expressed by ≥1.8-fold. Among these, 20 genes were directly bound by TFAP4, as confirmed by ChIP-seq (current study), and 9 of the 20 genes were shared TFAP4 and MYC targets (37) in Eμ-Myc B
cells (Figure 2.10A-C). Despite the lack of clear genotype-associated clustering of tumor samples (Figure 2.9E), an expression pattern of 17 of these 29 genes was preserved in Tfap4-deficient tumors, suggesting their expression is maintained, or selected for, during tumorigenesis (Figure 2.9F).

To further examine gene expression programs modules that were altered specifically in Tfap4-deficient compared to Tfap4-proficient pre-B tumors, we conducted gene set enrichment analysis (GSEA). Among curated gene sets in the Molecular Signature Database (MSigDB, https://www.gsea-msigdb.org/gsea/msigdb/) (44-46), genes related to MYC-targets and E2F targets—were significantly enriched in Tfap4-deficient/haploinsufficient tumors (Figure 2.10D-E). Gene sets associated with MYC and E2F targets were also significantly enriched in human BL samples harboring somatic TFAP4 mutations (Figure 2.10D-E). These findings suggest that TFAP4 functions as a negative feedback factor that restricts proliferative states of tumorigenic cells associated with high c-MYC and E2F activity. Unsupervised clustering of differentially expressed genes in pre-B cell tumors with distinct Tfap4 genotypes revealed two clusters specifically associated with reduced Tfap4 expression (Figure 2.10F, Clusters II/III). These clusters were enriched for genes associated with cancer/p53-related pathways (Figure 2.9G). In addition, a gene signature associated with upregulated KRAS signaling pathway, which has been associated with the Em-Myc model (47-49), was enriched specifically in Eμ-Myc Tfap4+/− tumors (Figure 2.10F, Cluster VIII; Figure 2.9G). Consistently, whole-exome sequencing and targeted sequencing of the tumor samples, which were mostly clonal or oligoclonal (Figure 2.11A), revealed frequent Kras mutations in Eμ-Myc Tfap4+/− tumors, despite similar overall mutational burdens (Figure 2.11B-D). Overall, transcriptomic and genetic data suggest that TFAP4-
dependent tumor suppression is mediated by the regulation of persistent engagement of cell proliferation via specific gene pathways rather than protection against global genomic instability.

**Restriction of the proto-oncogene Erg is required for tumor suppression by TFAP4**

TFAP4 supports the continued proliferation of normal lymphocytes by supplementing an early decay of c-MYC by maintaining the expression of numerous c-MYC target genes but is dispensable when c-MYC is expressed (15, 16). However, during tumorigenesis, our data showed that TFAP4 counteracts c-MYC-mediated transformation of developing B cells, likely through control of its unique targets. Among the unique TFAP4 targets in Figure 4B, Erg, encoding an ETS-family transcription factor, has been implicated in hematopoietic malignancies. Erg is also necessary to maintain the stemness of HSCs (50). ERG overexpression in mouse hematopoietic progenitor cells causes lymphoid and megakaryocytic cancers (51-54). In humans, although impaired ERG function has been associated with a small subset of B-ALL specifically harboring a DUX4 rearrangement (54, 55), ERG was overexpressed in a majority of B-ALL cases compared to other tumor types and normal BM cells across multiple studies (56-60) (Figure 2.12A-E). Despite the limited sample number with gene expression data available, a human T-ALL case with a TFAP4<sup>E57K</sup> substitution and an LMO2-LYL1 translocation in PeCan expressed ERG at a level higher than the 95% confidence interval of ERG expression in other T-ALL samples with an LMO2-LYL1 or LMO1-LMO2 translocation (Figure 2.12F-G), suggesting that elevated ERG is associated with functionally defective TFAP4.

In Eμ-Myc Tfap4<sup>+/-</sup> pre-transformed pro/pre-B cells, Erg expression was elevated by 3.8-fold compared to controls (Figure 2.13A). Erg was further upregulated in pro/pre-B tumors lacking TFAP4 (Figure 2.13B). TFAP4 bound an intronic region of the Erg locus, colocalizing
with H3K27ac-marked accessible chromatin in Eμ-Myc B cells, whereas no c-MYC binding was detected in the genomic region encompassing the Erg locus (Figure 2.13C). These results suggest that Erg is a direct TFAP4 target. Erg is co-expressed with Myc and Tjap4 in pro-B cells before the Igh checkpoint at the population level but is downregulated in proliferating large pre-B cells as they differentiate into small pre-B cells (Figure 2.13D). However, within c-kit+ pro-B cells, expression of c-MYC protein and Erg mRNA was inversely correlated, with Erg expression enriched in c-MYC− pro-B cells and significantly downregulated as cells upregulate c-MYC during the transition to large pre-B cells (Figure 2.13E). Therefore, we hypothesized that Erg must be regulated in c-MYC+ B cells in a TFAP4-dependent manner to restrict the risk of tumorigenesis as both c-MYC and ERG are required for B cell development but are also oncogenic. Accordingly, Erg was elevated in Tjap4+/− compared to Tjap4++ c-MYC+ pro-B cells (Figure 2.13F). Furthermore, TFAP4-mediated Erg suppression was even more apparent in Eμ-Myc mice as Erg was completely suppressed in Eμ-Myc compared to wild-type B220+ IgM+ cells (Figure 2.13G). The Erg suppression was partially reversed in Eμ-Myc Tjap4+/− and completely lost in Eμ-Myc Tjap4− pre-transformed pro/pre-B cells (Figure 2.13G). These results indicate that TFAP4 prevents simultaneous expression of the two proto-oncogenes, c-MYC and ERG, in differentiating B cells.

To directly determine whether Erg de-repression accelerates tumorigenesis, we deleted one Erg allele in Eμ-Myc Tjap4+/− mice since the complete loss of Erg arrests B cell development prior to the pre-B cell stage (Figure 2.14) due to defective Igh rearrangement (61). Erg haploinsufficiency significantly delayed tumor onset and extended survival of Eμ-Myc Tjap4+/− mice (Figure 2.13H). Furthermore, B cell tumors developed in Erg+/− mice were skewed toward the mature phenotype lacking expression of Rag1 and Vpreb1 (Figure 2.13I), indicating
prolonged latency to transformation after MYC was turned-on. These results indicate that TFAP4 suppresses the MYC-mediated transformation of B cell precursors, at least in part, through the regulation of Erg.

**TFAP4 suppresses tumorigenesis by coupling c-MYC-driven proliferation and differentiation of B cell progenitors**

ERG maintains the undifferentiated state of HSCs by inhibiting c-MYC targets, while c-MYC antagonizes self-renewal in favor of differentiation (50, 62). Since our data demonstrated that c-MYC suppresses Erg through TFAP4, we hypothesized that TFAP4 prevents B cell progenitors from maintaining their undifferentiated state or stemness while rapidly proliferating, thus restricting transformation. To test this, we adoptively transferred a mixture of Tfaq4+/+ and Tfaq4+/- CD19+ cells transduced with c-MYC marked by distinct reporters into sub-lethally irradiated congenic mice and assessed their expansion and differentiation (Figure 2.15A). Three weeks after transfer, Tfaq4+/- cells became dominant among the total transduced, donor-derived B220+ cells (Figure 2.15B-C). In addition to superior expansion and persistence of MYC+ Tfaq4+/- cells, frequencies of IgM+ cells were significantly lower in Tfaq4+/- compared to Tfaq4+/- cells (Figure 2.15D). These results imply that Tfaq4+/- pro/pre-B cells maintain their undifferentiated state during c-MYC-driven proliferation due to high Erg expression. Indeed, reducing Erg expression to 50% in Tfaq4+/- pro/pre-B cells diminished the expansion of overall donor-derived cells mainly due to the reduced expansion of Tfaq4+/- cells relative to Tfaq4+/- cells. Reduction of Erg in Tfaq4+/- had no additional effect on the expansion of transferred cells (Figure 2.15C, E). Additionally, reducing Erg expression rescued differentiation of transferred Tfaq4+/- MYC+ pro/pre-B cells as determined by frequencies of IgM+ cells (Figure 2.15F).
Although a previous study suggested ERG alone is insufficient to cause transformation of pre-B cells\textsuperscript{33}, we asked whether over-expression of both Myc and Erg is sufficient. While retroviral expression of c-MYC alone in Tfap4\textsuperscript{+/−} pro/pre-B cells, similar to c-MYC transduction in Tfap4\textsuperscript{+} pro/pre-B cells (Figure 2.3D), resulted in tumorigenesis, co-expression of c-MYC and ERG showed a trend for further acceleration (Figure 2.16A). However, the co-expression was insufficient to cause robust tumorigenesis by WT pro/pre-B cells (Figure 2.16B). Therefore, c-MYC-induced AP4 engages multiple tumor-suppressive pathways, including restriction of Erg, to protect MYC-expressing developing B cells from transformation.

TFAP4 is a positive prognosis factor in MYC-high B-ALL.

So far, our data have demonstrated that TFAP4 is mutated in human B cell malignancies, and reduced TFAP4 functionality predisposes proliferating B cells to transformation in preclinical models. To gain insights into the role of TFAP4 in c-MYC-driven B cell malignancies in humans, we reanalyzed the expression of c-MYC and TFAP4 in human pediatric B-ALL samples from the TARGET Phase II study (Figure 2.17A). Comparing patients with top 25% and bottom 25% TFAP4/MYC ratios did not show a difference in the overall survival (data not shown). However, when we specifically compared samples with high MYC expression (z-score > 2), overall survival of the patients with high TFAP4 expression was better than that of TFAP4\textsuperscript{lo} patients (Figure 2.17B-C). There was no difference in the relative ERG expression between the two groups of patients, possibly reflecting selection of ERG\textsuperscript{high} clones during tumorigenesis (Figure 2.17D). These results suggest that upregulation of TFAP4 by c-MYC is not only a decisive factor for predisposition to B cell malignancies but also associated with the prognosis of B-ALL patients' survival.
2.5 Discussion

Our study provides compelling evidence that c-MYC engages a tumor-suppressive program in B cells by inducing its direct downstream factor, TFAP4. Coding mutations of TFAP4 prevail in lymphoid malignancies, particularly in a significant proportion of Burkitt lymphomas. Our mouse models reveal substantial impacts on tumor onset by deletion or reduced expression of TFAP4 in developing B cells and mature B cells in the face of aberrant Myc expression. The tumor suppression is mediated, at least in part, by restricting the stemness factor ERG in immature B cells since the reduced expression of ERG was sufficient to rescue developing B cells from aberrant expansion and accelerated transformation.

During hematopoiesis, HSCs give rise to various mature subsets through defined pathways. Since several proto-oncogenes are essential for normal hematopoiesis, their expression levels and timing must be tightly regulated to restrict the risk of leukemogenesis. Nonetheless, clonal hematopoiesis carrying oncogenic translocations is found in healthy individuals at a higher incidence than that of corresponding leukemias, including the t(8;14) translocation modeled by Eμ-Myc mice (63-65). Therefore, appropriate engagement of tumor suppression is essential to keep these clones from transformation. HSCs and undifferentiated progenitors generate greater numbers of mature progenies through proliferation tightly linked to differentiation and at the cost of their self-renewing potential. Thus, perturbation of normal differentiation could result in a decoupling of the loss of self-renewal capacity and proliferation, which has been associated with hematopoietic malignancies (66-72). In HSCs, the antagonistic interplay between c-MYC and ERG is essential for balancing self-renewal and differentiation (50). ERG is essential for self-renewal of undifferentiated HSCs through suppression of c-MYC
target genes, and accordingly, loss of ERG closely recapitulates c-MYC overexpression in HSCs, resulting in depletion of undifferentiated HSCs (50, 62). We demonstrate that the balance between MYC and ERG controls self-renewal and differentiation of B cell progenitors. Insufficient TFAP4 induction and subsequent Erg persistence selectively impair the differentiation program by c-MYC, allowing c-MYC-expressing cells to continue proliferation with an undifferentiated phenotype and eventually form tumors. Although a subset of B-ALL is characterized by reduced ERG activity, likely caused by an independent mechanism, elevated ERG was noted in a majority of B-ALL that also express elevated c-MYC.

In the human B-ALL cases, the frequency of TFAP4 mutation is rare. However, within MYC-hi B-ALL cases, the level of TFAP4 expression is associated with patients’ prognosis. Since increased TFAP4 expression is associated with poor prognosis in some cancers (73-75), lymphocytes may have established unique gene expression circuitry that converts the c-MYC-TFAP4 axis from a pro-proliferative/tumorigenesis module into a tumor-suppressive module by restricting their distinct targets. Similar counter-regulation of MYC-driven proliferation may also be involved in the suppression of lymphomagenesis in GCB cells. GCB cells retain high proliferative potential similar to pro/pre-B cells and TFAP4 may analogously function as a tumor suppressor by tightly coupling the expression of MYC and restriction of continued proliferation. Thus, the induction of TFAP4 by MYC not only impacts the predisposition of B cell malignancies but also likely contributes to disease progression. A therapeutic benefit for B cell malignancies marked by high MYC expression and low TFAP4 could be achieved by inhibiting SCF ubiquitin ligase, leading to not only the general cell cycle inhibition but also specific stabilization of TFAP4, thus increasing the level of TFAP4 and balancing MYC and TFAP4
levels (76, 77). We uncover that perturbation of the c-MYC-initiated and TFAP4-mediated feedback mechanism dramatically accelerates tumorigenesis.
2.6 Acknowledgments

We thank Vivek Durai, Sunnie Hsiung, Tenzin Yangdon, Jason Walker, and Mike White for technical support, Barry Sleckman for MYC-GFP mice, Kenneth Murphy, Eugene Oltz, Jacqueline Payton, and Dan Littman for discussion and critical reading of the manuscript. We thank the Maxim Artyomov lab for the development of RNA-seq analysis tool Phantasus and the Genome Technology Access Center (GTAC) in the Department of Genetics at Washington University School of Medicine for help with genomic analysis. **Funding:** This study was supported by US National Institutes of Health (NIH) grants R56AI114593-01A1 (T.E.), R01AI130152-01A1 (T.E.), and R01AR046000 (M.I), the Leukemia and Lymphoma Society Scholar Award #1349-18 (T.E.), the Siteman Investment Program Research Development Award (T.E.) and Hu and Zeng Predoctoral Scholarship (E.T.). Washington University Rheumatic Diseases Research Resource-based Center was supported by an NIH grant P30AR073752. The GTAC is partially supported by NCI Cancer Center Support Grant P30CA91842 to the Siteman Cancer Center and by ICTS/CTSA Grant UL1TR000448 from the National Center for Research Resources (NCRR), a component of the NIH, and NIH Roadmap for Medical Research.

2.7 Authorship contributions

T.E. conceptualized the project. E.T., C.C., and T.E. designed the experiments, interpreted the results. E.T., Y.T., C.C., Y.X., M.H., C.F., S.R, and T.E. performed the experiments. G.S.C. helped with the analysis of whole-exome sequencing data, M.I. provided *Erg*-flox mice. E.T. and T.E. wrote the manuscript with editorial comments from all other co-authors.
2.8 References


Figure 2.1. The transcription factor TFAP4 is induced by MYC in developing B cells and GC B cells, and mutated in lymphoid cancers.

(A) A z-score heatmap showing expression of ≥3 fold differentially expressed genes (DEGs) by pro/pre-B cells from Eμ-Myc− Tfacp4+/− (non-Tg) (n=3) and Eμ-Myc Tfacp4+/+ (n=5) mice determined by RNA-Seq. (B) A z-score heatmap showing expression of ≥3 fold DEGs by c-MYC+ versus c-MYC− mouse splenic GCB cells determined by microarray (GSE38304). (C)
Overlap of differentially expressed genes between (A) and (B). (D) A list of overlapping genes from (C) involved in gene regulation with direct c-MYC binding to each genomic region and mutation frequencies in hematopoietic tumors in COSMIC. The list is sorted by numbers of point mutations in hematopoietic tumors after prioritization of c-MYC bound genes. (E) qRT-PCR (left) and immunoblot analysis (right) of Tfap4 mRNA and TFAP4 protein in BM B220+ IgM− cells of Eμ-Myc− Tfap4+/+ (non-Tg) and Eμ-Myc+ Tfap4+/+ and Tfap4+−/− mice. Tfap4 mRNA expression was normalized to spike-in control RNA, ERCC-00108. Histone H3 serves as a loading control in immunoblot. (F) Frequency of coding TFAP4 mutations registered across Burkitt lymphoma subtypes (26). (G) Mapping of recurrent (≥4 independent) somatic missense mutations of TFAP4 identified in primary human tumors from TCGA, PeCan and COSMIC databases. Each mutation is shown by an asterisk and mapped to its position of the TFAP4 protein, with PeCan lymphoid tumors shown in blue and Duke BL cases in red. The basic region (b) functioning as a DNA binding domain and the helix-loop-helix (HLH) domain are depicted in the diagram. A caret indicates the R129W mutation found outside of this region. (H) A list of recurrent somatic mutations in the DNA binding region and their cancer types in (G). (I) (Left) an assay to determine the function of somatic TFAP4 variants in upregulation of CD25, which is a direct TFAP4 target (Supplemental Figure 1C-E), in Tfap4−/− CD8 T cells. (Right) expression of CD25 in Tfap4−/− CD8 T cells retrovirally expressing each TFAP4 somatic variant. Data are shown as representative of three independent experiments.
Figure 2.2. The transcription factor TFAP4 is mutated in lymphoid cancers and directly upregulates the expression of Il2ra, encoding the CD25 subunit of interleukin-2 receptor.

(A) PCA of RNA-seq data from patient samples across sporadic, HIV positive, and endemic subtypes of Burkitt lymphoma. (B) List of coding TFAP4 mutations in Burkitt lymphoma samples from (A). (C) ChIP-sequencing data showing binding of AP4, MYC, and p300, deposition of H3K27ac, and accessible chromatin states in a genomic region containing the Il2ra
locus in activated CD8 T cells. AP4 binding colocalizes with chromatin accessibility within the Il2ra locus and p300 binding, which is predictive of enhancer activity. Y-axes show tag counts in 10^7 mapped reads. (D) Expression of CD25 in adoptively transferred Tfap4−/− OT-I cells compared to co-transferred Tfap4+/+ OT-I cells in congenic recipient mice at different time points after Listeria monocytogenes expressing ovalbumin (Lm-Ova) infection. (E) Mean fluorescence intensity (MFI) of CD25 on Tfap4−/− OT-I cells and Tfap4+/+ OT-I T cells after Lm-Ova infection. Data are representative of three independent experiments. (F) Microarray data showing Il2ra mRNA expression in Tfap4−/− OT-I cells compared to Tfap4+/+ OT-I cells on days four and six post Lm-Ova infection as in (E).
Figure 2.3. TFAP4 is a c-MYC-induced, cell-intrinsic tumor suppressor in developing B cells.

(A) Survival analysis of Eμ-Myc mice crossed to Tfap4+/+, Tfap4+/− or Tfap4−/− mice (Tfap4+/+, n=68, median survival: 134 days; Tfap4+/−, n=50, median survival: 62 days; Tfap4−/−, n=6, median survival: 36 days). (B) Frequencies of surface Ig− Rag1+ VpreB1+ (immature) and Ig+ Rag1− VpreB1− (mature) B cell tumors in Eμ-Myc mice with distinct Tfap4 genotypes (Eμ-Myc Tfap4+/+, n=10; Eμ-Myc Tfap4+/−, n=14; Eμ-Myc Tfap4−/− mice, n=2). (C) Survival of Eμ-Myc mice with specific deletion of the Tfap4 allele in developing B cells using Cd79a^{cre} (Tfap4^{F/+} or Tfap4^{F/F}^{cre}(−), n=59, median survival: 104 days; Tfap4+/+ Cd79a^{cre/+}, n=16, median survival: 113 days; Tfap4^{F/+} Cd79a^{cre/+}, n=17, median survival: 59 days; Tfap4^{F/F} Cd79a^{cre/+}, n=6, median survival: 42 days). (D) Tumorigenesis of Tfap4+/+ or Tfap4−/− BM CD19+ cells retrovirally transduced with mouse c-MYC (T58A) and adoptively transferred into sublethally irradiated CD45.1 mice (Tfap4+/+, n=10, median survival: undefined days; Tfap4−/−, n=12, median survival: 83.5 days; two independent experiments combined). (E) Survival of mice
receiving T\textit{fap}4\textasciitilde{+/+} or T\textit{fap}4\textasciitilde{+/-} BM CD19\textasciitilde{} cells transduced with human p190 \textit{bcr-abl} expressing retrovirus and adoptively transferred into sublethally irradiated CD45.1 mice (T\textit{fap}4\textasciitilde{+/+}, n=9, median survival: 32 days; T\textit{fap}4\textasciitilde{+/-}, n=10, median survival: 19.5 days; two independent experiments combined). (F) Survival of lethally irradiated CD45.1 mice reconstituted with BM cells from C\textgamma{1}-cre \textit{R26Stop}^{\text{LSL-MYC}} x \textit{R26Stop}^{\text{LSL-P110r}} mice of indicated T\textit{fap}4 genotypes following immunization with sheep red blood cells. Data are combined from four independent experiments. (T\textit{fap}4\textasciitilde{+/+}, n=12, median survival: 88 days; T\textit{fap}4\textasciitilde{F/+}, n=14, median survival: 74.5 days; T\textit{fap}4\textasciitilde{F/F}, n=15, median survival: 27 days). **P<0.01, ***P<0.001, by log-rank test adjusted for multiple comparisons where "ns" or absence of asterisks or indicates no statistical significance.
Figure 2.4. AP4 is a cell-intrinsic suppressor in c-MYC-induced tumorigenesis of developing B cells.

(A) A representative gating strategy for pre-transformed pro/pre-B cells in the BM and spleen of Eμ-Myc mice, or mature B and pro/pre-B cell tumor cells, based on surface expression of B220, IgM, and IgD. Transformed mature B cells can be found in the BM (second column), and, similarly, transformed pro/pre-B cells can be found at a high frequency with an almost complete lack of surface Ig+ cells in peripheral lymphoid organs (last column). (B) Representative expression of Rag1 and VpreB1 mRNA, measured by qPCR, in mature B and pro/pre-B (immature) tumor cells. (C) A representative gating strategy for subpopulations of developing B cells in the bone marrow defined as follows: early pro-B (B220+IgM−IgD−c-Kit+CD25−CD24−BP-1−), late pro-B (B220+IgM+IgD−c-Kit−CD25+CD24+BP-1+), large pre-B (B220+IgM+IgD−c-Kit−CD25+CD24+large), small pre-B (B220+IgM+IgD−c-Kit−CD25+CD24+small), immature B (B220+IgM+IgD−), and mature B (B220+IgM+IgD+). (D) Number of developing B cells in populations defined in (C) from 3-week old Eμ-Myc Tfap4+/+(n=7) and Eμ-Myc Tfap4+/−(n=5) mice. EdU (E) and Annexin V (F) staining of developing B cells from bone marrow of 3-week old mice in (D). (G,H) An in vitro survival assay of BM CD19+ pro/pre-B cells of 3-4 week old Eμ-Myc mice with indicated Tfap4 genotypes in an FBS-containing medium without (G) or with 10ng/μl of recombinant IL-7 (H). Data shown are combined from two independent experiments. (I-K) Frequencies of surface Ig− (immature) or Ig+ (mature) B cells from tumor models in Figure 2 C-E, respectively. (I) Eμ-Myc Tfap4+/+, n=8; Eμ-Myc Tfap4+/+ Cd79αcre, n=5; Eμ-Myc Tfap4+/+ Cd79αcre n=8; and Eμ-Myc Tfap4+/+ Cd79αcre mice, n=1. (J) Tfap4+/−+RV-MYC, n=8; no Tfap4+/− donor derived tumors developed in this model. (K) Tfap4+/−+RV-P190 bcr-abl, n=9; Tfap4+/−+RV-P190 bcr-abl, n=9. **P<0.01, ****P<0.0001 by one-way ANOVA followed by Tukey’s multiple comparisons test (D-E).
**Figure 2.5. AP4 is a cell-intrinsic tumor suppressor.**

(A) A scheme for competitive mixed BM chimera experiments. BM cells from 3-week old mice of indicated genotypes were mixed at a 1:1 ratio and transferred into lethally irradiated CD45.1 recipients. (B) Analysis of CD45.2/2 tumor free survival of mice in (A) (CD45.2 Eμ-Myc Ttap4+/+ recipients, n=15; CD45.2 Eμ-Myc Ttap4+/– recipients, n=16). (C), Analysis of overall survival of mice in (A) (Eμ-Myc Ttap4+/+, n=17; Eμ-Myc Ttap4+/–, n=16). (D) Frequencies of CD45.2 or CD45.1/2 B cell tumors in mice from (A) (Eμ-Myc Ttap4+/+, n=12; Eμ-Myc Ttap4+/–, n=16). Data are combined from two independent experiments in B, C, and D. **P<0.01, ****P<0.0001 by log-rank test (B,C).
Figure 2.6. Direct binding of c-MYC in the Tfad4 locus is essential for tumor suppression in Eμ-Myc mice.

(A) Binding of c-MYC to the Tfad4 locus in Eμ-Myc B cells, CD40L-activated B cells, and activated T cells. Histograms of sequenced tags pulled down with anti-c-MYC or anti-H3K27ac antibody are shown, as well as ATAC-seq histograms for Hardy's fractions B, C, D, and mature splenic B cells from the Immgen database. Y-axes show tag counts in 10^7 mapped reads. (B) Survival of mice with one allele deletion of the 1kb region containing the two c-MYC consensus binding sites in the Tfad4 locus (∆MYCBS1kb), and those harboring one allele with a 32-nucleotide deletion encompassing the 3' MYC binding site (∆MYCBS2). Eμ-Myc Tfad4^+/+, n=28, median survival: 161 days; Eμ-Myc Tfad4^∆MYCBS1kb+, n=11, median survival: 64 days; Eμ-
Myc $Tfap^4\Delta_{MYCBS2/+}$, n=10, median survival: 122 days. (C) Expression of $Tfap4$, $Coro7$, $Dnaja3$, and $Runx1$ in B220+ IgM- pro/pre-B cells from 3-4 week old Eμ-Myc mice with indicated genotypes, prior to tumor development. **$P<0.01$, ***$P<0.001$ by log-rank test, adjusted for multiple comparisons.
Figure 2.7. Low levels of TFAP4 endow interclonal competition of pre-transformed B cell precursors with aberrant c-MYC expression.

(A) Expression of Tfap4 mRNA in pre-transformed B220+IgM− BM (pre-tumor) or B220+ IgM− tumor cells harvested from Eμ-Myc mice with indicated Tfap4 genotypes. Tfap4 mRNA expression measured by qRT-PCR was normalized to Hprt1 expression. (B) Genomic PCR detecting the region of the Tfap4 allele encompassing exons 2-4 in tumor DNA (T) and tail DNA (GL: germline) of each corresponding mouse. A genomic region containing Runx1 located on the same chromosome was used as an internal control. Three out of five lymphomas with low Tfap4 mRNA expression were analyzed by genomic PCR. (C) A diagram showing detection of LOH using Tfap4-mCherry reporter knock-in mice. (D) Top, detection of TFAP4-mCherry fusion protein in peripheral blood B cells from healthy 4-week old Eμ-Myc Tfap4mC/+ and Eμ-Myc Tfap4mC− mice. Bottom, loss of TFAP4-mCherry expression in Eμ-Myc Tfap4mC− tumor B cells with LOH or CD11b+ cells as internal mCherry− control. Numbers next to the legend indicate unique mouse IDs.
Figure 2.8. Continuous AP4 expression is sufficient to suppress transformation of Eμ-Myc AP4-haploinsufficient B cells.

(A) Survival of Tfap4-heterozygous or -homozygous Eμ-Myc mice with B cell-specific overexpression of TFAP4 under the control of the Rosa26 locus (Tfap4F/+ or Tfap4F/F cre−, n=12, median survival: 114 days; Tfap4F/+ Rosa26LSL-AP4/+ Cd79aicre/+, n=4, median survival: 151 days; Tfap4F/+ Cd79aicre/+, n=6, median survival: 63 days; Tfap4F/F Rosa26LSL-AP4/+ Cd79aicre/+, n=5, median survival: 58 days; Tfap4F/F Cd79aicre/+, n=17, median survival: 44 days). The top panel shows a schematic for a conditional activation allele expressing TFAP4 with a mCherry reporter.

(B) Generation of a degradation resistant mutant of AP4 by substituting alanine for serine at position 139 (S139A). Stability of the mutant was assessed in activated Tfap4−/− CD8 T cells reconstituted with WT AP4 or AP4(S139A) and cultured in the presence of cycloheximide. (C) Expression of TFAP4 detected by mCherry fluorescence in peripheral blood B cells prior to tumor development, but its loss in developed tumor cells in Eμ-Myc Tfap4F/+ mice. Data are representative of two Tfap4F/+ Rosa26LSL-AP4/+ Cd79aicre/+ mice and three Tfap4F/F Rosa26LSL-AP4/+ Cd79aicre/+ mice. *P<0.05 by log-rank test, adjusted for multiple comparisons (A).
Figure 2.9. Gene expression changes in Eμ-Myc Tfp4+/+, Eμ-Myc Tfp4+/- and Eμ-Myc Tfp4-/- pre-transformed and transformed pro/pre-B cells.

(A) Usage of variable gene segments of Igh in B220+ IgM+ BM cells sorted from 3-4-week-old Eμ-Myc Tfp4+/+ (non-Tg) (n=3), Eμ-Myc Tfp4+/- (n=5) and Eμ-Myc Tfp4-/- (n=5) mice. A fraction of individual Ighv reads over total read counts for all Ighv within each sample are shown where each color represents expressed mRNA for distinct Ighv. (B) Principal component analysis
(PCA) of RNA-seq data in (A), following filtering of genes expressed at RPKM>2 in any given sample. (C-D) A z-score heatmap (C) and relative transcript frequencies (D) for anti-apoptosis-, cell cycle-, pro-apoptosis- and senescence-associated genes in Eµ-Myc Tfap4+/+ and Eµ-Myc Tfap4+/- BM B220+ IgM pre-transformed cells (Eµ-Myc Tfap4+/+, n=5; Eµ-Myc Tfap4+/-, n=5 mice). (E) PCA of RNA-seq data of Eµ-Myc Tfap4+/+ (n=4), Eµ-Myc Tfap4+/- (n=5), Eµ-Myc Tfap4+/- with LOH (n=5), and Eµ-Myc Tfap4−/− (n=2) pro/pre-B tumor cells. (F) A z-score heatmap showing expression of differentially expressed genes defined in Figure 4B by tumor pro/pre-B cells from mice with indicated Tfap4 genotypes. (G) GSEA of genes in Figure 4D using selected signatures enriched in indicated clusters.
Figure 4.

A

Expression ratio (Log2(Heq/WT))

P-value

B

Eμ-Myc pre-transformed

C

Eμ-Myc Tlpat4−/−
Eμ-Myc Tlpat4−/−

mRNA/RPKM

C366
Cacna2d1
H2-DMB2
S100as6
Msr-a1
Sod4a3
Erg
Kcnk1

mRNA/RPKM

C366
Cacna2d1
H2-DMB2
S100as6
Msr-a1
Sod4a3

D

Mouse pro-B tumor

BL sporadic

BL Endemic

Myc targets v1

<0.001

Myc targets v2

0.001-0.009

0.01-0.049

0.05-0.1

>0.1

E2F targets

Hallmark p53 pathway

Net Enrichment Score

1.5

2.5

3.5

E

Myc targets v1

Nes= 2.22

Padj = 1e-10

Myc targets v1

Nes= 1.64

Padj = 8.2e-5

Myc targets v1

Nes= 2.77

Padj = 1.0e-10

F

Eμ-Myc pro-B tumors

Cluster

I

II

III

IV

V

VI

VII

VIII

IX

X

row min

row max
Figure 2.10. Gene expression changes associated with c-MYC target gene signature are enriched in mouse Eμ-Myc Tfap4<sup>+/−</sup> tumors and TFAP4-mutated BL patient samples.

(A) A volcano plot showing gene expression changes between pre-transformed Eμ-Myc Tfap4<sup>+/−</sup> and Eμ-Myc Tfap4<sup>+/−</sup> B220<sup>+</sup> IgM<sup>−</sup> BM cells. Log<sub>2</sub>-fold change and P-values were calculated using a limma R package through a Phantasus application. Red dashed lines indicate cut-off thresholds for fold-change >1.8-fold and P<0.05. The top five most differentially expressed genes are labeled on the graph. (B) Annotation and a z-score heat map of genes differentially expressed by >1.8-fold in (A). Genes bound by Tfap4 (red) or co-bound by Tfap4 and Myc (blue) determined by ChIP-seq are indicated by corresponding colors. (C) Expression of differentially expressed genes defined in (B) by pre-transformed pro/pre-B cells from mice with indicated Tfap4 genotypes. Top: upregulated genes; bottom: downregulated genes. (D) GSEA of Tfap4-deficient/mutated vs. Tfap4-WT/non-mutated mouse tumor B cells or patient Burkitt Lymphoma samples relative to indicated gene set signatures. Dot sizes, colors, and color gradients indicate the net enrichment score, positive or negative enrichment, and adjusted p-value, respectively. (E) GSEA illustrating the selective upregulation of MYC target genes in Tfap4-deficient/haploinsufficient mouse tumors (top) and TFAP4-mutated human sporadic (middle) or endemic (bottom) Burkitt Lymphoma samples. (F) K-means clustering (10 clusters) of genes expressed in pro/pre-B tumor cells from Eμ-Myc mice with indicated Tfap4 genotypes.
Figure 2.11. Tfap4+/- Eμ-Myc pro/pre-B tumor cells harbor comparable overall mutation burden but frequent oncogenic Kras mutations.

(A) Usage of variable gene segments of IgH in B220+ IgM− tumor B cells sorted from Eμ-Myc Tfap4+/+ (n=5), Eμ-Myc Tfap4+/− (n=5), Eμ-Myc Tfap4+/− LOH (n=5), and Eμ-Myc Tfap4−/− (n=2) mice. A fraction of individual Ighv reads over total read counts for all Ighv within each sample are shown where each color represents expressed mRNA for distinct Ighv. (B) Frequencies of coding mutations of pro/pre-B tumor cells from three Eμ-Myc Tfap4+/+, three Eμ-Myc Tfap4+/− and combined three Eμ-Myc Tfap4+/− mice with LOH and one Eμ-Myc Tfap4−/− mouse determined by whole exome sequencing. (C) List of coding mutations that were detected at the variant allele frequencies ≥10%. (D) Frequencies of Kras mutations in pro/pre-B tumor cells of
indicated genotypes. Mutations found in Eμ-Myc Tjap4+/– tumor cells are italicized. * indicates a mutation detected in one out of six sequenced clones.
Figure 2.12. *ERG* is upregulated in B-ALL compared to other human cancers.

(A-D) Violin plots of log2 median-centered *ERG* expression determined by microarray-based gene expression profiling of various cancer types in indicated studies. Bladder urothelial carcinoma (BL), breast adenocarcinoma (BR), colorectal adenocarcinoma (CR), prostate adenocarcinoma (PR), pancreatic adenocarcinoma (PA), endometrial adenocarcinoma (EA), ovarian adenocarcinoma (OV), pleural mesothelioma (ME), renal cell carcinoma (RE), lung adenocarcinoma (LU), diffuse large B-cell lymphoma (DLBCL). Data presented here were obtained and analyzed through the Oncomine database (www.oncomine.org, Thermo Fisher Scientific, Ann Arbor, MI). (E) A violin plot of *ERG* expression in pediatric B-ALL and T-ALL, normalized to *GAPDH* from RNA-seq data from the TARGET database. (F) *ERG* expression in a T-ALL sample with an E57K AP4 mutation and an LMO2-LYL1 translocation (red) and
control T-ALL samples of LMO2-LYL1 and LMO1_LMO2 subtypes without a somatic AP4 mutation (black). Data are shown with a mean and a 95% confidence interval. (G) List of samples in (F) with the subtype and sample identifiers. The first sample has E57K AP4 mutation. RNA-seq data were obtained from the TARGET website and is also available in dbGaP under accession number phs000218. (H) Representative flow cytometry plots of CD19-enriched BM cells from WT, Tfap4<sup>+/−</sup>, Tfap4<sup>+/−</sup>Erg<sup>+/−</sup> and Erg<sup>+/−</sup> mice ~24hrs post infection with MYC-GFP and MYC-Thy1.1 viruses.
Figure 2.13. Repression of Erg by TFAP4 in MYC+ developing B cells is required for tumor suppression.

(A-B) Expression of Erg in pre-transformed B220+ IgM− pro/pre-B cells from Eμ-Myc Tβap4+/+ and Eμ-Myc Tβap4−/− mice (A) and pro/pre-B tumor cells from Eμ-Myc Tβap4+/+, Eμ-Myc Tβap4+/−, and Eμ-Myc Tβap4−/− mice (B) as determined by RNA-seq. (C) Colocalization of TFAP4 binding and accessible chromatin in the Erg locus (red vertical lines indicate exons and black horizontal lines introns) in developing B cells; pre-B (B220+ IgM−); pro/pre-B (CD19+); Fr.BC (CD19+ IgM− CD43+ CD24+); Fr.D (CD19+ IgM− CD43− B220+). Y-axes show tag counts in 10^7 mapped reads. An GEO accession number for each dataset from NCBI GEO is indicated. (D) Expression of Tβap4, Myc, and Erg mRNA in developing B cells from the Immgen database. (E) Sorting strategy for MYC+ and MYC− pro-B cells in MYC+/MYC-GFP protein reporter mice and expression of Erg mRNA in MYC+ vs. MYC− pro-B cells in MYC−MYC-GFP protein reporter mice.
mRNA expression measured by qRT-PCR was normalized to Hprt1. (F) Expression of Erg in MYC+ pro-B cells from Tfap4+/+, Tfap4+/− and Tfap4−/− mice harboring the MYC-GFP reporter. (G) Expression of Erg in CD19+ IgM− pro/pre-B cells from Eμ-Myc− Tfap4+/+ (non-Tg), Eμ-Myc Tfap4+/+, Tfap4+/− and Tfap4−/− mice prior to tumor development. (H) Survival of Tfap4-haploinsufficient Eμ-Myc mice with restricted Erg expression (Cd79aicre/+ n=20, median survival: 131 days; ErgF/+ Cd79aicre/+, n=8, median survival: 93.5 days; Tfap4F/+ ErgF/+ Cd79aicre/+ n=17, median survival: 94 days; Tfap4F/+ Cd79aicre/+, n=9, median survival: 62 days). (I) Frequencies of immature (Rag1 and/or VpreB1 expressing and surface Ig−) and mature (Rag1 and VpreB1 not-expressing and surface Ig− or Ig+) B cell tumors in Eμ-Myc mice with indicated genotypes (Eμ-Myc Tfap4F/+ Cd79aicre/+, n=3; Eμ-Myc Tfap4F/+ ErgF/+ Cd79aicre/+, n=6). *P<0.05, **P<0.01, ****P<0.0001 by unpaired t-test (A and E), one-way ANOVA with Tukey's post-hoc test (B, F-G), and log-rank test, adjusted for multiple comparisons (H) where "ns" or absence of asterisks or indicates no statistical significance.
Figure 2.14. *Erg* is required for B cell development at the pro-B to pre-B cell transition. (A) Representative flow cytometry plots showing expression of B220 and CD43 by CD11b− gated BM cells in *Erg*+/+ (n=6), *Cd79a*+/+ (n=4), *Erg*+/+ *Cd79a*icre+/+ (n=10), and *Erg*FF *Cd79a*icre+/+ (n=3) mice. (B) Quantification of data in (A). (C) Representative flow cytometry plots showing CD24 and BP-1 expression by B220+ CD43+ BM cells and IgM and IgD expression by B220+ CD43− BM cells of indicated mice as in (A). Numbers in plots show frequencies of gated cells in the corresponding parental cell population. (D) Quantification of data in (C). Fr.A: CD24+ BP-1−; Fr.B: CD24+ BP-1−; Fr.C: CD24+ BP-1+; Fr.D: IgM− IgD−; Fr.E: IgM+ IgD−; Fr.F: IgM+/IgD+. (E) Total BM cellularity of indicated mouse strains. Data are pooled from three independent experiments. *P<0.05 **P<0.01, ***P<0.001 ****P<0.0001 by one-way (E) or two-way (B,D) ANOVA and Tukey’s post-hoc test.
Figure 2.15. TFAP4-mediated restriction of Erg is required for coupling of c-MYC-dependent proliferation and differentiation.

(A) A scheme for in vivo assessment of expansion and differentiation of Tfap4+/+, Tfap4+/–, Tfap4+/– Erg+/– and Erg+/– CD19+ pro/pre B cells transduced with distinct retrovirus encoding mouse c-MYC (T58A). Cells from 6-14 week-old mice (age matched within the experimental groups) of indicated genotypes were enriched for CD19+ pro/pre-B cells, infected with MYC-ires-GFP-expressing or MYC-ires-Thy1.1-expressing retrovirus, respectively, and co-transferred into sub-lethally irradiated CD45.1 recipients at a 1:1 ratio. Three weeks later, mice were euthanized, and proportions of each transduced cell population and their differentiation status were analyzed. (B) Representative flow cytometry plots showing frequencies of Tfap4+/+ (WT) and Tfap4+/– (left), WT and Tfap4+/– Erg+/– (middle), and WT and Erg+/– (right) donor-derived B220+ cells in the BM of recipient mice 20 days after transfer. (C) Ratios of c-MYC-expressing Tfap4+/+ to WT, Tfap4+/– Erg+/– to WT, and Erg+/– to WT cells in the BM of recipient mice 20 days after transfer. (D) Percentages of surface IgM+ transduced Tfap4+/+ B220+ cells compared to Tfap4+/– B220+ cells in the same recipient mouse. (E) Percentages of CD45.2+ cells in the BM of mice in (A). (F) Percentages of surface IgM+ transduced Tfap4+/– B220+ cells compared to...
Tfap4^{+/−} Erg^{+/−} or Erg^{+/−} B220^{+} cells. (WT + Tfap4^{+/−} n=21; WT + Tfap4^{+/−} Erg^{+/−} n= 23, WT + Erg^{+/−} n= 9, three independent experiments combined). *P<0.05 by paired t-test, **P<0.01 by paired t-test (C, D), ***P<0.01, ****P<0.0001 by Kruskal-Wallis test with Dunn’s multiple comparison test (F).
Figure 2.16. Retroviral expression of ERG and c-MYC in Tfap4<sup>+/–</sup> BM B cells accelerates tumor formation.

(A) Tumorigenesis by Tfap4<sup>+/–</sup> BM CD19<sup>+</sup> cells that were retrovirally transduced with c-MYC only or c-MYC+ERG and adoptively transferred into sublethally irradiated CD45.1 mice (n=21 for both groups, five independent experiments combined, \(P=0.1316\)).

(B) Tumorigenesis by Tfap4<sup>+/+</sup> BM CD19<sup>+</sup> cells that were retrovirally transduced with c-MYC only or c-MYC+ERG, and adoptively transferred into sublethally irradiated CD45.1 mice (RV-MYC + RV-empty, n=8; RV-MYC + RV-ERG, n=7, two independent experiments combined, \(P=0.8815\)). **\(P<0.01\) by log-rank test.
**Figure 2.17. TFAP4 is a positive prognosis factor in B-ALL.**

(A) A plot showing normalized MYC and TFAP4 expression from 104 B-ALL patient samples in the TARGET phase 2 study. (B-C) Survival analysis of deceased patients with high versus low TFAP4 expression among patients with high MYC expression (z-score > 2) (TFAP4<sub>lo</sub> median survival: 1413 days; TFAP4<sub>hi</sub> median survival: undefined). (D) Normalized ERG expression from B-ALL patient samples in (B).
Chapter 3:

Discussion and future directions
3.1 Discussion and future directions

MYC function in normal cells and contribution to cancer development have been the subject of much research over the last four decades, resulting in numerous studies that have shown that MYC has a hand in almost every aspect of cellular function, in addition to driving proliferation. In this dissertation work, we sought to uncover novel MYC-driven tumor-suppressive mechanisms. Our approach focused on a subset of immune cells—B lymphocytes because c-MYC-driven cell proliferation is a hallmark of their function and necessary for effective immune responses. Additionally, lymphocytes are thought to be cancer-prone due to their rapid progression through rounds of cell cycling and endogenous gene rearrangements. However, the incidence of lymphocyte-derived cancers in the population suggests they may concurrently engage an active tumor-suppressive program. Here we have uncovered such a c-MYC-initiated feedback loop that protects developing MYC\textsuperscript{hi} B lymphocytes from transformation. A c-MYC downstream factor, TFAP4, maintains this feedback, in part, by restricting co-expression of protooncogenes c-Myc and Erg, each of which is essential for B lymphocyte development.

We have previously shown that TFAP4 is induced by c-MYC in activated lymphocytes, supporting their proliferation through the maintenance of expression of many c-MYC target genes (1, 2). Others have reported that TFAP4 contributes to intestinal tumor formation in mice (3) and is associated with poor patient survival in some cancers (4-7). Hence, while our findings are somewhat surprising, the tumor-suppressive role of TFAP4 in lymphocytes could be cell-type specific. A similar specificity has been reported for the cell cycle regulator CDK4 between colon cancer and lymphomagenesis (8). Thus, lymphocytes might have evolved to temporally
restrict their dependency on c-MYC during proliferation by utilizing an alternative program for continued proliferation. An example of such a program could be TFAP4-mediated proliferation observed in activated CD8 T cells and germinal center B cells (1, 2). While in these cells c-MYC and TFAP4 shared half of their target genes, this also suggests that single targets of TFAP4 could be responsible for its tumor-suppressive function as an additional layer of protection.

Indeed, we find that TFAP4-mediated restriction of another protooncogene Erg was necessary to prevent early oncogenesis in MYC-driven B cell malignancy (Figure 3.1). c-MYC and ERG both have essential roles in HSC and B lymphocyte differentiation. In HSCs their antagonistic interplay balances the need for maintenance of self-renewal capacity while allowing sufficient differentiation to support hematopoiesis (9). Thus, co-expression of c-Myc and Erg could lead to decoupling differentiation from the loss of self-renewal capacity, which has been connected to hematopoietic malignancies. Somewhat surprisingly then, ERG is necessary for successful B lymphocyte differentiation from pro-B cell to pre-B cell stage, and expression of c-Myc and its target genes in these progenitors (10, 11). However, we found that Erg and c-MYC were no longer co-expressed at the large pre-B cell stage; Erg was downregulated in these cells while c-MYC persisted. Thus, while Erg is necessary for B cell commitment and initial stages of differentiation, it then needs to be repressed while c-Myc is maintained for differentiation to progress. In agreement with this idea, overexpression of ERG in pro-B cells is sufficient to inhibit downregulation of c-Kit and differentiation into pre-B cells (12). In the presence of insufficient TFAP4 then, c-MYC expressing cells had inappropriately high expression of Erg and maintained their undifferentiated state while actively proliferating, leading to the establishment of immature B cell cancer. As expected then, restriction of deregulated Erg
suppressed the short-term aberrant expansion of pre-transformed \( T_fap^{+/-} \) cells and also delayed the onset of c-MYC-driven B cell malignancy.

Elevated c-MYC expression is insufficient for the transformation of normal cells. Additional mutations and gene expression alterations are required for cells with aberrant c-MYC to persist and become cancerous. Accordingly, primary B-cell leukemia samples with elevated c-MYC also had upregulated \( Erg \) compared to other lymphoid or solid cancer samples (13-16). Here, we show that perturbation of the c-MYC-driven AP4 expression dramatically accelerated tumorigenesis in mice. This process is possibly involved in the transformation of lymphoid cancers with somatic TFAP4 mutations in the DNA binding domain. Despite relatively low frequencies of TFAP4 mutations across all cancer types overall, its deregulation indeed seems deleterious in the presence of tumor-promoting gene alteration such as high c-MYC in Burkitt lymphoma or B-ALL. Moreover, it has been reported that the population-wide frequency of oncogenic chromosomal translocations, often involving \( c-Myc \), found in non-neoplastic cells is higher than the incidence of corresponding malignancies. This is believed to be due to the insufficiency of these translocations to cause malignant transformation by themselves. Thus, activation of tumor-suppressive pathways is necessary to keep the cells with such translocations from becoming clonogenic founders of lymphoid cancers.

Similarly to \( c-Myc \), others have found that ERG overexpression alone is not sufficient for B cell leukemia induction (12). Surprisingly though, we found that ERG overexpression even with elevated c-MYC was also not sufficient for B cell leukemia induction. However, ERG overexpression in \( T_fap^{4-} \)-haploinsufficient pre-B cells slightly accelerated tumor formation. Furthermore, TFAP4 also suppressed c-MYC-driven tumorigenesis in mature B cells, which do
not usually express Erg. These findings together suggest that TFAP4 engages additional tumor-suppressive pathways.

Since we did not detect increased mutational burden in Tfat^{+/−} immature tumors, it is unlikely that TFAP4 regulates genomic stability. However, there could be additional cooperating TFAP4 targets, such as ERG-independent regulators of self-renewal or cell proliferation. Recent studies using single-cell analysis demonstrated that leukemic cells alter the hematopoietic microenvironment for their own outgrowth and suppression of normal hematopoiesis (17, 18). TFAP4-deficient pre-transformed cells could similarly reprogram their bone marrow niche to promote tumorigenesis. Future work will be focused on identifying such additional candidate genes from the gene expression data of pre-transformed and transformed B cells to find additional targetable pathways for therapeutics.

Targeting TFAP4 itself is a potential therapeutic strategy in B cell malignancies with high MYC. Tfat-deficient mice are grossly normal and do not have impaired B lymphocyte differentiation suggesting that TFAP4 is not necessary for normal development. This makes it an attractive therapeutic target that could have few side effects on hematopoiesis and minimize contribution to immunodeficiency that often accompanies chemotherapy. One such approach is to stabilize TFAP4 specifically by inhibition of SCF ubiquitin ligase and thus re-balance MYC and TFAP4 levels as relative levels of c-MYC and AP4 have been reported to determine cell fate between cell death and senescence (19, 20).

Identification of a MYC-driven tumor-suppressive program in lymphocytes reported here unlocks the possibility that there could be additional MYC-driven tumor suppressors in lymphocytes and maybe even other cell types. It would be thus interesting to explore this possibility further by targeting other MYC-driven genes in future studies. Genes induced by c-
MYC in developing and germinal center B cells listed in Figure 2.1D are viable candidate genes for such future studies using CRISPR/Cas9 technology.
3.2 References


Figure 3.1. TFAP4-mediated restriction of protooncogene Erg is necessary for prevention of early oncogenesis in MYC-driven B cell malignancy.

c-MYC induces TFAP4 in pro-B cells with normal and aberrant Myc expression. TFAP4 represses the expression of Erg, thus reducing the stemness of differentiating B cells during MYC-driven proliferation. This feedback mechanism suppresses B lymphocyte tumorigenesis in cells with aberrant MYC by coupling loss of self-renewal capacity with differentiation and proliferation. In the absence of or reduced TFAP4, repression of Erg is lost, leading to maintenance of undifferentiated state in actively proliferating B cell progenitors and accelerated establishment of immature B cell malignancy.