UNIVERSITY OF CALIFORNIA, SAN DIEGO

Post-translational modifications of the E2A gene products and their roles in B lymphocyte development

A Dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

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Biology

by

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The Dissertation of Robert Thomas Teachenor is approved, and it is acceptable in quality and form for publication on microfilm and electronic submission:

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DEDICATION

To my grandmother,

for making the beginning of all of this possible,

and to my parents,

for making me who I am today.

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ABSTRACT OF THE DISSERTATION

Post-translational modifications of the E2A gene products

and their roles in B lymphocyte development

by

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E-proteins constitute a highly conserved family of helix-loop-helix proteins that modulate the developmental progression of a wide variety of developmental pathways. Prominent among the E-proteins are the E2A gene products, E12 and E47. E2A proteins play a critical role throughout B cell development, including specification, commitment and developmental progression. How the activity of E2A proteins is regulated has been a topic of intense investigation during the past two decades. However, previous studies have been mostly focused on a distinct class of helix-loop-helix proteins, named the Id proteins. Here we have examined another level of regulation involving the role of E47 phosphorylated serine residues in B-lineage development. Specifically, we have examined the role of phosphorylated residues in the E2A transactivation domains as well as a putative AKT substrate site in E47. Replacement of E2A phosphorylated serine residues in the E2A transactivation domains modestly but significantly affected B cell development. On the other hand, mutation of the E47 AKT substrate site in the mouse

germ-line substrate site did not grossly affect B cell development neither in the bone marrow nor in the peripheral lymphoid organs. Finally, we found that whereas depletion of Id4 only modestly interfered with loss-of-PTEN mediated lymphomagenesis, forced E47 expression suppressed the development of lymphoma in PTEN-deficient mice. We propose a model in which the PI3K-AKT axis and E47 are linked but that AKT acts on multiple functionally redundant components of the E-protein machinery in order to modulate developmental progression and cell growth.

I.

General Introduction

Early hematopoiesis

The entire spectrum of hematopoietic cells arises from a common precursor cell, known as the hematopoietic stem cell (HSC). HSCs are characterized by their ability to self-renew and to remain pluripotent (Spangrude, 1989). During early hematopoiesis LT-HSCs give rise to multipotent progenitor cells (MPPs). MPPs, in turn, develop into erythroid and megakaryocytic progeny. Erythroid and megakaryocytes develop from an intermediate cell type named the premegakaryocytic/erythroid progenitor (PreMegE) (Pronk et al., 2007). The adaptive and innate immune system, arise from the lymphoid-primed multipotent progenitor compartment (LMPP). LMPPs, in turn, give rise to lymphoid as well as macrophage and granulocytic cell progeny (Adolfsson et al., 2005; Lai et al., 2005). Based on the expression of cell surface markers and functional analyses a wide ensemble of intermediate compartments have been identified. Among these are the CLPs, GMPs and early thymic progenitors (ETPs). The CLPs have the ability to differentiate into B, T or NK cells (Kondo et al., 1997). GMPs have the potential to give rise to the granulocytic, neutrophilic and macrophage compartments. ETPs are the immediate precursors of committed T-lineage cells.

Developmental progression of B-lineage cells

The developmental progression of B-lineage cells follows a multi-step process that begins at the CLP cell stage. The CLP compartment can be segregated into the Ly6D- and Ly6D+ stages. The Ly6D- compartment can give rise to B, T and NK cells. Thus, this compartment is not yet committed to the B cell lineage. On the other hand, within the Ly6D+ compartment specification to the B cell lineage begins (Inlay et al.,

2009). B cell development can be defined on the basis of the rearrangement status of the immunoglobulin heavy (*IgH*) and light chain loci. At the pro-B cell stage, *IgH* DJ rearrangement is initiated prior to that of VDJ DNA recombination. Upon the assembly of an in-frame VDJ gene joint a pre-BCR is expressed which antagonizes the expression of the RAG1 and RAG2 genes. The pre-BCR also induces the expansion of the pro-B cell compartment. Subsequently the expansion phase is followed by cell cycle arrest. In resting G1 small pre-B cells, *RAG* gene expression is re-activated to initiate Ig light chain gene rearrangement (Bassing et al., 2002; Rooney et al., 2004). In the presence of auto-reactivity, Ig light chain DNA rearrangement will continue and replace primary *Igκ* VJ rearrangements, producing BCRs that lack self-reactivity (Quong et al., 2004). Once a BCR is expressed that lacks auto-reactivity, tonic signaling by the BCR suppresses *RAG1* and *RAG2* transcription (Manz et al., 1988). Next immature-B cells leave the bone marrow and differentiate into mature-B lineage cells. Once resident in the spleen or lymph nodes mature-B cells, upon interacting with pathogens, undergo class switch recombination, somatic hypermutation and developmentally progress into either plasma or memory B cells.

Developmental progression of T-lineage cells

Thymocyte development has been defined based on the expression of two cell surface markers, named CD4 and CD8, and the status of TCR gene rearrangements (Rothenberg et al., 2008). T cells arise from early thymocyte progenitors that lack CD4 and CD8 expression. This compartment is also termed as the double negative population (DN). The DN compartment can be further segregated into four subsets that are based on

the expression of cell surface proteins, named CD25 and CD44. The DN1 (CD44+CD25-) stage comprises the ETPs as well as dendritic and B cells. T cell specification begins within the DN2 (CD44+CD25+) compartment, but commitment is only ensured upon developmental progression into the DN3 stage (CD44-CD25+). The rearrangement of the TCR beta locus is initiated and completed at the DN3 cell stage. The generation of an in-frame TCR beta VDJ locus rearrangement leads to the expression of a pre-TCR complex. Committed T cells that traverse through the pre-TCR checkpoint expand and differentiate into the CD4+CD8+ (DP) compartment. Only cells that express a pre-TCR complex will differentiate and proliferate into the DP cell stage. Subsequently, DP cells developmentally progress into either CD4 or CD8 single positive (SP) cells (David-Fung et al., 2006; Carpenter et al., 2010; Rothenberg et al., 2008).

Helix-loop-helix proteins

Specification, commitment, expansion and developmental progression of B cells is dependent on transcriptional regulators. Prominent among these are the E2A, FOX1, EBF, Bcl11A and Pax5 proteins (Schebesta et al., 2002). The E2A locus encodes for two proteins, named E12 and E47. E12 and E47 are generated through alternative splicing of two exons encoding for the DNA binding and dimerization domains. E12 and E47 are members of a class of helix-loop-helix (HLH) proteins, termed E-proteins. E proteins comprise a class of basic helix-loop-Helix (bHLH) protein containing two activation domains, a basic region responsible for DNA-binding, and a dimerization domain known as the HLH domain. In mammals, four E-proteins exist, including the E2A proteins, E12 and E47 as well as HEB and E2-2 (Murre, 2005). HEB and E2-2 are represented by

distinct isoforms generated through alternative initiation of transcription (Wang et al., 2006; Kolligs et al., 2002). All E proteins bind as a dimer to the canonical E-box sequence CANNTG, where N represents any nucleotide. These sequences are present in a wide variety of tissue-specific enhancers. E-proteins activate or repress down-stream target gene expression. They contain a HLH dimerization domain, a basic DNA binding region, positioned N-terminal to the HLH region as well as two transactivation domains, named AD1 and AD2. E-proteins have the ability to interact with other HLH proteins to form either homodimers or heterodimers (Murre et al., 1989).

Regulating the DNA-binding activity of the E proteins is another class of HLH protein, the Id proteins, or Class V HLH proteins. Id proteins are also known as "inhibitors of differentiation" or "inhibitors of DNA-binding." As they lack a basic region, they have no capacity to bind to DNA, and their method of action is to hetero-dimerize with the E proteins, preventing their DNA-binding, as E proteins can only bind to DNA as a dimer. They act at almost every stage of lymphocyte development to promote commitment, expansion, survival and developmental progression of maturing lymphocytes (Benezra et al., 1990).

Lineage-specific HLH proteins

E-proteins are frequently referred to as Class I HLH proteins. Other relevant bHLH subsets include the class II and III HLH proteins, which are typically tissue specific transcription factors. Class II HLH proteins hetero-dimerize with E-proteins to regulate tissue-specific development in erythroid, muscle and the neuronal cell lineages. Prominent among these proteins are MyoD, myf5, MRF4, and myogenin that play critical

roles in myogenesis (Braun et al., 2000); (Rhodes et al., 1989; Wright et al., 1989). Within the context of neuronal development, NeuroD and Neurogenin are critical effectors (Miyata et al., 1999; Ma et al., 1996). The bHLH protein TAL1 plays an important role in erythrocyte development (Hsu et al., 1994).

Class III bHLH proteins include the myc family of transcription factors, well-known for their roles in the regulation of growth control (Zimmerman et al., 1986). The myc family of proteins is in turn regulated by Mad and Max gene products that comprise the Class IV family of bHLH proteins. Also of note are Hairy and Enhancer of Split, Class VI bHLH proteins characterized by the presence of a proline in their basic region.

The transcriptional control of hematopoiesis

Mice carrying mutations for a homozygous deletion of E2A typically have a high rate of post-natal death and those surviving to adulthood have a high incidence of thymic lymphomas. Other hematopoietic lineages, the granulocyte, macrophage, and erythroid lineages, are intact and display normal phenotypic characteristics (Bain et al., 1997). The E2A proteins are required in the HSC compartment where they act to maintain the HSC cell population (Semerad et al., 2010). They next act to promote the developmental progression of HSCs into LMPPs. In the CLPs they promote the differentiation into the pre-pro-B cell compartment (Dias et al., 2009). E2A acts in concert in the LMPP and CLP compartments with PU.1 and Ikaros, to promote developmental maturation. E2A-deficient mice exhibit a significant block at CLP cell stage and a complete block in the pro-B cell compartment (Bain et al., 1994).

As discussed above B cell development in E2A-ablated mice is blocked prior to the pro-B cell stage and the initiation of *IgH* DJ gene rearrangement. The majority of B cell specific genes involved in early B cell development, including RAG1, lambda 5, V-preB, Iμ, Igα, and Pax5, are not expressed in E2A null mutants (Bain et al., 1994). FOXO1- and EBF-deficient mice exhibit a similar block (Lin et al., 1994; Dengrel et al., 2009). Genome-wide studies have recently shown that E2A acts upstream of FOXO-1 and EBF to initiate B cell development at the pre-pro-B cell stage (Lin et al., 2010). E2A, FOXO1 and EBF then act in concert to induce the expression of PAX5 to promote commitment to the B cell lineage (Lin et al., 2010). Furthermore, forced EBF expression in E2A-deficient progenitors rescues the developmental arrest observed at the pre-pro-B cell stage (Seet et al., 2007). Collectively, these observations indicate that the block in B cell development in E2A-deficient mice is caused by the lack of FOXO-1 and EBF expression in the CLP cell stage. The E2A proteins also play an important role in enforcing maintenance of the B cell lineage, since conditional deletion of the E2A locus resulted in an inability to maintain the expression of EBF and PAX5 at the pro-B cell stage (Kwon et al., 2008). Furthermore, the arrest at the pre-pro-B cell stage in E2A-deficient bone marrow was also rescued by forced PAX5 expression (Kwon et al., 2008). As mice carrying one copy of E2A have a 50% reduction in the overall number of B cells, this implies that E2A levels are highly important in regulating development of the B lineage (Quong et al., 2004). Finally, a recent global network has revealed a wide spectrum of genes at the pro-B cell stage to be modulated by the combined activities of E2A, EBF and FOXO1, indicating that it is the combined activities of E2A, EBF and Pax5 that promote the B cell fate (Lin et al., 2010).

HEB and E2-2 similarly have roles in B cell development, albeit less critical than that of E2A. Mice lacking a functional copy of either gene typically die within 1-3 weeks of birth, but have a 50% reduction in overall B cell numbers (Zhuang et al., 1996). Of interest is the ability of HEB to partially restore B cell development in a knock-in mouse model carrying HEB alleles in the place of E2A (Zhuang et al., 1998). These findings and those previously mentioned suggest that the expression levels of E proteins are critical at several stages of B cell differentiation.

E2A and B cell maturation

Recent studies have provided additional insights into the role of E2A during the developmental progression of B cells. Briefly, upon the assembly of a pre-BCR, E47 protein levels decrease temporarily (Quong et al., 2004). On the other hand, Id3 levels are increased upon pre-BCR mediated signaling. In small pre-B cells, E47 levels are elevated again to promote light chain gene rearrangement. E47 abundance remains high at the immature B cell stage to promote receptor revision (Quong et al., 2004). Upon developing into the mature-B cells E47 levels decline. In summary, upon exposure to auto-antigens, E47 abundance remains high in order to activate Ig light chain gene rearrangement. The expression of a BCR that lacks self-reactivity then results in a decline of E47 abundance, antagonizing continued Ig light chain rearrangement and promoting the differentiation towards the mature B cell stage.

E12 and E47 activate light chain gene rearrangement by directly occupying binding sites located across the Ig light chain gene enhancers as demonstrated by mutation of the E2A binding sites in mutant mice (Inlay et al., 2006). These data

provided direct and unambiguous evidence for an important function of E-box sites in Ig kappa enhancer function. Furthermore, overexpression of E47 or E12 activates immunoglobulin light chain locus accessibility to the VDJ recombinase in 293 cells and E2A-deficient pre-B cells exhibit a significant block in their ability to promote light chain gene recombination (Romanow et al., 1998). Finally, receptor revision is significantly perturbed in E12 and E47 null mutant mice (Quong et al., 2004). E2A abundance rapidly increases to very high levels upon activation in peripheral B cells to promote class switch recombination (CSR) of the Ig isotype of activated B cells (Quong et al., 1999). Abrogation of the secondary Ig isotype phenotype can be accomplished by ectopic expression of Id3 in B cells undergoing activation in cell culture (Quong et al., 1999). The defect in CSR in cells expressing Id3 was caused by the absence of Activation Induced cytosine Deaminase (AID) expression, suggesting that E-proteins directly regulate the expression of AID (Sayegh et al., 2003).

E-proteins and thymocyte development

E2A and HEB also play important roles in T cell development. E2A abundance is high in the DN2 and DN3 compartments where they induce TCR beta V(D)J gene rearrangement (Agata et al., 2004). The E2A proteins also activate the expression of a subset of genes encoding for components involved in the Notch- and pre-TCR signaling (Ikawa et al., 2008). Finally, they act to suppress the proliferation of DN3 cells (Engel et al., 2006). E47 abundance remains high in DN4 but declines upon developing into the ISP compartment. During the positive selection process they decline further to barely detectable levels (Engel et al., 2004). The expression pattern of the E-protein antagonist

named Id3 is quite distinct. Id3 abundance increases during beta- and positive-selection (Bain et al., 2002). High levels of E2A suppress developmental progression while declining levels of E2A and higher levels of Id3 promote progression through the pre-TCR and TCR checkpoints (Murre, 2008).

The role of E2A in cell cycle progression and lymphomagenesis

In addition to their key roles in developmental progression, the E2A proteins also control cell cycle progression and act as tumor suppressors (Engel and Murre, 2004). Forced E47 expression in E2A-deficient lymphoma cell lines has been shown to inhibit cell growth. E47 acts to directly regulate p21, p27Kip, Cdk6 and cyclin D3 expression indicating that E47 modulates cell cycle progression by regulating the expression of cell cycle effectors (Murre, 2005; Schwartz et al., 2006).

E-proteins and the Id gene products are closely involved with the development of lymphoma. E2A and E47 deficient mice readily develop lymphoid malignancies. Furthermore, ectopic expression of either Id1 or Id2 in T cell progenitors rapidly leads to lymphomagenesis (Kim et al., 1999; Morrow et al., 1999). Since the E-proteins modulate the transcription of genes associated with cell cycle progression such as p21, p27Kip, cdk6 and cyclin D3, it is generally assumed that the Id proteins control cell cycle progression by regulating the DNA binding activity of E-proteins, which then in turn directly or indirectly, modulate the expression of cell cycle regulators (Engel and Murre, 2004; Schwartz et al., 2006).

In humans, a substantial proportion of patients associated with human acute T lymphoblastic leukemia, bear chromosomal translocations involving the bHLH TAL loci

(Park et al., 1999). Furthermore, elegant studies have shown that forced Tal-1 expression readily leads to the development of lymphoma by antagonizing E-protein mediated transactivation (Palomero et al., 2006). Consequently, it has been suggested that the E2A proteins function as tumor suppressors (Schwartz et al., 2006).

The E2A proteins are also associated with the development of pediatric pro-B and pre-B cell leukemias (Look, 1997; Armstrong and Look, 2005). A proportion of these malignancies are caused, at least in part, by chromosomal translocations in which the E2A bHLH domain is replaced with DNA binding domains derived from either a leucine zipper (HLF) or a homeodomain (Pbx1) containing proteins.

The PI3-kinase signaling pathway

There is now ample evidence that the phosphoinositide 3-kinase (PI3K)-AKT pathway modulates cell growth, metabolism, differentiation, migration, survival, and proliferation of a wide variety of cell types (Engelman et al., 2006; Sinclair et al., 2008). Within the context of hematopoiesis, signaling by PI3K plays critical roles in modulating cell growth, proliferation, and survival of hematopoietic progenitor cells (Yilmaz et al., 2006; Zhang et al., 2006). Misregulation of this signaling pathway can cause several disorders, including leukemia and/or autoimmunity or alternatively immune deficiencies (Di Cristofano et al., 1999; Suzuki et al., 2001).

The PI3K family includes several isoforms that can be divided into three distinct classes. The three classes include class I, class II, and class III. Typically, class IA PI3K is activated by receptor tyrosine kinase associated receptors, including cytokine receptors, co-stimulatory receptors, and antigen receptor mediated signaling (Vanhaesebroeck et al.,

1997; Okkenhaug & Vanhaesebroeck, 2001). The regulatory subunit (p85α, p85β, or p85γ) binds to the catalytic subunit (p110α, p110β, or p110γ) to stabilize and inactivate its kinase activity. When stimulated by growth factors, the YxxM motifs of Receptor Tyrosine Kinases (or other substrate proteins) are phosphorylated, allowing the p85 subunit to dock via its SH2 domain. The P110 subunit is thereby relieved from inhibition and is now recruited to the plasma membrane (Okkenhaug & Vanhaesebroeck, 2001). Upon activation, P110α converts phosphatidylinositol-1,4,5 bisphosphate (PIP2) into phosphatidylinositol-1,3,4,5 trisphosphate (PIP3). PIP3 acts to localize a variety of intracellular enzymes containing a Pleckstrin Homology (PH) domain to the plasma membrane. Prominent among these enzymes are Protein Kinase B (PKB/Akt), Src Homology 5' Inositol Phosphatase 1 (SHIP1), Bruton's Tyrosine Kinase (Btk), and Phosphatidylinositol Dependent Kinase 1 (PDK1). Localization to the plasma membrane brings PDK1 in close proximity to Akt, allowing phosphorylation and activation of Akt by PDK1 (Cully et al., 2006). Akt, in turn, phosphorylates a wide variety of downstream effectors, including the FoxO family of transcription factors (Brunet et al., 1999; Burgering et al., 2003).

Lymphocyte development and PI3K signaling

As aforementioned, development along the B-cell lineage normally follows an ordered progression, typically characterized by distinct molecular markers. While the molecular mechanism is not completely understood, antigen-BCR (B cell receptor) stimulation activates PI3K (Astoul et al., 1999). SYK, a tyrosine kinase, then phosphorylates both the co-receptors CD19 and B-cell PI3K adaptor (BCAP), which in

turn recruit PI3K, causing its activation (Okada et al., 2000). Mice with a deficiency or mutation in p85 or p110 have similar phenotypes, with partial blocks at the pro-B cell stage (Fruman et al., 1999; Suzuki et al., 1998). Deletion of either p85α or p110δ in murine B-cells causes a drastic decrease in IgM-specific antibody-stimulated Akt phosphorylation. These observations suggest that p85α or p110δ are the main downstream components of BCR signaling in B-cells. Additionally in the p85α or p110δ ablated mice, antibody-stimulated proliferation is severely affected (Fruman et al., 1999; Okkenhaug, 2001; Clayton et al., 2002).

Unlike in the phenotype in B-cells, deletion of either P85α or p110δ in murine T-cells does not appear to cause any defect in T-cell development or CD3-stimulated proliferation of mature T-cells. However, LY294002 (a PI3K inhibitor) does inhibit CD3-stimulated proliferation of T-cells (Fruman et al., 1999; Okkenhaug, 2001).

PTEN and the control of hematopoiesis

Phosphatase and Tensin Homologue (PTEN) is the major regulator of PI3K signaling. It acts by removing the phosphate group added by PI3K at the 3' hydroxyl group of phosphatidylinositol 1,3,4,5 trisphosphate. The PTEN phosphatase is inactivated in a wide variety of human cancers, and is common in melanomas, prostate cancers, lung cancers, sporadic glioblastomas, breast tumors, and lymphomas (Suzuki et al., 1998; Cully et al., 2006). The critical role of PTEN has been confirmed in several mouse models using both germline and conditional mutations (Di Cristofano et al., 1999; Vivanco & Sawyers, 2002). Mice having a complete knockout (PTEN-/-) fail to develop beyond E7.5 to E9.5 (depending on the type of deletion used) day of embryonic

development, typically because of failure of fusion of the chorion and allantois layers of the embryo (Suzuki et al., 1998). Mice having one allele of PTEN (PTEN+/-) readily develop carcinomas in thyroid, lymphoid, breast, colon, and prostate tissues (Suzuki et al., 1998; Di Cristofano et al., 1999). The mechanisms of these carcinomas have yet to be elucidated.

PI3K signaling has been implicated in the proliferation and survival of multiple cell types. Acute deletion of PTEN in the hematopoietic stem cells (HSCs) of adult mice causes a rapid increase in the proliferation of HSCs, followed by a reduction in the overall size of the stem cell pool. PTEN-ablated mice similarly develop fatal myeloproliferative disease and T-cell lymphomas (Yilmaz et al., 2006; Zhang et al., 2006). Conditional deletion of PTEN via Cre recombinase in B-cells results in preferential generation of marginal zone B-cells over follicular B-cells as well as creating hyper-IgM syndrome, as proper PI3K signaling is important for CSR (Omori et al., 2006; Anzelon et al., 2003). In early developing T-cells, ablation of PTEN, results in lymphoproliferative disease (Suzuki et al., 1998). Akt-mediated signaling also promotes developmental progression of immature T lineage cells (Webb et al., 2005; Juntilla & Koretzky., 2008).

FoxO transcription factors

A critical effector mediating PI3K signaling involves the FOXO proteins. The FoxO family consists of four transcription factors, FoxO1, FoxO3a, FoxO4, and FoxO6. FoxO1 is important for the proper development of endothelial cells (Furuyama et al., 2004; Hosaka et al., 2004). FOXO3a ablated female mice are sterile due to increased

cycling and eventual burnout of the ovarian follicle (Castrillon et al., 2003; Hosaka et al., 2004). FoxO4 is the least well characterized; little is known of its function, except that it acts as a redundant tumor suppressor gene with FoxO1 and FoxO3a (Paik et al., 2007). FoxO6 is active primarily in neural development (Jacobs et al., 2003; van der Heide et al., 2005).

FoxO transcription factors function as tumor suppressors and are involved in mediating a response to oxidative stress in HSCs (Tothova et al., 2007). The nuclear location and abundance of FOXO proteins is regulated by AKT-mediated phosphorylation. Phosphorylation of FoxO proteins leads to inhibition of their DNA binding activity and expulsion from the nucleus (Brunet et al., 1999).

Post-translational modifications and their roles in regulating the activities of HLH proteins

Previous studies have indicated that the Myc and Max class of HLH proteins (class IV bHLH) is regulated by Casein Kinase II mediated phosphorylation. Signaling by these enzymes interferes with the formation of Max homodimers and thereby promoting the Myc/Max heterodimerization (Berberich & Cole, 1992). Similarly, phosphorylation of E47 by Casein Kinase II blocks the formation of E47 homodimers and facilitates the formation of E47-MyoD heterodimers in developing muscle cells (Johnson et al., 1996). A recent study has demonstrated that the E2A proteins are phosphorylated by p42/p44 ERK Map kinase activity (Nie et al., 2003). Interestingly, it was demonstrated that the ERK-mediated phosphorylation, in conjunction with Notch signaling, resulted in the association of E47 with the E3 ubiquitin ligase SCFSkp2,

followed by proteasome-dependent degradation (Nie et al., 2003). These studies indicated the importance of post-translational modifications in the control of E-protein activity.

Conclusion

It is now well established that the Id proteins are the primary regulators of the activity of E-proteins. Less is known, however, about the roles of post-translational modifications in modulating the localization, dimerization and transcriptional activity of E-proteins. In this thesis we aimed to examine how post-translational modifications affect E2A activity in a physiological setting.

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II.

Phosphoproteomic and biochemical analysis identifies E47 as an AKT substrate

Summary

The PI3K-AKT signaling cascade acts to modulate the activity of multiple effectors to regulate developmental progression and cell growth. Here we demonstrate that the helix-loop-helix protein, E47, is phosphorylated at multiple serines including a residue, in a region containing a highly conserved AKT substrate site. The phosphorylated AKT site is present in E47 and HEB but not in E12 and is highly conserved. However, replacement of the putative AKT substrate site of E47 in the mouse germ-line does not grossly affect B cell development neither in the bone marrow nor in the peripheral lymphoid organs. We propose a model in which the PI3K-AKT axis and E47 are linked but that AKT acts on multiple functionally redundant components of the E-protein machinery in order to modulate developmental progression and cell growth.

Introduction

The phosphoinositide 3-kinase (PI3K)-AKT pathway controls diverse aspects of cell metabolism and cell growth and is activated in a wide spectrum of human cancers (Cully et al., 2006). PI3Ks are activated by growth factor receptor tyrosine kinases (RTKs) (Engelman et al, 2006). Once activated, PI3Ks catalyze at the cell membrane the synthesis of phosphatidylinositol lipid second messengers, including $PI(3,4,5)P_3$ (PIP3). PIP3 then activates a diverse set of targets, including, albeit indirectly the serine-

threonine protein kinase AKT. The levels of PIP3 are tightly regulated and are controlled by a subset of lipid phosphatases, including the phosphatase and tension homologue, PTEN. PTEN has the ability to convert PIP3 back into PIP2 to suppress PI3K-mediated signaling (Cantley et al., 1999).

The PI3K/AKT signaling cascade promotes cell growth through a set of critical targets. The FOXO members are among the best characterized. The human and murine genomes contain four FOXO members, named FOXO1, FOXO3a and FOXO4 and FOXO6, which are closely related to the C. elegans DAF-16 gene product (Biggs et al., 2001, Burgering et al, 2003). The FOXO members are directly phosphorylated by AKT at three conserved residues (Brunet et al., 1999). Phosphorylated FOXO members interact with 14-3-3, resulting in their cytoplasmic localization and subsequent degradation (Burgering et al, 2003).

The PI3K/AKT pathway acts through FOXO proteins to modulate cell cycle progression by regulating cyclin D1 transcription and induction of p27kip1 and p130Rb2 expression (Burgering et al., 2003, Dijkers et al, 2000). FOXO proteins also have recently been demonstrated to act as redundant tumor suppressors in developing T-lineage cells and to mediate stress resistance in hematopoietic stem cells (Paik et al, 2007, Tothova et al, 2007).

PI3K-AKT signaling promotes cell survival by phosphorylating the pro-apoptotic factor Bcl2-family member, BAD, as well as the I-kB kinase (Engelman et al., 2006, Ozes et al., 1999, Romashkova et al., 1999). Cell cycle progression is regulated by AKT signaling, at least in part, by suppressing the activity of GSK3, which in turn, modulates the stability of cyclin D1 and c-myc (Cross et al., 1995). Finally, AKT signaling

phosphorylates Mdm2, a negative regulator of p53 function, while PTEN transcription is regulated by p53 (Trotman et al., 2003). The regulation of cell size by AKT is mediated by direct phosphorylation of TSC2 (tuberous sclerosis complex) (Inoki et al., 2002). Akt phosphorylation of TSC2 leads to mTOR activation, which in turn, results in the phosphorylation of ribosomal p70S6 kinase and initiation factor 4E binding protein-1, elevating protein synthesis (Hay et al., 2005).

PI3K/AKT-mediated signaling has been implicated in the survival and proliferation of multiple cell types. In hematopoietic stem cells, PTEN loss of function mutations, initially lead to an increase in stem cell proliferation but ultimately result in a loss of the stem cell pool (Yilmaz et al., 2006, Zhang et al., 2006). During T-lineage development, AKT-mediated signaling has been shown to promote the developmental progression of immature-T lineage cells that express a TCRb chain (Webb et al., 2005, Shiroki et al., 2007). During B-lineage development, PI3-K mediated signaling acts to regulate class switch recombination and plasma cell differentiation (Susuki et al., 1999, Susuki et al., 2003, Omori et al., 2006).

Aberrant activation of the AKT pathway has been implicated in a wide variety of cancers. Loss of function mutations in the PTEN gene is common among melanomas, prostate cancers, lung cancers, sporadic glioblastomas, breast tumors and lymphomas (Sakai et al., 1998). Mouse cancer models have recapitulated the effects of the absence of PTEN on tumorigenesis (Di Crisofano et al., 1998, Vivanco et al., 2002). Mice heterozygous for PTEN readily develop cancers in thyroid, lymphoid, breast, prostate and other tissues (Kishimoto et al., 2003). When targeted for deletion in the hematopoietic

compartment, PTEN-ablated mice develop myeloproliferative disease and T cell lymphoma (Cantley et al., 1999, Zhang et al., 2006, Hagenbeek et al., 2004).

B- and T-lineage development is regulated, in part, by a distinct class of helix-loop-helix (HLH) proteins, termed E-proteins (Bain et al., 1998, Benezra et al., 1990). Four E-proteins, E12, E47, E2-2 and HEB, are expressed in developing lymphoid cells. E12 and E47 are encoded by the E2A gene, and arise through differential splicing in an exon encoding the DNA binding and dimerization domain, named the helix-loop-helix (HLH) region. E-protein DNA binding activity during lymphoid development is regulated by the Id gene products (Benezra et al., 1990, Yokota et al., 1999, Pan et al., 1999, Rivera et al., 2000). Id proteins contain an HLH domain but lack a basic region and inactivate the DNA binding activity of E-proteins upon dimerization (Benezra et al., 1990). Four Id proteins are expressed in mammalian cells, named Id1-4.

E-proteins play multiple roles during lymphoid development (Benezra et al., 1990). E47 levels are high in developing B-lineage cells until they reach the mature B-cell stage (Quong et al., 1999, Quong et al., 2004). Once an antigen receptor has been generated that lacks auto-reactivity, E47 protein levels decline (Quong et al., 2004). In activated B-lineage cells, however, E47 abundance is elevated again to induce AID expression and class switch recombination (CSR) (Quong et al., 1999, Sayegh et al., 2003). During T-lineage maturation, E47 activates the expression of a subset of genes involved in Notch- and pre-TCR mediated signaling (Barndt et al., 2000, Ikawa et al., 2006). E47 at this stage also enforces the pre-TCR checkpoint (Engel et al., 2001, Kim et al., 2003). Once a pre-TCR has been generated, pre-TCR mediated signaling acts to decrease E47 protein levels and to induce the expression of Id3, to promote

developmental progression and cellular expansion (Engel et al., 2001, Kim et al., 2003). Id2 and Id3 gene expression in lymphoid cells is regulated by Erk MAP kinase and TGF-b mediated signaling (Bain et al., 2001, Kee et al., 2001). Id4 expression in developing thymocytes is suppressed by FOXO expression (Paik et al., 2007).

E2A and HEB act as tumor suppressors in the T cell lineage (Bain et al., 1997, Yan et al., 1997, Engel et al., 2002, Engel et al., 1999, O'Neil et al., 2004). E2A deficient mice readily develop T cell lymphoma and enforced expression of Id1 and Id2 in transgenic mice rapidly leads to lymphomagenesis (Morrow et al., 1999, Kim et al., 1999). Overexpression of E47 into cell lines adapted from lymphomas that developed in E2A-ablated mice leads to apoptosis, whereas ectopic expression of E47, in conjunction with Bcl-2, results in cell cycle arrest and a reduction in cell size (Engel et al., 2002, Kim et al., 1999, Engel et al., 2004, LLorian et al., 2007). The precise mechanism by which E47 and HEB act as a tumor suppressor remains to be determined.

Here we show that E47 is phosphorylated by AKT at a residues located immediately N-terminal of the HLH region. However, we find that mutation of the E47 AKT substrate site in the mouse germ-line does not significantly affect B cell development. We propose a model in which the PI3K-AKT axis and E47 are connected but that AKT acts on multiple functionally redundant components of the E-protein machinery in order to modulate developmental progression and cell growth.

Results

Interaction of E47 with 14-3-3

To identify proteins that interact with E47 in primary B cells, pro-B cells were isolated from bone marrow, purified using magnetic beads that were conjugated with a B220 specific antibody and cultured for a one week period in the presence of interleukin-7 and SCF. Nuclear lysates were prepared from the expanded pro-B cell population and E47 was purified using a monoclonal E47-antibody. Associating proteins were eluted, digested with trypsin and analyzed for protein identification by mass spectrometry. The peptide sequences were used to infer the identities of the E47-associating proteins components (Table 2.1). Among these are DNA methyltransferase (cytosine-5), PCNA, replication factor C, PAK2, chromosome condensation related SMC-associated protein 1, histone H1, histone H4, SWI-related actin dependent regulator of chromatin members 1 and c2, ubiquitin enzyme E1 and E2, ubiquitin B, ubiquitin-like 1, proteasome subunit 6 and alpha type 7, proteasome activator subunits 2, splicing factor 3b and Rpl7a-like. Interestingly, 14-3-3 proteins were also associated with E47, raising the possibility that E47 is modified at serine or threonine residues in response to AKT-mediated signaling.

Phosphorylation of E47 at Multiple Serine and Threonine Residues

To determine whether E47 contains a potential AKT substrate recognition site, a motif search program at the MIT Scansite website was used, allowing identification of potential phosphorylation sites (www.scansite.mit.du). This search identified a potential

highly conserved potential AKT substrate recognition site (RARTSS) present in the E47 HLH domain.

To examine whether E47 was phosphorylated at serine 528, E47 was purified as described from above murine pro-B cells in the presence of phosphatase inhibitors, digested with trypsin and subjected to mass spectrometry. This analysis identified five serine residues (137, 140, 142, 377 and 528), two tyrosine residues (194 and 201), and one threonine residue (169) that were phosphorylated (Figure 2.1). Residue serine 528 was intriguing since it is located in the putative 14-3-3 and AKT substrate target site. Comparison of the AKT-substrate motif shows its high degree of conservation (Figure 2.2). Interestingly, serine residue 528 is also present in HEB but not in E12 and E2-2 (Figure 2.2).

To confirm that the AKT-substrate motif was phosphorylated in human E47, E47 was FLAG and HIS-tagged at the C-terminus. The tagged protein was expressed from a hormone-inducible (Ponasterone A) promoter in 293T cells and affinity purified. Purified human E47 was digested with trypsin and subjected to mass spectrometry. The analysis identified three residues that were phosphorylated in human E47, including threonine 162, serine 373 and serine 524 (Figure 1A; lower diagram). The putative E47 AKT substrate recognition site originally identified in murine E47, was also phosphorylated in human E47 (Fig. 2.1)

Upon further inspection, we observed that the majority of cDNAs encoding E47 contained two serine residues within the AKT-substrate motif (Fig. 1C). However, a minor fraction of ESTs that were analyzed contained a single serine residue. Thus either RTRTSSTDE or RTRTSTDE were present in E47 (Fig. 2.3). The presence of an extra

serine residue in the AKT-recognition site raised the possibility that it was generated by alternative splicing since NAGNAG splice acceptor sites are known to result in alternative splicing at tandem 3' splice sites. Indeed, two NAGNAG splice acceptor splice sites are located at the 5' boundary of the E47 exon (Fig. 2.3). In contrast, the E12 exon lacks the CAGCAG tandem splice acceptor sites (Fig. 2.3). Thus, choosing the first acceptor splice site would lead to the insertion of an extra serine, whereas choosing of the second acceptor splice site would result in a single serine residue present in the 14-3-3/AKT substrate recognition site. The result is two distinct E47 proteins and one E12 protein generated by differential splicing of the E2A gene.

E47 is Phosphorylated at Serine Residue 524

To verify that E47 was phosphorylated in 293T cells at serine residue 524, nuclear extracts were generated from 'empty vector' control 293T cells or 293T cells expressing his- and flag-tagged E47. E47 was affinity purified and analyzed by Western blotting for the presence of E47 as well as putative 14-3-3 and AKT substrate recognition sites. Affinity-purified E47 reacted with antibodies recognizing the 14-3-3- and AKT-substrate sites (Fig. 2.4).

To examine that phosphorylated E47 at serine residue 524 interacts specifically with the AKT-substrate antibody, serine 524 was substituted for either a glutamate ($E47^{S524D}$) or an alanine ($E47^{S254A}$) residue. Expression vectors encoding wild type E47, $E47^{S524D}$ and $E47^{S524A}$ were transfected into 293T cells. Subsequently, nuclear extracts were derived from 293T cells transfected with wild type and mutant E47, immunoprecipitated using an E47-antibody and analyzed by Western blotting using an

AKT substrate antibody. Substitution for a serine to either glutamate or alanine substitution at residue 524 interfered with the ability of the AKT-substrate antibody to interact with purified E47 (Fig. 2.5).

To determine whether AKT activity is required to phosphorylate serine residue 524, 293T cells expressing E47 were transfected with vectors expressing either constitutively active AKT (caAKT) or a dominant-negative (dnAKT) form of AKT. Expressing the constitutive active form of AKT did not lower the fraction of E47 containing phosphorylated S524 (Fig. 2.6, lane 3). On the other hand, the proportion of S524 phosphorylated E47 was substantially reduced upon enforced expression of a dominant-negative form of AKT (Fig. 2.6, lane 4). Collectively, these data indicate that E47 serine residue 524, is phosphorylated in cells expressing an activated form of AKT.

Regulation of E47 by the PI3K/AKT Pathway in Developing B-lineage Cells

The data described above raise that question as to how phosphorylation of serine 524 by AKT signaling modulates E47 activity. Previous observations have indicated striking similarities involving the PI3K pathway and E-protein deficient mice. These include: (1) the inability of both E-protein and PTEN- and FOXO-deficient B-lineage cells to undergo class switch recombination (CSR) (Omori et al., 2006, Quong et al, 1999). (2) the marginal versus follicular zone B cell fate decision (Omori et al., 2006, Quong et al, 2004).

To examine the function of the putative AKT site in B cell maturation, mice were generated in which the serine residue was replaced by an alanine amino acids using insertional mutagenesis (Fig. 3A). ES cells were targeted for replacement using a vector

in which the conserved serine residue was substituted by alanine. From transfected cells genomic DNA was isolated and examined by Southern blotting using the appropriate probes. ES cells that carried the expected recombinants were generated from cells carrying the mutated E2A alleles (Fig. 3B). Subsequently, mice were crossed with *Ella-cre* transgenic mice in order to excise the *loxP* elements. To verify that the alanine mutation was properly inserted, mRNA was isolated from splenic B cells, reverse transcribed and sequenced (Fig. 4). As expected the codon encoding for the serine residue was replaced by an alanine amino acid (Fig. 4). Mice were backcrossed into the C57Bl/6 background. Mice carrying the serine to alanine substitution were subsequently crossed with $E2A^{+/-}$ mice in order to generate $E2A^{S528A/-}$ mice. Mice that were generated were viable, bred with normal Mendelian frequencies and did not exhibit gross abnormalities.

Analysis of the role of the E2A AKT substrate site in B cell development

Since it is conceivable that the effects of the replacement of $E47^{S258}$ would only be detectable in E2A heterozygous mice, mice were generated that were hemizygous for E2A and carried the serine 528 substitution. Consequently B-lineage cells derived from wild-type, $E2A^{S528A/S528A}$ as well as $E2A^{+/-}$ and $E2A^{S528A/-}$ mice were examined for abnormalities in B cell development (Fig. 3B). Specifically, bone marrow cells derived from wild-type and mutant mice were stained with antibodies directed against CD19, c-kit and CD25. We found that both the pro-B and pre-B cell compartments were not significantly reduced in $E47^{S528/-}$ null mutant mice (Fig. 5). Consistent with previous observations, $E2A^{+/-}$ mice showed a significant decrease in the pre-B cell compartment

(Quong et al, 2004). However, replacement of S528 by an alanine residue in conjunction with a loss-of-function allele did not further decrease the proportion of pre-B cells as compared to $E2A^{+/-}$ mice (Fig. 5). The transitional and mature-B cell compartments in the spleen were also not affected by the replacement of serine residue 528 (Fig. 6). Cellularity was also not significantly perturbed in $E47^{S528A}$ as compared to wild-type mice (Figs. 5 and 6). Taken together, these data indicate that phosphorylation of E47 serine residue 528 is not essential for proper B cell development.

Discussion

Previous observations have shown striking similarities between mice carrying mutations in components of the PI3K-mediated signaling and E2A activity. Among these are the common roles of PI3K signaling and E2A in receptor editing, the inability of E2A- and PTEN-deficient B-lineage cells to promote class switch recombination, the enforcement of PTEN and E2A of the pre-TCR checkpoint and the activities of PTEN, FOXO and E2A to act as tumor suppressors (Susuki et al., 2003, Omori et al., 2006, Hagenbeek et al., 2004, Quong et al., 1999, Quong et al., 2004, Engel et al., 2001, Morrow et al., 1999). These data have raised the question as to how PI3K mediated signaling and E-protein activity are linked. Recent studies have provided some insight into this question. It was shown that E2A proteins act directly upstream of FOXO1 to modulate is expression (Lin et al., 2010). Additionally, E2A and FOXO1 appeared to act collaboratively to induce a pro-B lineage specific program of gene expression. Furthermore, the FOXO proteins were shown to directly repress the transcription of both Id1 and Id4. Here, we have identified an additional link involving PI3K-mediated

signaling and E47 activity. Specifically, we show that activated AKT directly phosphorylates E47 at serine residue 524.

Thus, AKT signaling and E-protein activity appear to act at multiple levels by promoting: (1) Direct regulation of FOXO1 gene expression by E47. (2) Regulation of gene expression by the combined activities of E47 and FOXO1 at common enhancer elements. (3) Modulating Id1 and Id4 transcription. (4) Phosphorylation of a highly conserved E47 immediately upstream of the bHLH domain. We note that that putative AKT substrate site is also present in HEB located upstream of the HLH domain and although still to be proven it is conceivable that HEB is also phosphorylated by AKT. These data bring into question as to whether the activities of the PI3K signaling cascade and E-protein activity are physiologically linked. We show here that mutation of the putative E47 AKT phosphorylation site does not significantly impact B cell development. Similarly, mutation of serine residue 528 or ablation of Id4 does not interfere with the development of lymphoma in PTEN-deficient mice (unpublished observations). It is conceivable that the PI3K-PTEN-AKT signaling pathway and E47 are not directly linked. However, we would like to suggest that the PI3K-AKT axis and E47 are directly connected but that AKT acts on multiple functionally redundant components of the E- and Id-protein components in order to modulate developmental progression and cell growth. This may involve HEB as well as members of the Id gene family and it will be important to generate compound mutant mice that carry mutations in E47, HEB as well as members of the Id gene family in order to further evaluate the relationship between the PI3K pathway and E-protein activity.

Experimental Procedures

Expression of E47 in 293T

Sequences encoding FLAG and HIS peptides were fused to the N-terminus of E47 and constructed in a vector containing a ponasterone inducible promoter. A stable cell line was generated in EcR293T cell line (Invitrogen). A clone was isolated, expressing high levels of E47 upon proE47-13 C2 cell line was cultured in DMEM with 10% fetal bovine serume plus 400ug/ml Zeocin and 1mg/ml Geneticin. E47 expression was induced using 5mM Ponasterone A.

E47 protein purification and mass spectrometry analysis

Nuclear extracts were purified using EZview Red ANTI-FLAG M2 Affinity GEL (Sigma) and eluted twice with 100mg/ml FLAG peptide. The eluted fractions were combined and subjected to a second purification step using TALON Metal Affinity Resins (Upstate). Tagged E47 was eluted using 200mM imidazole, in 300mM sodium chloride and 50mM sodium phosphate. The eluted fractions were combined, reduced and alkylated using 2 mM Tris(2-carboxyethyl) phosphine (Fisher, AC36383) for 30 minutes at 37°C, followed by another 30 minutes incubation in the presence of 5 mM iodoacetamide (Fisher, AC12227) at 37°C. The proteins were digested with 1 mg trypsin (Roche, 03 708 969 001) at 37°C overnight.

Automated 2D nanoflow LC-MS/MS analysis was performed using LTQ tandem mass spectrometer (Thermo Electron Corporation, San Jose, CA) employing automated data-dependent acquisition. Raw data were extracted and searched using Spectrum Mill

(Agilnet, version A.03.02.060b). MS/MS spectra with a sequence tag length of 1 or less were considered as poor spectra and discarded. The rest of the MS/MS spectra were searched against the IPI (International Protein Index) database limited to mouse taxonomy. A concatenated forward-reverse dataset was used to calculate the in-situ identification false positive rates (FDR). The enzyme parameter was limited to full tryptic peptides with a maximum mis-cleavage of 1. All other search parameters were set to SpectrumMill's default settings (carbamidomethylation of cysteines, +/- 2.5 Da for precursor ions, +/- 0.7 Da for fragment ions, and a minimum matched peak intensity of 50%).

Western Blotting

Nuclear extracts were resolved on 4-12% Bis-tris gradiant gel (Invitrogen) and transferred to PVDF membranes. The blots were then blocked with 5% nonfat dry milk in 1xPBS with 0.1% tween-20) for 1 hour (room temperature) or O/N (4^oC). Then the blots were incubated in anti-E47 antibody 271 (BD Pharmingen) at a dilution of 1:5000 for 1 hour (room temperature) or O/N (4^oC). The blots were washed with 1xPBS with 0.1% tween-20 3 X 5'. The blots were then incubated in goat anti-mouse-HRP secondary antibody (Santa Cruz Biotechnology) at a dilution of 1:5000 for 1 hour at room temperature. The blots were then washed with 1xPBS with 0.1% tween-20 3 X 5'. The blots were then developed and exposed to x-ray film using ECL-plus (Amersham).

Generation of $E47^{S528A}$ mice

The linearized targeting vectors, carrying the S528A replacements were electroporated into 129Sv/Ev ES cells. Targeted ES cell clones were identified by Southern blotting using the appropriate probes. ES cells were injected into blastocysts and chimeric mice carrying the targeted S528 mutation were generated. Mice were subsequently crossed with *Ella-cre* mice in order to generate $E47^{S528A}$ mutant mice. Mice were subsequently backcrossed into C57Bl/6 mice. All animal studies were performed as outlined by the IACUC UCSD.

FACS analyses

FACS studies were performed with isolated bone marrow and spleen of mice that were between 6-12 weeks of age. Single cell suspensions were generated, incubated and stained with fluorochrome-conjugated antibodies purchased from either eBioscience or BD Biosciences. Data were collected with the LSRII Flow Cytometer (BD Biosciences) and were analyzed using FlowJo software (Tree Star).

Statistical Analysis

Statistical significance was calculated using two-tailed Student's t-test. All p-values of less than 0.05 were considered significant.

Acknowledgements

We would like to acknowledge Lilyan Wright for the bulk of the biochemistry work performed on the Akt substrate site. Several of the figures presented herein are the product of her work, including Figures 1.2, 1.4, 1.5, and 1.6. We would also like to acknowledge Kristina Beck for creating the $E47^{S528A}$ mice. Figures 1.7, 1.8, 1nd 1.9 are attributable to her work and diligence in the lab. Additionally, Zhouxin Shen and Steve Briggs were instrumental in performing proteomics analysis leading to discovery of proteins associating with E47, and discovery of post-translational modification sites of E47. Lilyan Wright initially created Table 1, with subsequent modification by Robert Teachenor.

Chapter 2, in part, has been submitted for publication of the material as it may appear in Molecular and Cell Biology, 2011, Teachenor, Robert; Beck, Kristina; Wright, Lilyan Y.T.; Shen, Zhouxin; Briggs, Steve; and Murre, Cornelis; 2011. The dissertation author was the primary investigator and author of this paper.

We are grateful to David Nemazee and Patrick Skogg (Scripps Research Institute) for PI3Kd deficient mice, Robert Rickert (Sanford-Burnham Medical Research Institute) for the CD19-Cre+ and $PTEN^{F/F}$ mice. We thank members of the Murre laboratory for reagents and stimulating discussions. R.T. and L.W. were supported by a training grant from the NIH (Cellular and Molecular Genetics). K.B was supported by the NIH. This work was supported to C.M. by grants from the NIH.

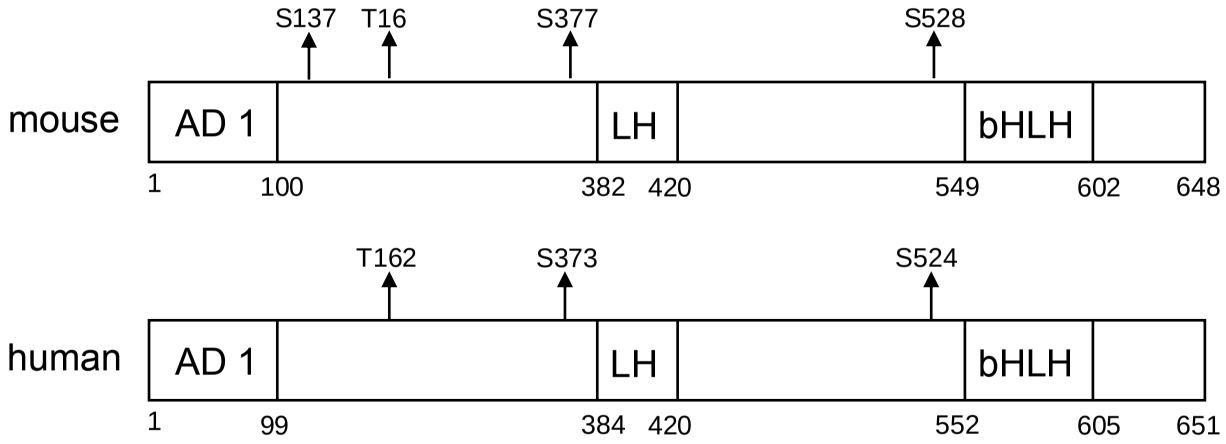


Figure 2.1 E47 is Phosphorylated at Multiple Serine and Threonine Residues. Murine and human E47 are phosphorylated at multiple serine and threonine residues. Murine E47 was purified from a pro-B cell culture using an E47 antibody. Human E47 was expressed in 293T cells and purified by affinity chromatography. Purified E47 was subjected to trypsin digestion and analyzed for modifications by mass spectrometry. Indicated are positions of phoshorylated serine and threonine residues. The transactivation domains AD1 and the LH region as well as the bHLH are depicted in the diagram.

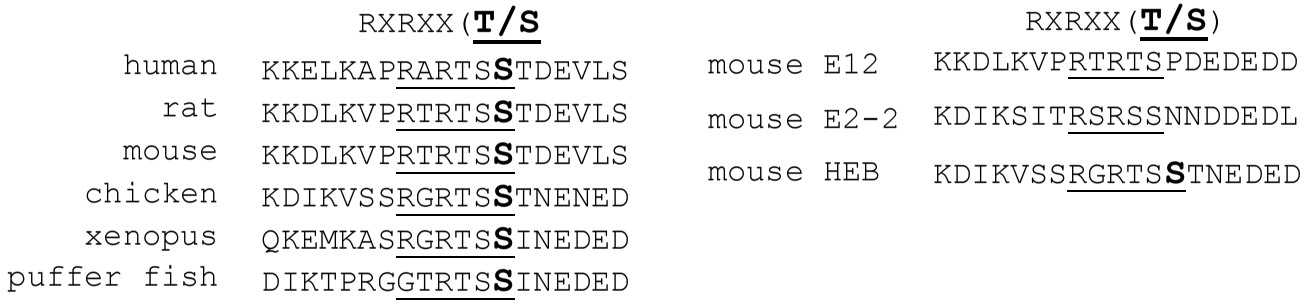


Figure 2.2 The putative AKT substrate site (RXRXXT/S) in human, rat, murine, chicken, Xenopus laevis, and puffer fish (left panel). The RXRXXT/S site is also indicated in E12, E2-2 and HEB. Note that the serine residue is conserved in HEB but not in E12 and E2-2 (right panel).

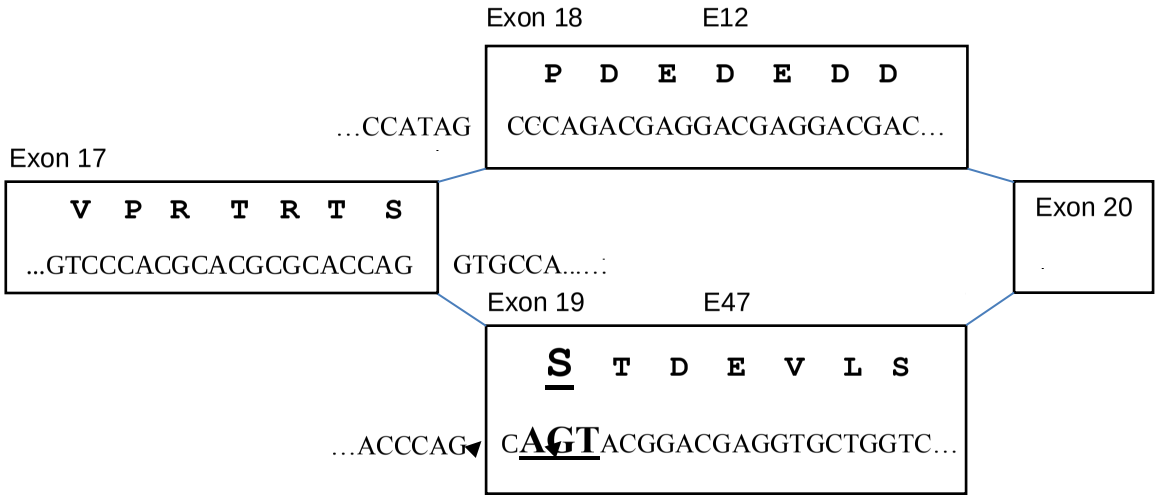


Figure 2.3 Diagram indicating the exon 17-20 organization of the E2A gene. Note that the AKT substrate serine residue in E47 is a replaced by a proline residue in E12. Either RTRTSSTDE or RTRTSTDE are present in the bHLH region of E47. Arrows indicate alternative splicing since NAGNAG splice acceptor sites are known to result in alternative splicing at tandem 3' splice sites. Note that the first acceptor splice site results in the insertion of an extra serine, whereas choosing of the second acceptor splice site generates a single serine residue present in the substrate recognition site. The alternative splicing mechanism results in three E2A gene products, encoding two distinct E47 proteins and one E12 protein.

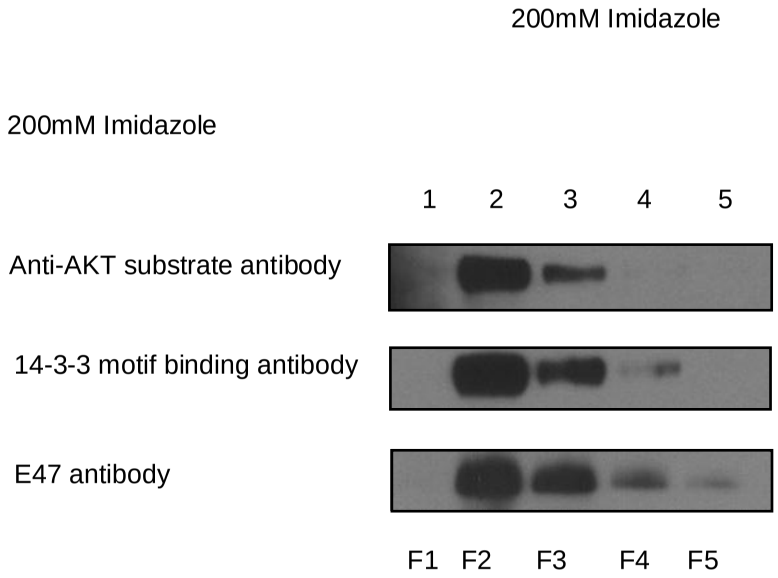


Figure 2.4 E47 is a substrate for AKT activity. E47 contains AKT and 14-3-3 substrate sites. Epitope-tagged E47 was affinity purified from 293 cells. Purified E47 was analyzed by Western blotting using antibodies with specificity for 14-3-3 and AKT substrate sites as well as E47. F1 indicates flow-through fraction eluted with 10mM imiddazole. F2-F5 indicate fractions eluted with 200mM imidazole containing purified E47.

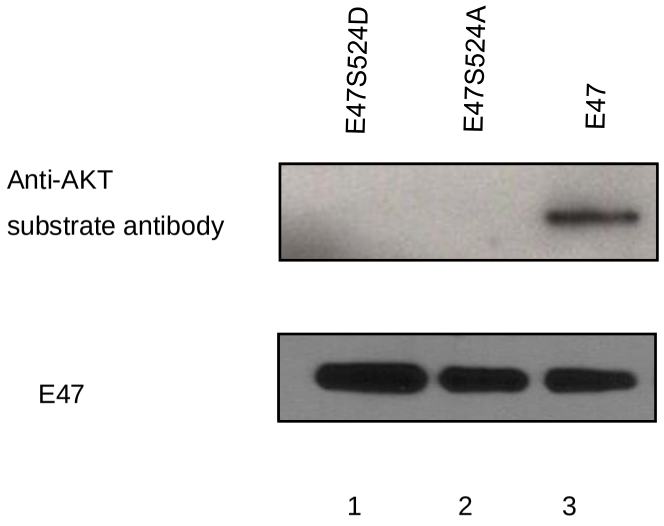


Figure 2.5 E47 is a substrate for AKT activity. AKT-substrate antibody recognized serine residue 524 in E47. 293 cells were transfected with either expression vectors expressing either E47, or $E47^{S524A}$, or $E47^{S524D}$. Lysates were derived from transfected cells, immunoprecipitated using an antibody recognizing E47 and immunoblotted using an Akt-substrate antibody.

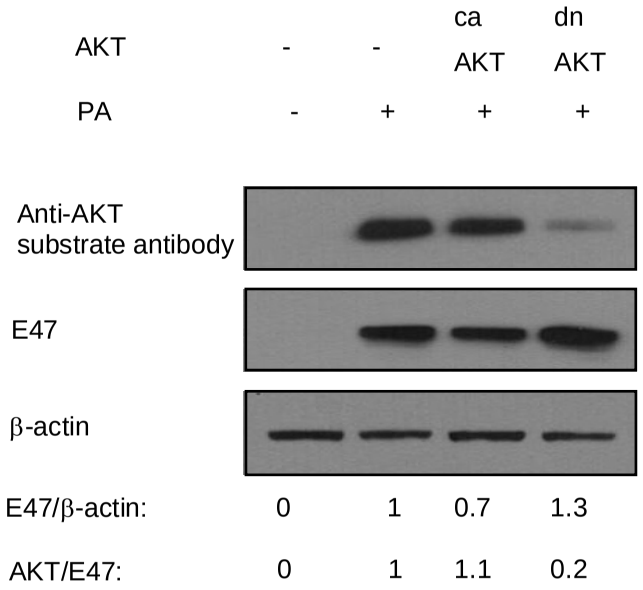


Figure 2.6 E47 is a substrate for AKT activity. Phosphorylation of E47 serine residue 524 is affected by AKT signaling. 293 cells were transfected with vectors expressing E47 in the absence or presence of a constitutively active form of AKT1 (ca-AKT) or a dominant-negative form of AKT1 (DN-AKT). Immunoblotting was performed with AKT substrate (upper panel), E47 (middle panel) and β-actin (lower panel) specific antibodies, respectively.

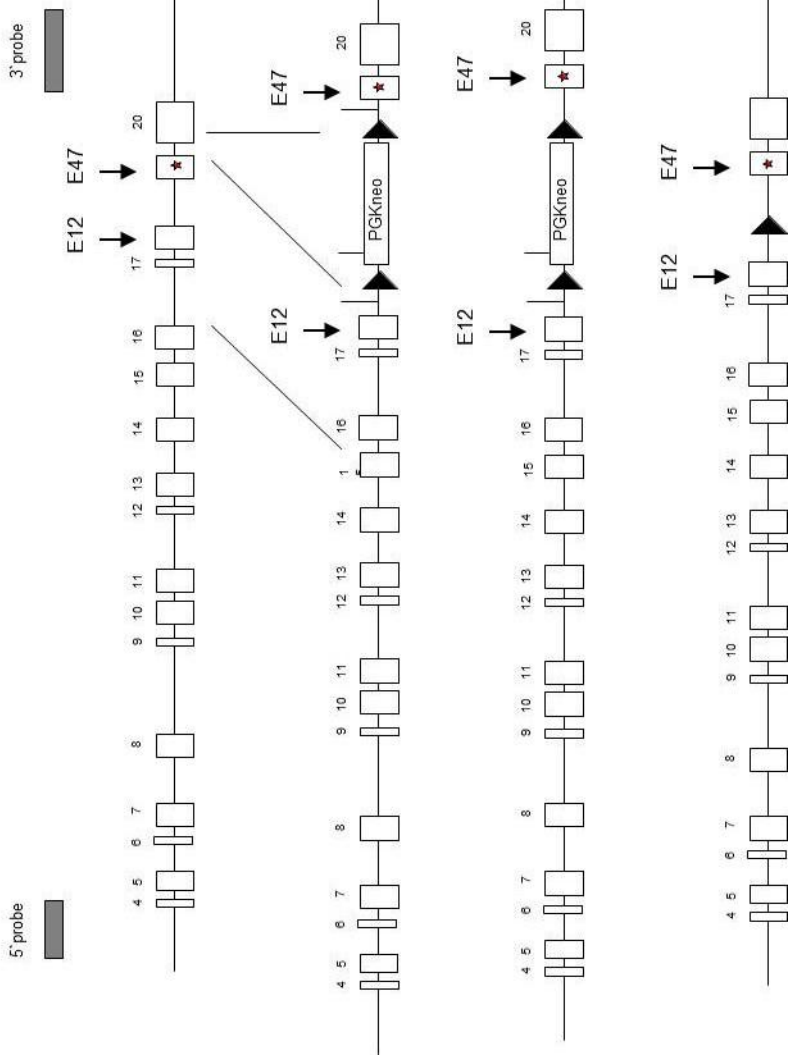


Figure 2.7 Replacement of residue serine 528 by an alanine amino acid. The substitution was introduced into the germ-line sequence by homologous recombination. Briefly, 129 embryonic stem cells were electroporated with the targeting vector as shown. Transient expression of Cre recombinase was used to excise the neomycin resistance cassette. Properly targeted ES were injected into the pseudo-pregnant mice.

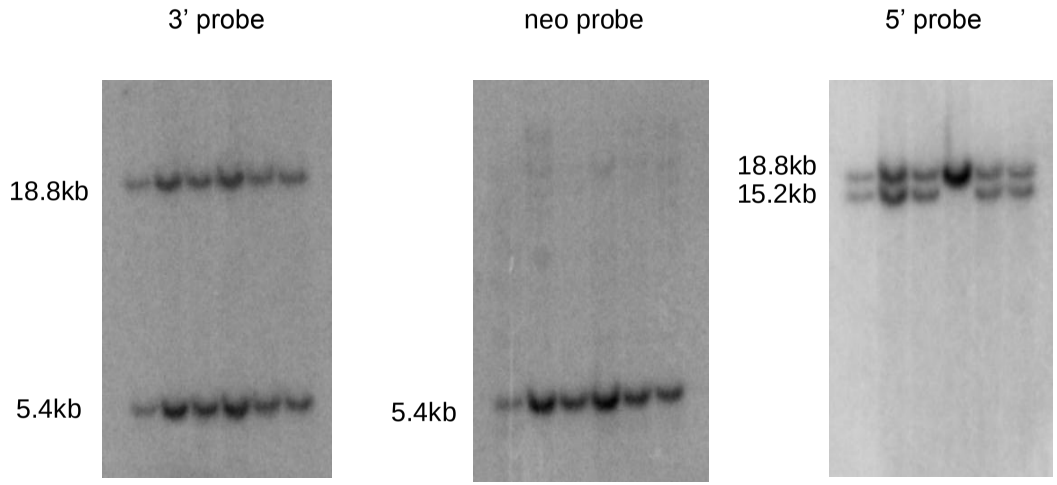
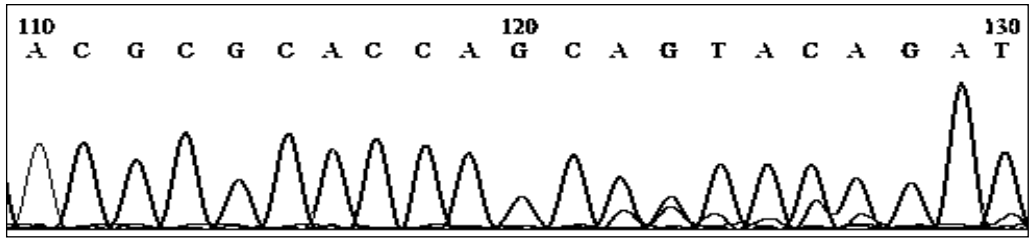


Figure 2.8 Replacement of residue serine 528 by an alanine amino acid. Offspring were analyzed by Southern blotting for germ-line transmission. Targeted allele using a 5’ probe (indicated in Figure 1A) was predicted to be 15.2 kbp in size whereas an 18.8 kbp fragment was predicted for the wild-type allele.

wt-mRNA:	ACG	CGC	ACC	AGC	**AGT**	ACA	GAT
S528A-mRNA:	ACG	CGC	ACC	AGC	**GCT**	ACA	GAT
wt-protein:	T	R	T	S	**S**	T	D
S528A-protein:	T	R	T	S	**A**	T	D

wt



S528A/A

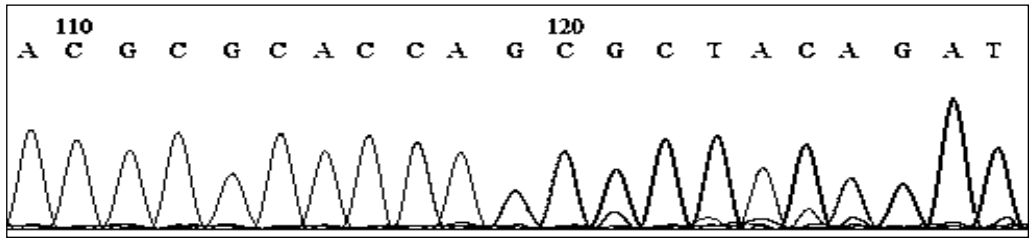


Figure 2.9 Verification of substitution of serine residue S528 in the mouse germ-line. mRNA was isolated from splenic B cells from the indicated mice, cDNA was generated using SuperscriptIII (Invitrogen) and random hexamers and a PCR to amplify the E47 transcript was performed using specific primers (the forward primer spans exon16/17 and the reverse primer lies in exon 20). Sequencing was performed on the PCR product using the

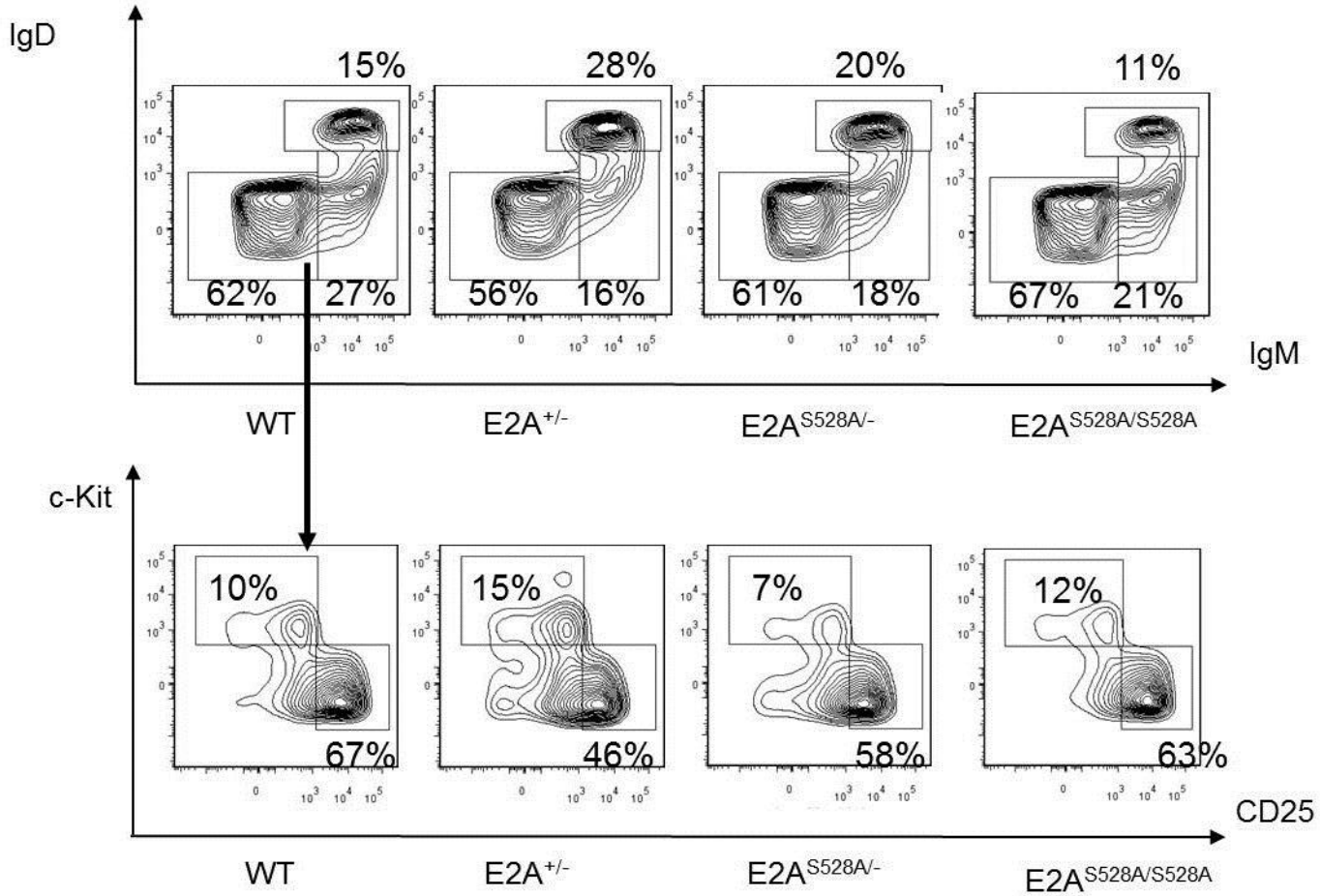
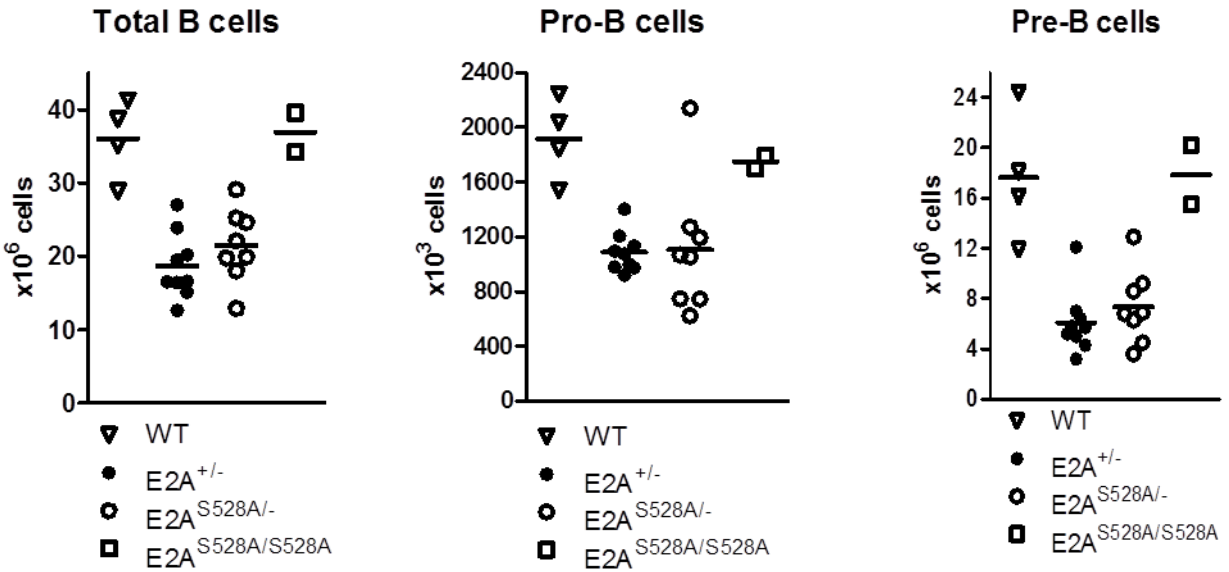


Figure 2.10 Flow cytometric analysis of the bone marrow derived from wild-type and S528 mutant mice. Bone marrow cells were isolated from wild-type, $E2A^{+/-}$ and $E2A^{S528/-}$ mice. FACS analysis is shown for c-kit and CD25 expression gated on the lineage-negative/CD19+ compartments derived from wild-type, $E2A^{+/-}$ and $E2A^{S528/-}$ mice.



WT:	$36.0 \times 10^6 \pm 5.4 \times 10^6$
$E2A^{+/-}$:	$18.6 \times 10^6 \pm 4.5 \times 10^6$
$E2A^{S528A/-}$:	$21.5 \times 10^6 \pm 5.4 \times 10^6$
$E2A^{S528A/S528A}$:	$36.9 \times 10^6 \pm 3.8 \times 10^6$

WT:	$1917.4 \times 10^3 \pm 299.1 \times 10^3$
$E2A^{+/-}$:	$1088.3 \times 10^3 \pm 148.3 \times 10^3$
$E2A^{S528A/-}$:	$1106.5 \times 10^3 \pm 477.7 \times 10^3$
$E2A^{S528A/S528A}$:	$1748.3 \times 10^6 \pm 65.7 \times 10^3$

WT:	$17.6 \times 10^6 \pm 5.2 \times 10^6$
$E2A^{+/-}$:	$6.1 \times 10^6 \pm 2.5 \times 10^6$
$E2A^{S528A/-}$:	$7.4 \times 10^6 \pm 2.9 \times 10^6$
$E2A^{S528A/S528A}$:	$17.9 \times 10^6 \pm 3.3 \times 10^6$

Figure 2.11 Flow cytometric analysis of the bone marrow derived from wild-type and S528 mutant mice. Total cell numbers are presented for the various compartments.

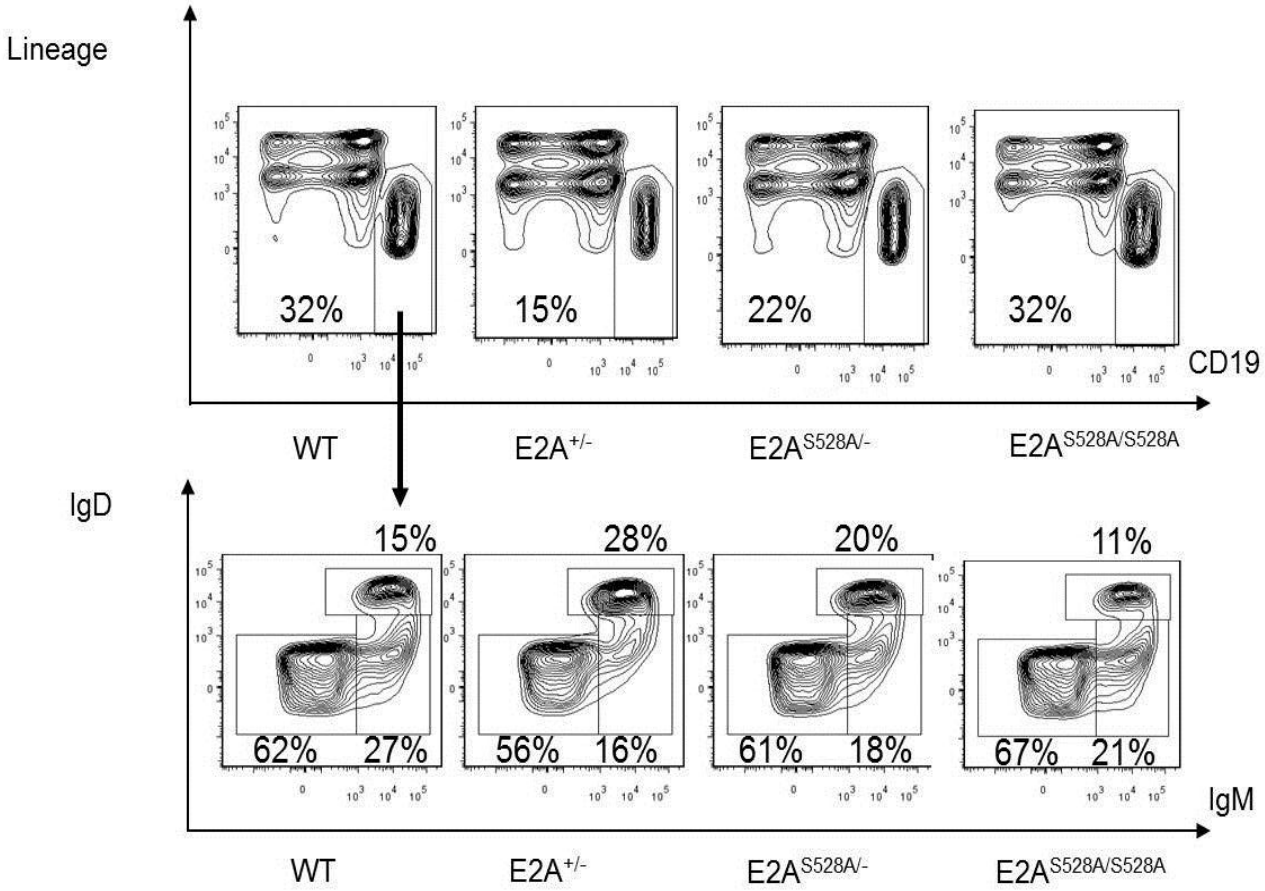


Figure 2.12 Flow cytometric analysis of the bone marrow derived from wild-type and S528 mutant mice. Bone marrow cells were isolated from wild-type, $E2A^{+/-}$ and $E2A^{S528/-}$ mice. FACS analysis is shown for IgD and IgM expression gated on the lineage-negative/CD19+ compartments derived from wild-type, $E2A^{+/-}$ and $E2A^{S528/-}$ mice.

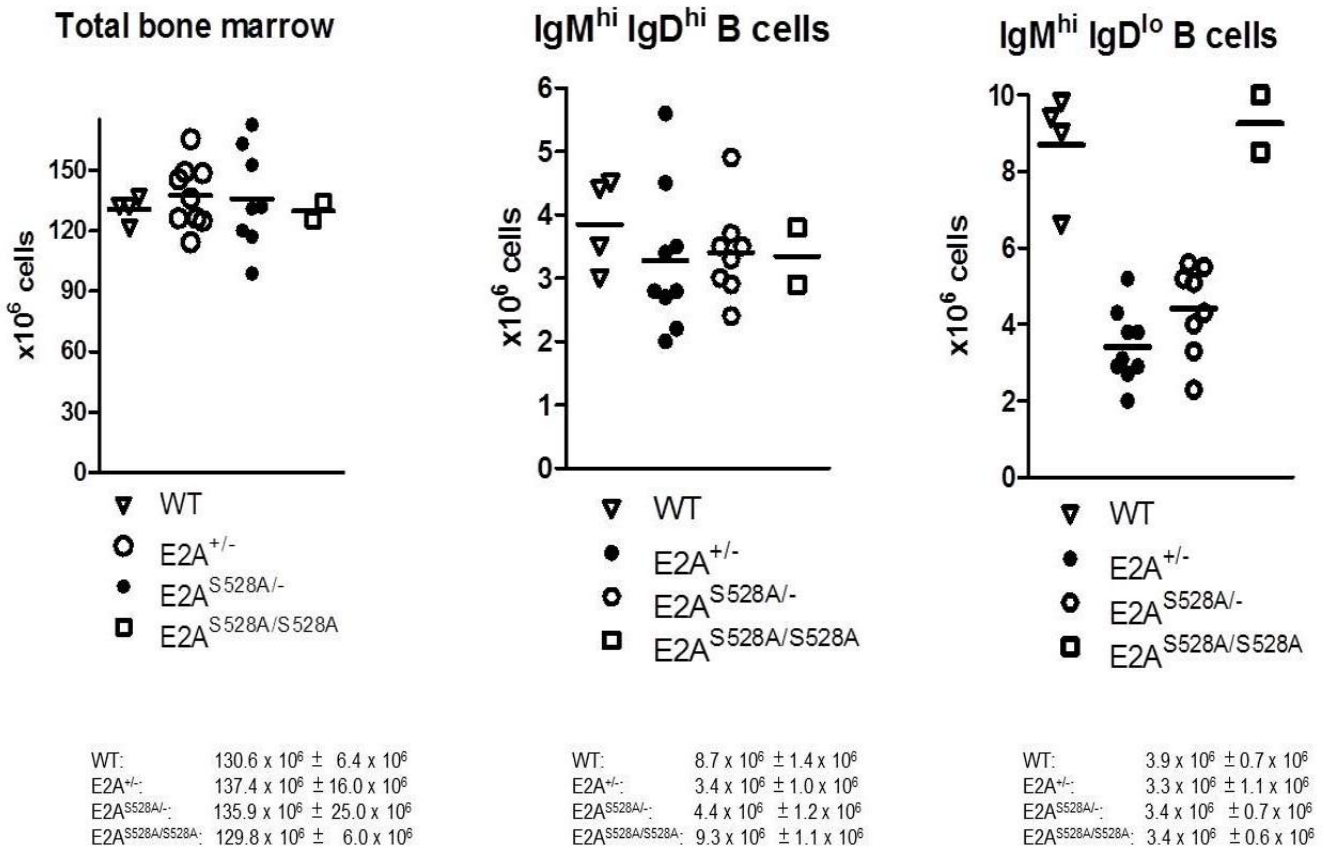


Figure 2.13 Flow cytometric analysis of the bone marrow derived from wild-type and S528 mutant mice. Total cell numbers are presented for the various compartments.

	WT	$E2A^{+/-}$	$E2A^{S528A/-}$
Pre-B: Pro-B:	9.18	5.58	6.64
Trans B: Pre-B:	0.49	0.56	0.53
Mature B: Transitional B:	0.44	0.96	0.86
Total B: Mature-B:	9.35	5.68	6.31
Total Bone Marrow: Total B:	3.63	7.37	6.36

Figure 2.14 Ratios of B cell compartments are shown for wild-type, $E2A^{+/-}$ and $E2A^{528A/-}$ mice.

Table 2.1 Proteomics panel of E47-associating proteins

Table 1 (Proteomics)	% a.a. coverage		% a.a. coverage
Nucleolin	22%	Proteasome 26S non-ATPase subunit 11	22%
Ribosomal protein S3	48%	Proteasome 26S non-ATPase subunit 13	17%
Ribosomal protein L27a	28%	Proteasome 26S non-ATPase subunit 2	13%
Ribosomal protein L4	19%	Proteasome activator subunit 3	16%
Nucleophosmin 1	36%	Ubiquitin-conjugating enzyme E2N	23%
Replication factor C (activator 1) 3	17%	Ubiquitin-conjugating enzyme E2I	8%
Laminin receptor 1 (ribosomal protein SA)	27%	Ubiquitin B	23%
Stratifin (14-3-3 protein sigma)	6%	Pre-mRNA processing factor 8	2%
14-3-3 protein theta	24%	PRP31	5%
Eukaryotic translation initiation factor 3	25%	Splicing factor (ASF/SF2)	18%
Eukaryotic translation initiation factor 4A1	38%	Splicing factor 3b, subunit 3	8%
Eukaryotic translation initiation factor 4A2	14%	snRNP core protein SMX5	14%
Eukaryotic translation initiation factor 1	43%	inhibitor of DNA binding 3	10%
DEAD (Asp-Glu-Ala-Asp) box polypeptide 5	16%	stress-induced phosphoprotein 1	10%
DNA methyltransferase (cytosine-5) 1	6%	proliferating cell nuclear antigen	37%
Non-POU-domain, octamer binding protein	23%	SWI/SNF related regulator of chromatin	8%
Histone 1, H4h	52%	Rpl7a protein	17%
Histone 4, H4	52%	Programmed cell death 6	29%
Histone 1, H4c	52%	P21-activated kinase 2	14%
Histone 2, H2ab	49%	Chromosome condensation protein G	5%
Histone 3, H2a	49%	Chromodomain helicase DNA binding 4	6%
Histone 1, H4k	52%	S-phase kinase-associated protein 1A	33%
TAF15 RNA polymerase II, TBP-associated	7%	Chromosome condensation-related SMC 1	4%
Retinoblastoma binding protein 4	16%	DJ-1 protein	22%
Retinoblastoma binding protein 7	7%	RNA polymerase II transcriptional coact	15%

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III.

Highly conserved phosphorylated serine and tyrosine residues in the E2A transactivation domains are required to promote efficient B-lineage maturation

Summary

There is now ample evidence that the E2A proteins promote B cell differentiation at multiple stages. In previous studies we have identified three highly conserved clustered phosphorylated serine residues 135, 140 and 142 as well as two distinct tyrosine residues flanking the E2A transactivation domains. To directly evaluate the role of these residues in B cell development we have generated E2A mutant mice in which this cluster of residues was mutated. The data indicate that these highly conserved serine residues do play a role, albeit a minor one, in early B cell development as well as in the transitional B cell compartment. Similarly, tyrosine residues located in the N-terminal portion of the E2A protein are involved in promoting the developmental progression of early B cell progenitors as well as the transitional B cell compartments in the peripheral lymphoid organs.

Introduction

Lymphoid development is controlled, in part, by a subset of helix-loop-helix (HLH) proteins, named E-proteins (Murre, 2005). There are four E-proteins, E12, E47, E2-2 and HEB, which are expressed in both the B and T cell lineages. E12 and E47 arise through differential splicing and are encoded by the E2A gene. A subset of HLH proteins lacking the basic region, named the Id proteins, modulates the DNA binding activity of E2A (Yokota et al., 1999; Pan et al. 1999; Rivera et al., 2000). Id proteins suppress upon

dimerizing with E-proteins the DNA binding activity (Benezra et al., 1990). Two Id gene products, Id2 and Id3, have been shown to play critical roles in lymphoid development.

The E2A proteins are involved during multiple stages in lymphoid development. E47 abundance is high in B cell progenitors (Quong et al., 2003). However, once an antigen receptor is expressed that lacks auto-reactivity, E47 protein abundance decreases (Quong et al., 2004). Upon activation, E47 abundance is elevated again in B cells to activate AID expression and promote class switch recombination (Quong et al., 1999; Sayegh et al., 2003). In developing T cells, E47 activates the expression of genes encoding components of the Notch- and pre-TCR signaling cascades (Barndt, et al. 2000; Ikawa et al., 2006). Once a pre-TCR is assembled, pre-TCR signaling activates the expression of Id3, to suppress E2A activity and to promote developmental progression (Engel et al., 2001; Kim et al., 2002). Interestingly, Id1 and Id4 expression is suppressed by members of the FOXO family (Paik et al., 2007).

E2A and HEB are powerful tumor suppressors (Bain et al., 1997; Yan et al., 1997; Engel and Murre, 2002; O'Neil et al., 2004). E2A as well as HEB deficient mice rapidly develop lymphoma. Furthermore, forced Id1 and Id2 expression leads to lymphomagenesis (Morrow et al., 1999; Kim et al., 1999). Forced E47 expression in cell lines leads to apoptosis, whereas expression of E47 with Bcl-2 promotes cell cycle arrest (Engel and Murre, 1999; Engel and Murre, 2004; Wojciechowski et al., 2007).

The N-terminal portion of the E2A gene products consists of two transactivation domains present. The transactivation domains are termed AD1 and AD2 (Aronheim et al., 1993; Quong et al., 1993; Massari et al., 1996). These domains are not only conserved, but also restricted to the E-proteins. Both of these domains have been shown

to activate transcription in both yeast and mammalian cells (Aronheim et al., 1993; Quong et al., 1993; Massari et al., 1996). Despite a complete lack of sequence homology, a α-helical structure present in both transactivation domains is important for their functional activities (Quong et al., 1993; Massari et al., 1996). AD1 has been shown to activate gene transcription via recruitment of histone acetyl-transferases via the SAGA and/or p300 co-activators (Massari et al, 1999). The mechanism by which this occurs remains to be determined.

It is now well established that phosphorylation affects the functional activities of a wide variety of HLH proteins. Casein Kinase II has been shown to affect the dimerization activity of Myc (a class IV HLH) and Max (class III HLH). In particular, phosphorylation of Max by Casein Kinase II prevents the binding of Max homodimers to DNA, but does not interfere with binding Myc/Max heterodimers (Berberich and Cole, 1992). Similarly, Casein Kinase II and Protein Kinase A phosphorylate E47 at serine residues 514 and 529, respectively, to prevent the DNA-binding of truncated E47 homodimers (Sloan et al., 1996). This phosphorylation event also promotes transcription driven by E47/MyoD heterodimers in developing muscle cells. Several known kinase consensus sequences match with peptide sequences in the first activation domain of the E2A gene products. The MAP Kinases relevant to these consensus sequences include p38 and Erk1, as well as the cyclin-dependent kinases Cdc2 and Cdk5, and Glycogen Synthase Kinase 3. Here we show that highly conserved residues located in the N-terminal portion of the E47 AD1 region play an important role for early B-lineage development. Specifically, we demonstrate that three clustered serine and two tyrosine residues are necessary to promote efficient developmental progression from the pro-B

cell stage to the pre-B cell stage as well as during the immature- to the mature-B transition.

Results

Replacement of three highly conserved serine residues flanking the transactivation domains of E2A in the mouse germ-line

To examine the function of the cluster serine residues in B cell maturation, mice were generated in which the clustered conserved serine residues were replaced by alanine amino acids using insertional mutagenesis (Figure 3.1). ES cells were targeted for replacement using a vector in which the conserved serine residues were substituted by alanine residues (Figure 3.2) From transfected cells genomic DNA was isolated and examined by Southern blotting using the appropriate probes. ES cells that carried the expected recombinants were generated from cells carrying the mutated E2A alleles. Subsequently, mice were crossed with Ella-*cre* transgenic mice in order to excise the *loxP* elements. Mice were backcrossed into the C57Bl/6 background. Mice carrying the serine to alanine substitutions were subsequently crossed with $E2A^{+/-}$ mice in order to generate $E2A^{SSSAAA/-}$ mice. Mice that were generated were viable, bred with normal Mendelian frequencies and did not exhibit gross abnormalities.

Roles of clustered serine residues in early B cell development

To determine whether the clustered serine residues play essential roles in mediating the activity of E2A in early B cell development, bone marrow cells derived from wild-type and $E2A^{SSSAAA}$ mice were examined for defects in early B cell

development. Specifically, B-lineage cells derived from wild-type, $E2A^{+/-}$ and $E2A^{SSSAAA/-}$ mice were isolated and examined for abnormalities in B cell development (Figure 3.3). Specifically, bone marrow cells derived from wild-type, E2A+/- and $E2A^{SSSAAA/-}$ mice were stained with antibodies directed against CD19, c-kit and CD25. We found that the pro-B cell compartment was not significantly reduced in $E2A^{SSSAAA/-}$ null mutant mice (Figure 3.2 and Figure 3.3). In contrast, the pre-B cell population appeared to be slightly decreased in $E2A^{SSSAAA/-}$ mice (Figure 3.3 and Figure 3.5). In contrast, the immature-B and mature-B cell compartments were not substantially altered (Figure 3.4). The transitional-B compartment in the spleen derived from $E2A^{SSSAAA/-}$ mice was also significantly affected by the replacement of the clustered serine residues by alanines (Figure 3.6). The pre-B versus pro-B ratio upon comparing E2A+/- and $E2A^{SSSAAA/-}$ mice was changed as expected (Figure 3.7). Taken together, these data indicate that the clustered serine residues in E2A play an important role in B cell development.

Replacement of two highly tyrosine serine residues flanking the transactivation domains of E2A in the mouse germ-line

To examine the function of two tyrosine residues in B cell maturation, mice were generated in which the clustered conserved serine residues were replaced by phenylalanine substitutions (Figure 3.8). ES cells were targeted for replacement using a vector in which the conserved tyrosine residues were substituted by phenylalanines. From transfected cells genomic DNA was isolated and examined by Southern blotting using the appropriate probes. ES cells that carried the expected recombinants were

generated from cells carrying the mutated E2A alleles. Subsequently, mice were crossed with Ella-*cre* transgenic mice in order to excise the *loxP* elements. Mice were backcrossed into the C57Bl/6 background. Mice carrying the tyrosine to phenylalanine substitutions were subsequently crossed with $E2A^{+/-}$ mice in order to generate $E2A^{YYFF/-}$ mice. Mice that were generated were viable, bred with normal Mendelian frequencies and did not exhibit gross abnormalities.

Roles of clustered serine residues in early B cell development

To determine whether the two tyrosine residues play essential roles in mediating the activity of E2A in early B cell development, bone marrow cells derived from wild-type and $E2A^{YYFF/-}$ mice were examined for defects in early B cell development. Specifically, B-lineage cells derived from wild-type, E2A+/- and $E2A^{YYFF/-}$ mice were isolated and examined for abnormalities in B cell development (Figure 3.9). Specifically, bone marrow cells derived from wild-type, $E2A^{+/-}$ and $E2A^{YYFF/-}$ mice were stained with antibodies directed against CD19, c-kit and CD25. We found that the pro-B cell compartment was not significantly reduced in $E2A^{YYFF/-}$ null mutant mice (Figure 3.9 and Figure 3.11). In contrast, the pre-B cell population appeared to be significantly decreased in $E2A^{YYFF/-}$ mice (Figure 3.9). The immature-B and mature-B cell compartments appeared unchanged (Figure 3.10). The transitional-B compartment in the spleen derived from $E2A^{YYFF/-}$ mice was also significantly affected by the replacement of the clustered tyrosine residues by phenylalanines (Figure 3.11). As predicted from the observations described above the pre-B versus pro-B ratios were significantly altered between $E2A^{+/-}$

and $E2A^{YYFF/-}$ mice (Figure 3.12). Taken together, these data indicate that the two tyrosine residues in E2A play a significant role in B cell development.

Discussion

It has been demonstrated in previous studies that the transcription factor E2A is required for specification and commitment to the B cell lineage. However, it is not merely indispensable for early B cell development but it also continues to play a critical function in maturing B cells, including that V(D)J rearrangement, receptor revision, follicular B cell development as well as class switch recombination. These data bring into question as to how the E2A proteins mediate such a diverse set of activities. As a first approach to this question we have mutated highly conserved residues that are located within the transactivation domains through the usage of the loxP system, permitting us to examine their roles during B cell maturation.

Previous observations have indicated that B cell development in E2A-ablated mice is completely blocked prior to the initiation of V(D)J gene rearrangement. The data described here indicate that the clustered serine residues as well as two tyrosine residues are not absolutely essential to promote B cell specification and commitment. B cells in $E2A^{SSSAAA}$ and $E2A^{YYFF}$ mice develop normally and exhibit no significant defects prior to the pro-B stage. Thus, phosphorylation of the clustered serine residues is dispensable for the specification and commitment of the B cell fate.

However, there are striking abnormalities observed in the pre-B cell compartment. Specifically, a significantly reduced pre-B cell compartment was detected in $E2A^{SSSAAA/-}$ and $E2A^{YYFF/-}$ mice. A similar phenotype was observed in the transitional compartments

of $E2A^{SSSAAA/-}$ and $E2A^{YYFF/-}$ mice. These data then raise the question as to how mutations of the clustered serine and tyrosine residues affect developmental progression from the pro-B to the pre-B cell stage. In recent studies it has become apparent that the expression of a subset of genes involved in pre-BCR signaling is directly regulated by E2A activity (Lin et al., 2010). Prominent among these genes are VpreB1, VpreB2, VpreB-3, BLINK and lambda-5. Based on these observations we would like to propose that the clustered serine and tyrosine residues play a particularly important role in E2A-mediated transactivation.

Additionally we suggest that the clustered serine and tyrosine residues play a role as well in regulating immunoglobulin light chain gene rearrangements. The E2A proteins were originally identified and characterized as proteins that bind to the κE2 site of the Igκ intronic enhancer (Inlay et al., 2004). Deletion of the E2A binding sites in the Ig kapp enhancer was demonstrated to severely affect immunoglobulin light chain locus rearrangement (Inlay et al. 2004). Enforced E2A expression in non-lymphoid cells was shown to readily initiate immunoglobulin light chain gene rearrangement (Romanow et al., 2000). Furthermore, immunoglobulin light chain gene rearrangements in E2A-deficient pre-B cells were shown to be substantial reduced (Quong et al. 2005). Together, these studies have demonstrated that the E2A proteins act as essential modulators of light chain gene rearrangement. Thus, it is conceivable that the clustered serine and tyrosine residues are also essential to promote Ig light chain gene rearrangement.

Recent observations have indicated that the E2A proteins function by modulating the pattern of H3K4me1 across putative enhancer elements (Lin et al. 2010). These data

have raised the question as to how the E2A proteins modulate the pattern of H3K4me1. We would like to consider the possibility that the E2A proteins act by recruitment of histone-methyltransferases. Such enzymes may act to increase the degree of H3K4me1 across putative enhancer elements. Interestingly, we have recently identified methyltransferases that associate with E2A by mass spectrometric analyses (data not shown). We speculate that the conserved clustered serine and tyrosine residues function to recruit histone modifiers to the appropriate target promoter in order to activate down-stream target gene expression.

Materials and Methods

Generation of E2A^{SSSAAA} and E2A^{YYFF} mice

The linearized targeting vectors, carrying the SSSAAA or YYFF replacements were electroporated into 129Sv/Ev ES cells. Targeted ES cell clones were identified by Southern blotting using the appropriate probes. ES cells were injected into blastocysts and chimeric mice carrying the targeted SSSAAA and YYFF mutations were generated. Mice were subsequently crossed with Ella-*cre* mice in order to generate E2A^{SSSAAA} and E2A^{YYFF} mutant mice. Mice were subsequently backcrossed into C57Bl/6 mice. All animal studies were performed as outlined by the IACUC UCSD.

FACS analyses

FACS studies were performed with isolated bone marrow and spleen of mice that were between 6-12 weeks of age. Single cell suspensions were generated, incubated and stained with fluorochrome-conjugated antibodies purchased from either eBioscience or

BD Biosciences. Data were collected with the LSRII Flow Cytometer (BD Biosciences) and were analyzed using FlowJo software (Tree Star).

Statistical Analysis

Statistical significance was calculated using two-tailed Student's t-test. All p-values of less than 0.05 were considered significant.

Acknowledgments

We would like to acknowledge Kristina Beck for creation of both of the $E47^{SSSAAA}$ and $E47^{YYFF}$ mouse strains depicted herein. Furthermore, Kristina initially created Figures 3.2 and 3.8, used here with permission.

We would like to acknowledge Lilyan Wright for the bulk of the biochemistry work performed on the Akt substrate site. Additionally, Zhouxin Shen and Steve Briggs were instrumental in performing proteomics analysis leading to discovery of proteins associating with E47, and discovery of post-translational modification sites of E47. Lilyan Wright initially created Table 1, with subsequent modification by Robert Teachenor.

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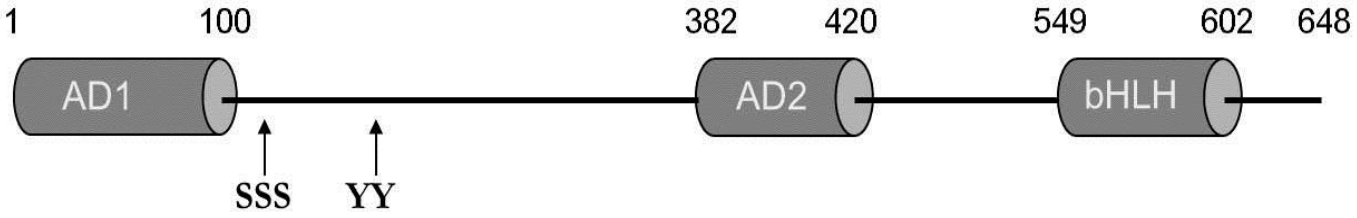

Amino acid residue	adjacent amino acid sequence
S135/S140/S142	S **S** P G P L **S** P **S** G
Y194/Y201	D S **Y** S R D A A A **Y** P S

Figure 3.1 Domains of the E2A gene products are depicted. Both E12 and E47 contain two activation domains, termed AD1 and AD2. They also contain a DNA binding and dimerization domain named the bHLH domain. Residues that are phosphorylated are indicated.

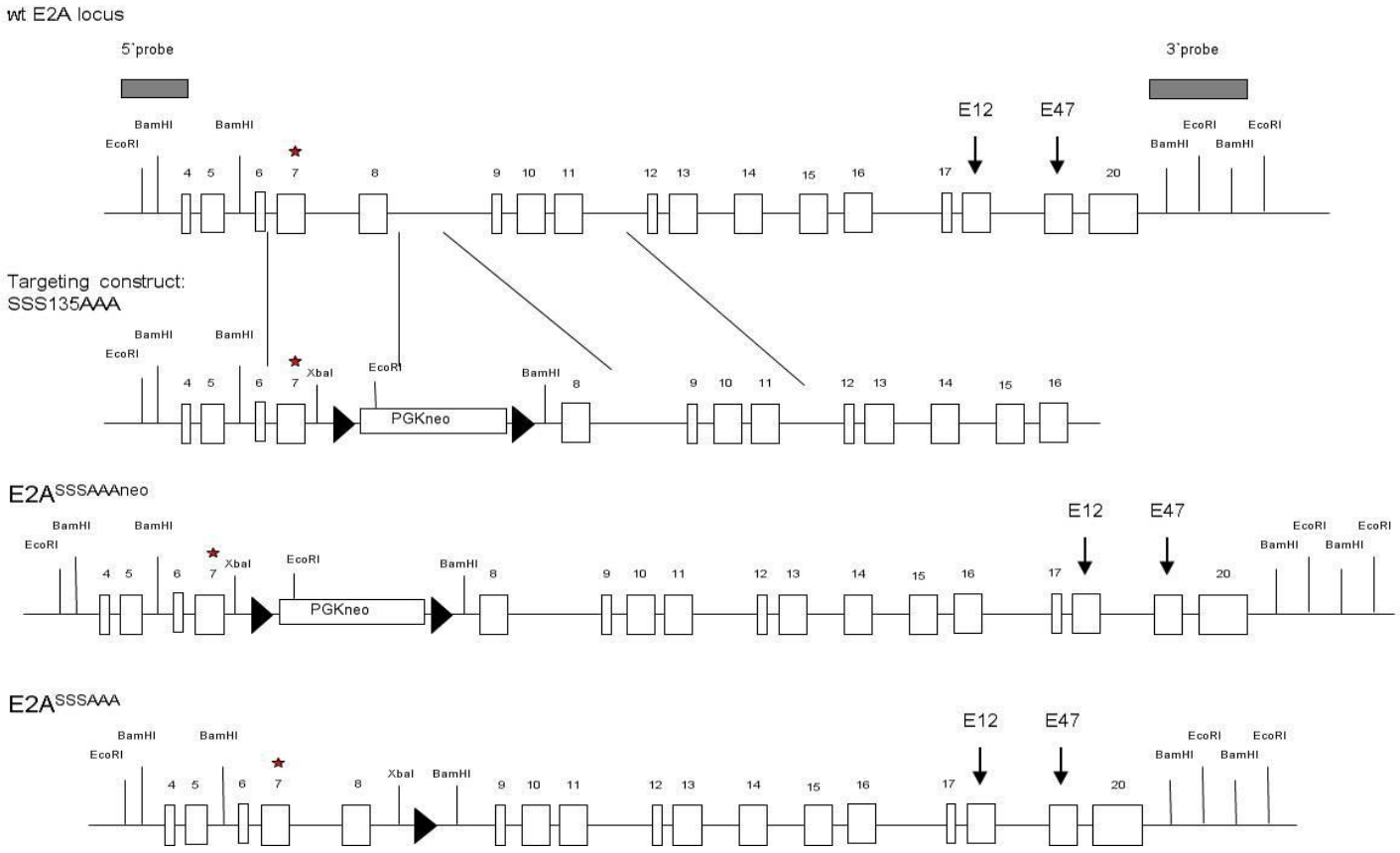

Figure 3.2 Replacement of residue serines 135, 140 and 142 by alanine amino acids. The substitutions were introduced into the germ-line sequence by homologous recombination. Briefly, 129 embryonic stem cells were electroporated with the targeting vector as shown. Transient expression of Cre recombinase was used to excise the neomycin resistance cassette. Properly targeted ES were injected into the pseudo-pregnant mice. Offspring were analyzed by Southern blotting for germ-line transmission.

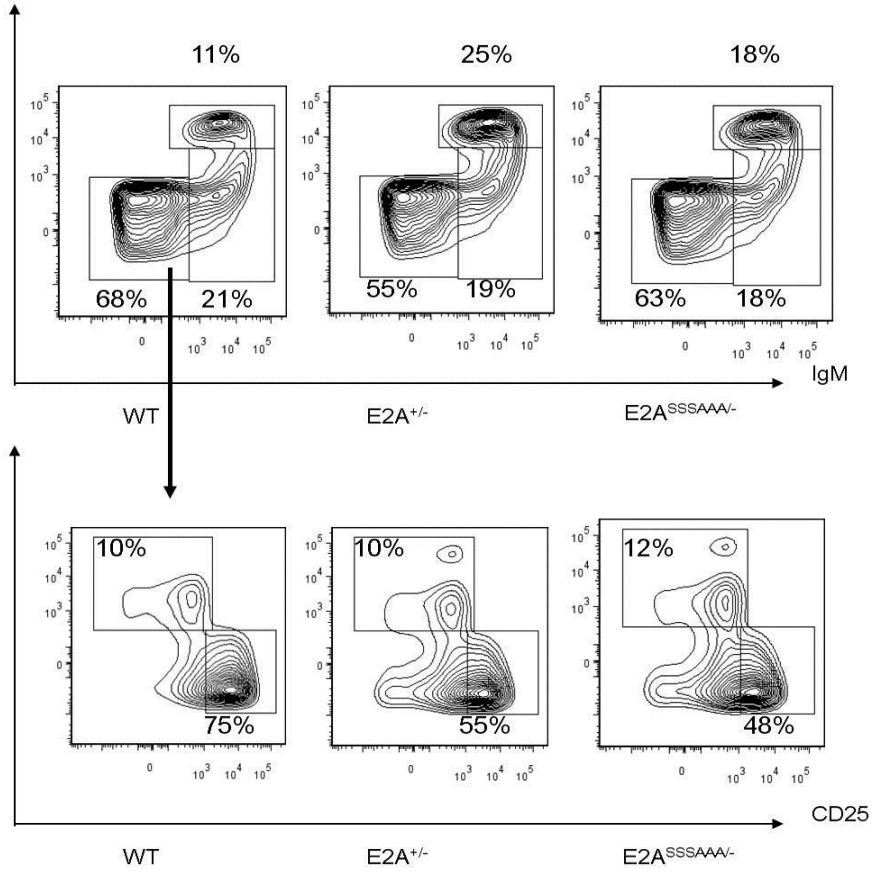

Figure 3.3 Flow cytometric analysis of wild-type and SSSAAA mutant mice. Bone marrow cells were isolated from wild-type, $E2A^{+/-}$ and $E2A^{SSSSAAA/-}$ mice. FACS analysis is shown for c-kit and CD25 expression gated on the lineage-negative/CD19+ compartments derived from wild-type, $E2A^{+/-}$ and $E2A^{SSSAAA/-}$ mice

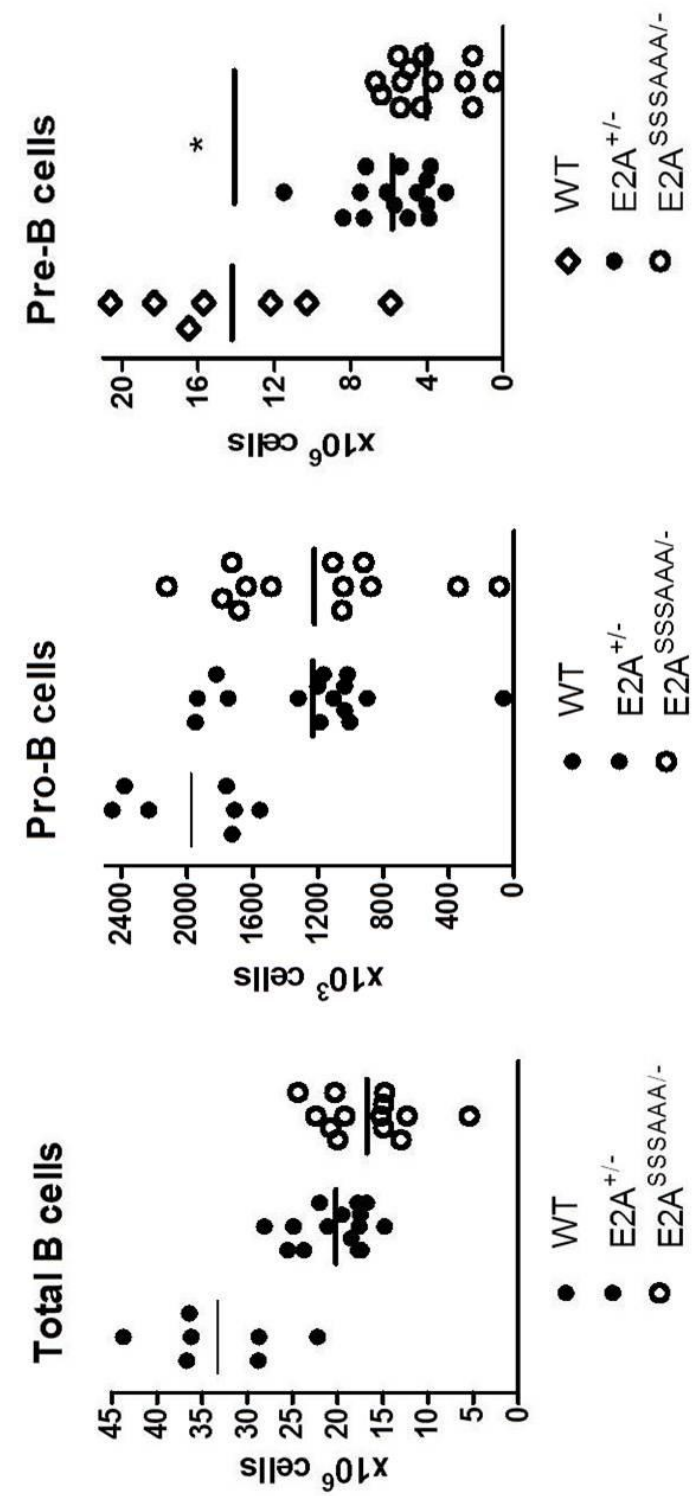

Figure 3.4 Absolute cell numbers are shown for wild-type, $E2A^{+/-}$ and $E2A^{SSSAAA/-}$ mice. Cell numbers are shown here for the B cell compartments.

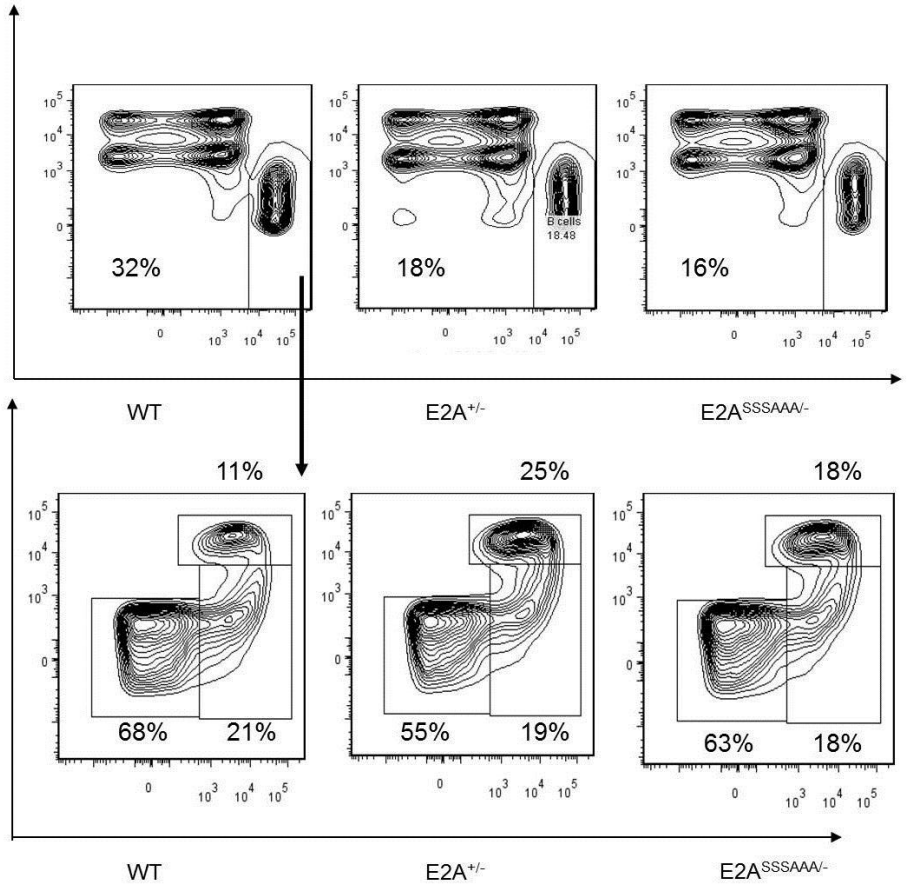

Figure 3.5 Flow cytometric analysis of wild-type and SSSAAA mutant mice. Bone marrow cells were isolated from wild-type, $E2A^{+/-}$ and $E2A^{SSSSAAA/-}$ mice. FACS analysis is shown for IgD and IgM expression gated on the lineage-negative/CD19+ compartments derived from wild-type, $E2A^{+/-}$ and $E2A^{SSSAAA/-}$ mice.

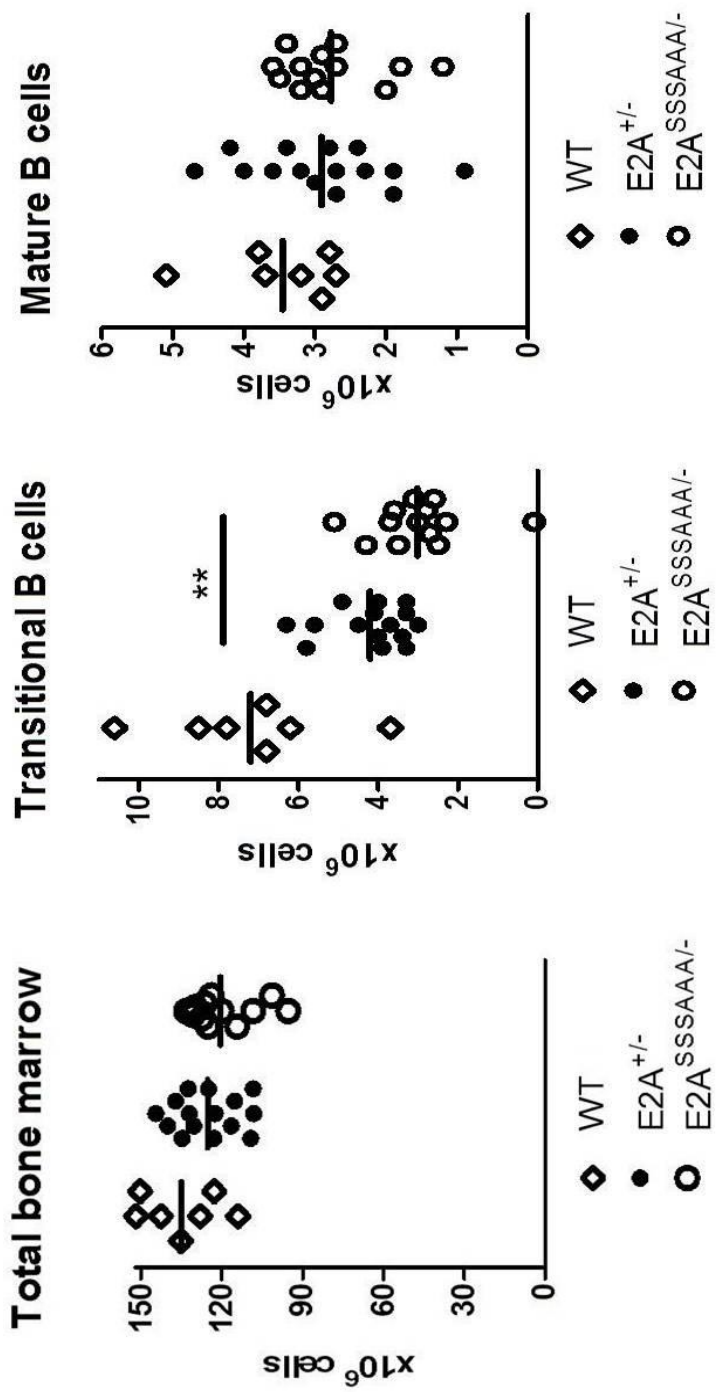

Figure 3.6 Absolute cell numbers are shown for wild-type, $E2A^{+/-}$ and $E2A^{SSSAAA/-}$ mice. Cell numbers are shown here for the B cell compartments.

	WT	$E2A^{+/-}$	$E2A^{SSSAAA/-}$
• Pre-B: Pro-B:	7.21	4.73	3.60
• Trans B: Pre-B:	0.51	0.72	0.76
• Mature B: Transitional B:	0.48	0.69	0.92
• Total B: Mature-B:	9.63	6.94	6.02
• Total Bone Marrow: Total B:	4.06	6.20	6.66

Figure 3.7 Ratios are shown for the wild-type , $E2A^{+/-}$ and $E2A^{SSSAAA/-}$ mice.

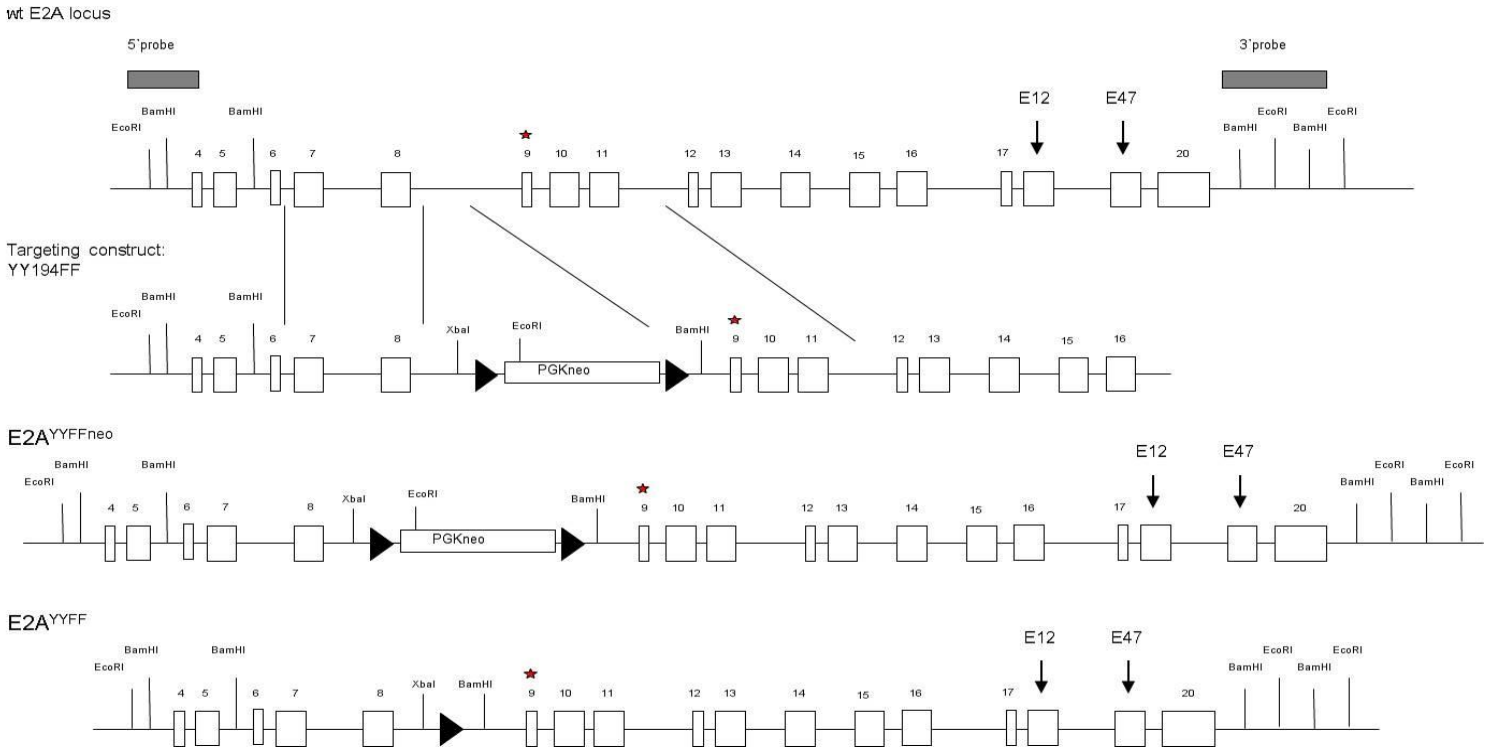

Figure 3.8 Replacement of residue serines 194 and 201 by alanine amino acids. The substitutions were introduced into the germ-line sequence by homologous recombination. Briefly, 129 embryonic stem cells were electroporated with the targeting vector as shown. Transient expression of Cre recombinase was used to excise the neomycin resistance cassette. Properly targeted ES were injected into the pseudo-pregnant mice. Offspring were analyzed by Southern blotting for germ-line transmission.

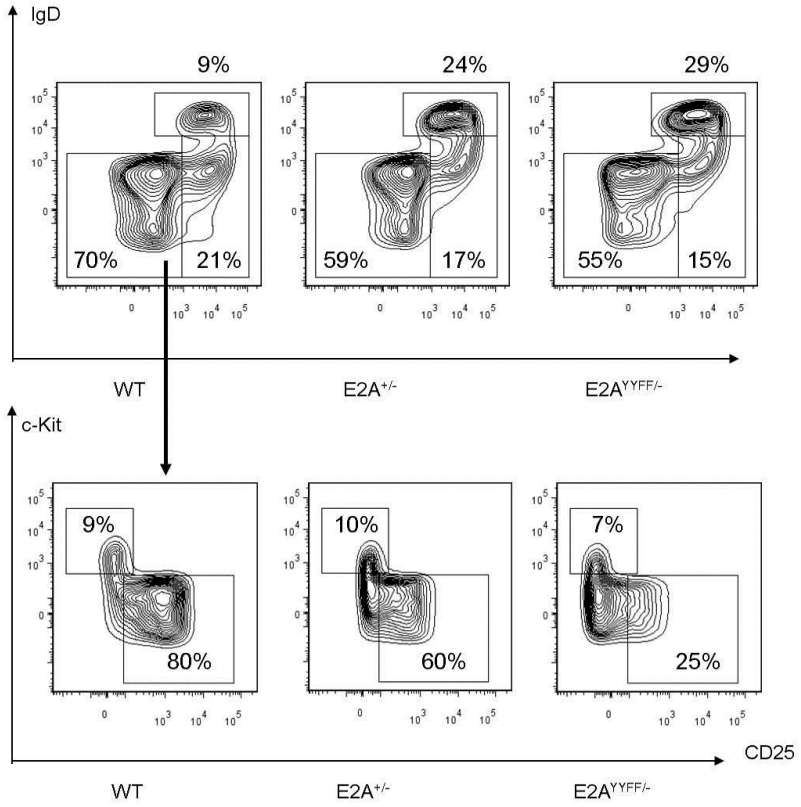

Figure 3.9 Flow cytometric analysis of wild-type and YYFF mutant mice. Bone marrow cells were isolated from wild-type, $E2A^{+/-}$ and $E2A^{YYFF/-}$ mice. FACS analysis is shown for c-kit and CD25 expression gated on the lineage-negative/CD19+ compartments derived from wild-type, $E2A^{+/-}$ and $E2A^{YYFF/-}$ mice.

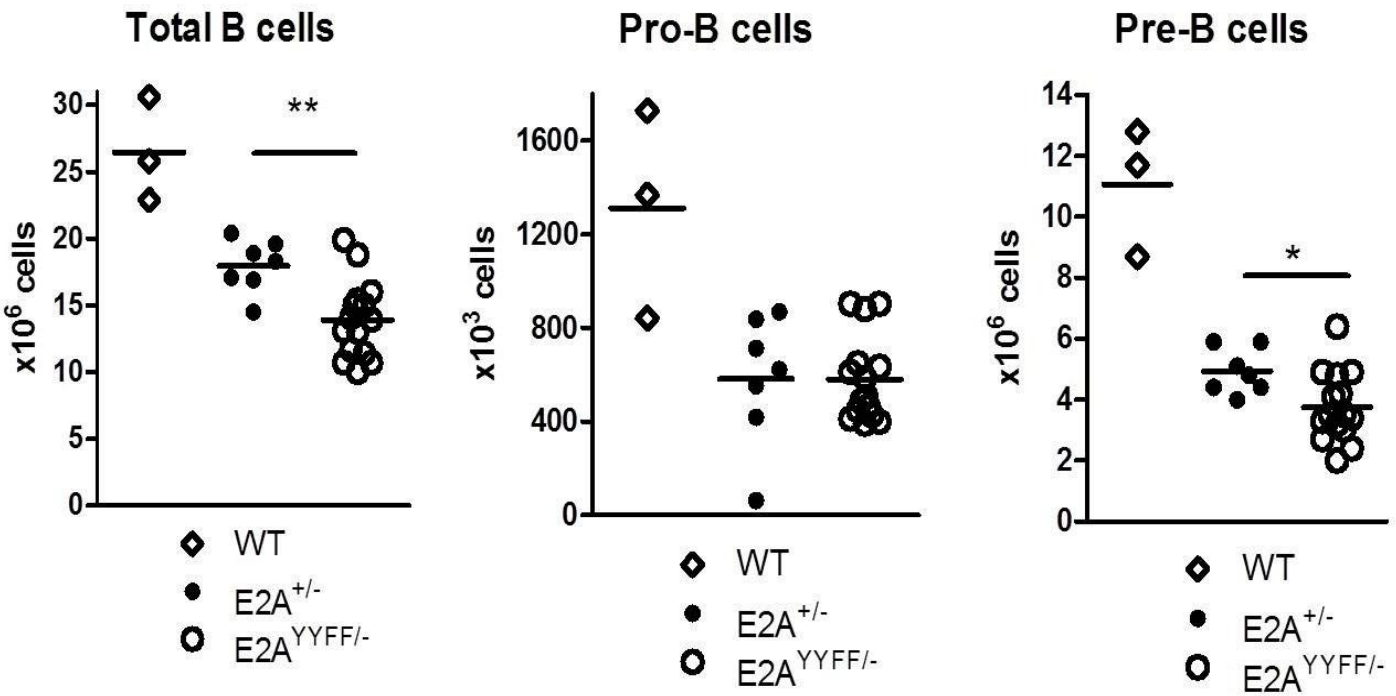

Figure 3.10 Absolute cell numbers are shown for wild-type, $E2A^{+/-}$ and $E2A^{YYFF/-}$ mice. Cell numbers are shown here for the B cell compartments.

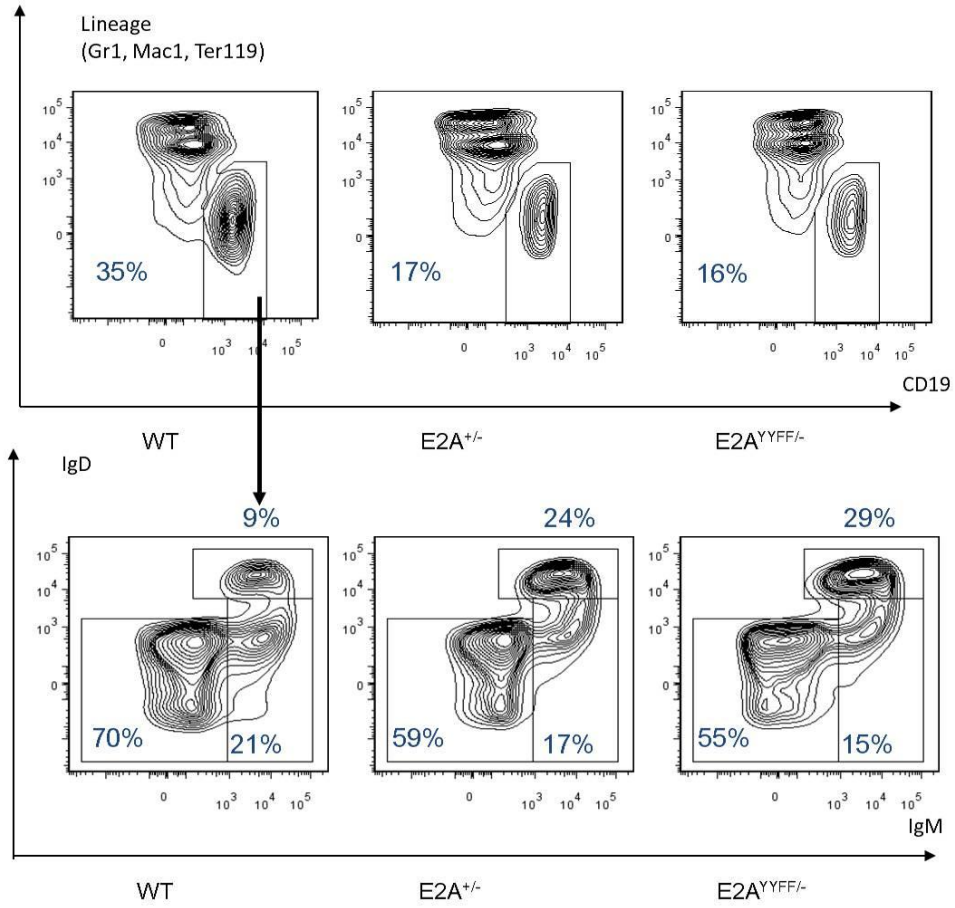

Figure 3.11 Flow cytometric analysis of wild-type and YYFF mutant. Bone marrow cells were isolated form wild-type, $E2A^{+/-}$ and $E2A^{YYFF/-}$ mice. Cells were stained using the appropriate antibodies as indicated. FACS analysis is shown for IgM and IgD expression gated on the lineage-negative/CD19+ compartments of wild-type, $E2A^{+/-}$ and $E2A^{YYFF/-}$ mice.

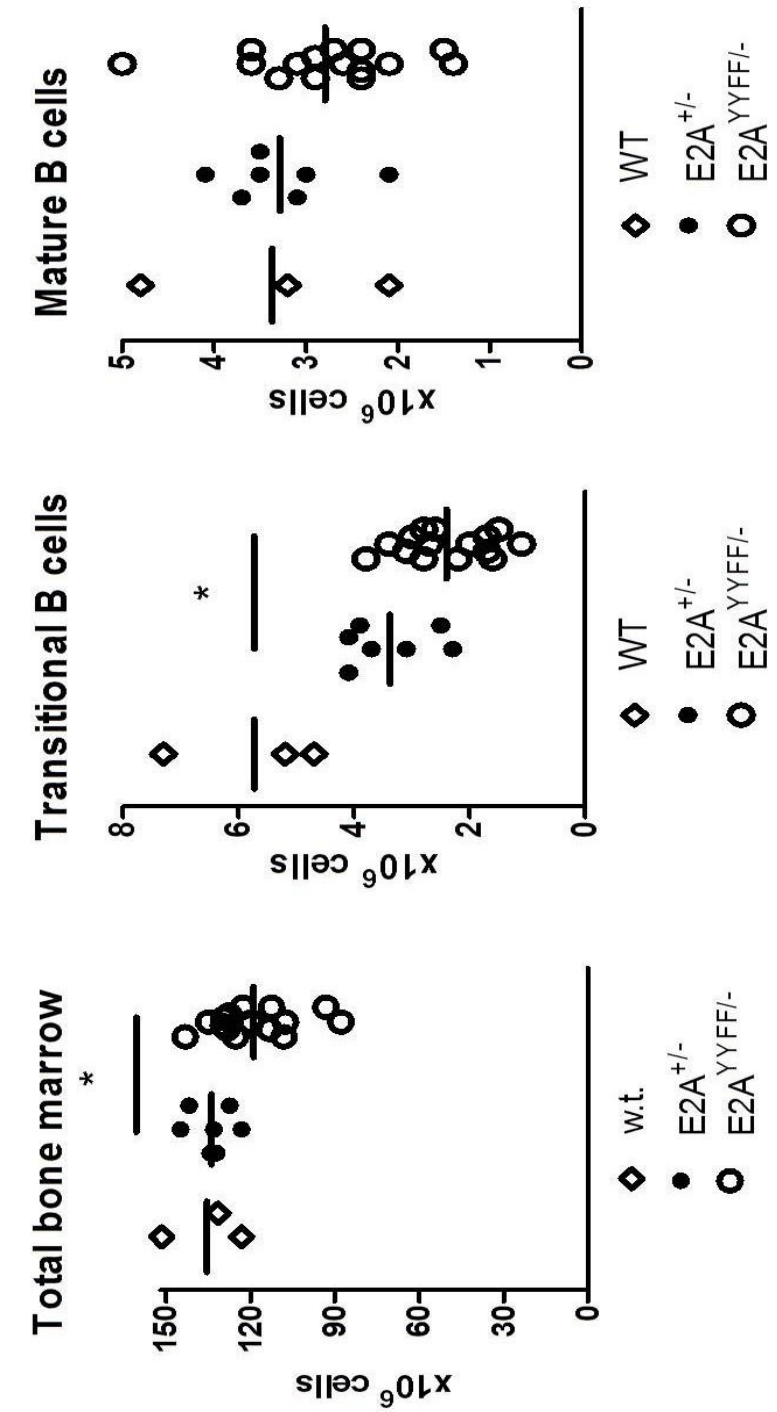

Figure 3.12 Total cells numbers of splenocytes, transitional B-cells and mature B-cells derived from wild-type, $E2A^{+/-}$ and $E2A^{YYFF/-}$ mice are indicated.

	WT	$E2A^{+/-}$	$E2A^{SSSAAA/-}$
• Pre-B: Pro-B	8.45	8.50	6.46
• Trans B: Pre-B	0.52	0.69	0.64
• Mature B: Transitional B	0.59	0.97	1.16
• Total B: Mature-B	7.84	5.49	4.99
• Total Bone Marrow: Total B	5.12	7.45	8.55

Figure 3.13 Ratios of B cell compartments are shown for wild-type, $E2A^{+/-}$ and $E2A^{YYFF/-}$ mice.

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Chapter IV.

The roles of E- and Id-proteins in PTEN-mediated lymphomagenesis

Summary

The Tumor Suppressor protein PTEN (Phosphatase and Tensin Homologue, deleted on chromosome 10) acts as a tumor suppressor in a wide variety of malignancies. Its role in suppressing the development of lymphoma is particularly well studied. Loss of PTEN is closely associated with the inactivation of members of the FOXO family. Recent observations have indicated that indeed loss of the FOXO members rapidly leads to the development of lymphoma. Among the critical targets of the FOXO proteins in thymocytes are the Id4 gene. Specifically, Id4 expression was demonstrated to be elevated in FOXO1, 3, 4 triple null mutant mice. Since previous studies have shown that forced Id-expression leads to the development of lymphoma similar as described for FOX1, 3, 4 triple null mutant mice we have explored the possibility that PTEN and FOXO act as tumor suppressors by inhibiting the expression of Id4 in the thymus. Here we show that E-protein activity is a critical target for PI3K-AKT mediated signaling. We show that loss of Id4 does not significantly interfere with lymphomagenesis in PTEN-deficient mice but that forced E47 significantly interferes with loss-of-PTEN mediated lymphomagenesis. Thus, these observations indicate that aberrant expression of Id4 in FOXO1, 3, 4 triple deficient mice is not a key factor in mediating lymphomagenesis but that forced expression of E47 significantly affects lymphoma development in PTEN deficient mice.

Introduction

The phosphoinositide 3-kinase (PI3K)-AKT pathway controls a diverse set of effectors. Among these are Phospholipase C, Protein Kinase B (Akt), Protein Kinase C, Inositol Triphosphate, Src Homology Inositol Phosphatase 1, Bruton's tyrosine kinase as well as the mammalian Target Of Rapamycin (mTOR) and Tuberous Sclerosis signaling complexes (TSC1 and TSC2). A main target of PI3K-mediated signaling is AKT, a serine/threonine kinase which itself modulates cell motility, growth, proliferation, metabolism, cell cycle, and apoptosis. Among the effectors of the Akt signaling pathway are Glycogen Synthase Kinase 3, the cell cycle inhibitors p21 and p27, the apoptotic factor Bad, and Mdm2. Akt is, in turn, regulated by the lipid phosphatase PTEN, which directly counteracts PI3K activity.

PI3K is primarily activated by receptor tyrosine kinases. In developing B cells, antigen-receptor interactions lead to the activation PI3K initiated by Syk which phosphorylates CD19 and the PI3K Adaptor (BCAP). CD19 and BCAP, in turn, activate and recruit PI3K to the plasma membrane to phosphorylate the 3' hydroxyl group of phosphatidyl inositol 4,5 bis-phosphate (PIP_2), a lipid found exclusively on the cytosolic face of the plasma membrane. This phosphorylation event converts PIP_2 into phosphatidyl inositol 3,4,5 tris-phosphate (PIP_3). PIP_3 is then able to act as a site for the recruitment of two serine/threonine kinases: Phosphatidylinositol Dependent Kinase 1 (PDPK1), and Akt. Once localized together, PDPK1 phosphorylates Akt at threonine residue 308, activating it and promote release from PIP_3 to diffuse into the cytoplasm and nucleus in order to phosphorylate its downstream effectors.

The Forkhead Box O (FoxO) binding also are regulated by AKT signaling. FOXO proteins bind the motif BBTRTTTTD. FOXO proteins were initially discovered for their role in extending the lifespan of the nematode C. elegans. FoxO transcription factors in B cells act to mediate cell cycle arrest, apoptosis and promote immunoglobulin class switching.

Aberrant activation of the PI3K-AKT pathway has been demonstrated to play an important role in a wide variety of cancers. Prominent among these are loss of function mutations in the PTEN gene in melanomas, prostate cancers, lung cancers, sporadic glioblastomas, breast tumors and lymphomas (Sakai, et al, 1998)(Suzuki et al., 1998)(Cully et al., 2006). Mouse cancer models have confirmed the critical role of PTEN in transformation(Di Cristofano & Pandolfi, 2000)(Vivanco & Sawyers, 2002). When targeted for deletion in hematopoietic cells, PTEN depletion readily results in myeloid leukemia as well as lymphomagenesis (Cully et al., 2006)(Yilmaz et al., 2006)(Zhang et al., 2006). Most recently critical targets of the PTEN-FOXO axis have been identified. Prominent among these are the HLH proteins Id1 and Id4 (Paik et al., 2007).

Id proteins act by suppressing the DNA binding activities of E-proteins(Yokota et al, 1999)(R. Rivera & C Murre, 2001)(R. Rivera & C Murre, 2001). Id proteins operate by their ability to heterodimerize with E-proteins, preventing E proteins from binding with relevant bHLH proteins (including other E-proteins) to form DNA-binding dimers. Id1 and Id3 are broadly expressed in a variety of tissues. Id2 and Id4 are more restricted in their expression patterns. Id proteins contain an HLH domain but lack a basic region

and inactivate the DNA binding activity of E-proteins upon dimerization (Benezra et al., 1990). Four Id proteins are expressed in mammalian cells, named Id1-4.

The Id4 HLH protein is less well characterized than Id1-3. It is highly expressed in brain, kidney, and the testis, and has a moderate level of expression in the brain (Riechmann et al, 1994). Mice that are homozygous for a germline deletion exhibit a high frequency (~50%) of in uteri or neonatal lethality by the age of weaning. Additional $Id4^{-/-}$ mice lost weight and died, leaving approximately 20% of mutants alive at adulthood. It is also known to be required for correct timing in neural differentiation, as the neural precursor cells (NPCs) of homozygous mutants have increased death by apoptosis. NPCs of Id4-/- mice differentiate prematurely into neurons, resulting in a thicker neocortex and smaller overall brain size (Bedford et al., 2005).

Enforced expression of Id1 and Id2 in transgenic mice rapidly leads to lymphomagenesis (Kim et al., 1999)(Morrow et al., 1999). Enforced expression of E47 into cell lines adapted from lymphomas that developed in E2A null mutant mice leads to apoptosis, whereas ectopic expression of E47, in conjunction with Bcl-2, results in cell cycle arrest and a reduction in cell size (Engel & Murre, 1999)(Engel & Murre, 2004). The precise mechanism by which E47 and HEB act as tumor suppressors remains to be determined.

Here we show that E-protein activity is a critical target for PI3K-AKT mediated signaling. We show that loss of Id4 does not significantly interfere with lymphomagenesis in PTEN-deficient mice but that forced E47 significantly interferes with loss-of-PTEN mediated lymphomagenesis.

Results

Ectopic expression of E47R interferes with the development of T cell lymphoma in PTEN-deficient mice

PTEN and E47-deficient mice rapidly develop lymphoma with similar kinetics. Previous observations indicated that the PTEN-FOXO axis activates Id4 expression in developing thymocytes. Hence, we examined whether increased Id4 abundance in PTEN-deficient cells results in lymphomagenesis in PTEN-ablated mice. To address this question, we forced the expression of E47S524A in PTEN-deficient T-lineage cells. Bone marrow cells were isolated from PTEN F/F; Lck-Cre mice that were treated with 5-fluoro-uracil, lineage-negative cells were purified and transduced with control virus or virus expressing E47S524A. Infected cells were isolated and transferred into irradiated recipients. During the first two months post-injection, Three mice that were injected with Lck-Cre;$PTEN^{F/F}$ bone marrow cells and transduced with control virus died within a three month period. On the other hand, mice transplanted with Lck-Cre;$PTEN^{F/F}$ bone marrow cells, transduced with virus expressing $E47^{S528A}$ all survived during these three months. Two and a half months post-transplantation, the mice carrying $PTEN^{-/-}$;$E47^{S528A}$ cells were assessed for their capability to reconstitute the T cell lineage. As expected, $PTEN^{-/-}$ progenitor cells expressing $E47^{S528A}$ exhibited efficient reconstitution.

The recipient mice injected with control virus showed greatly enlarged thymi (120-1500 million cells per thymus). On the other hand, mice injected with Lck-Cre;$PTEN^{F/F}$ mice carrying $E47^{S524A}$ virus did not exhibit enlarged thymi. Spleens isolated from the reconstituted mice showed similar results. Recipient mice harboring Lck-Cre;$PTEN^{-/-}$ bone marrow cells transduced with control virus showed large spleens

containing large numbers of white blood cells. Remarkable, the size of the spleen and white blood cell numbers were normal in mice transplanted with Lck-Cre;PTEN$^{-/-}$ bone marrow cells harboring E47^{S524A}. Furthermore, histological effacement was observed in recipient mice transplanted with PTEN-deficient progenitor that expressed control virus. In contrast, mice transplanted with PTEN-deficient progenitors carrying E47^{S524A} showed normal splenic histology. These observations indicate that E47^{S524A} expression interferes with the development of lymphoma in in PTEN-deficient T-lineage cells.

Absence of Id4 does not interfere with the development of lymphoma in PTEN$^{-/-}$ mice

Since in FOXO 1,3,4$^{-/-}$ mice rapidly develop T cell lymphoma with similar kinetics we considered the possibility that FOXO triple mutant mice target Id gene expression to promote lymphomagenesis. Consistent with this possibility were recent findings indicating that FOXO proteins directly suppress the expression of Id4 in developing thymocytes. To evaluate the importance of Id4 upregulation in the development of thymic lymphoma, we crossed mice with a conditional deletion of PTEN in developing thymocytes (PTEN$^{F/F}$ Lck-Cre^{+}) with mice homozygous for a germ-line deletion of Id4. Mice with deletions in both Id4 and PTEN that survive into adulthood show an average increase in lifespan of ~28%, ($p=0.02$) compared to mice lacking PTEN but having normal alleles of Id4 (Figure 4.4). Thus, these data indicate that the loss of Id4 only has a modest impact on the development of lymphoma in PTEN-ablated mice.

Discussion

E47 and PTEN-deficient mice rapidly develop T-cell lymphoma with similar kinetics. Furthermore, in previous studies it was shown that Id4 is a critical target of the FOXO proteins. This stimulated us to determine whether lymphomagenesis in PTEN-deficient mice can be abolished by enforced expression of E47 or the absence of Id4. We demonstrate here that expression of $E47^{S528A}$ suppressed lymphomagenesis in PTEN-deficient mice. We note, however, that forced E47 expression is not sufficient to completely block lymphomagenesis in PTEN deficient T-lineage cells. Few of the transplanted mice showed lymphomagenesis two months post injection. In contrast, recipient mice that were reconstituted with lymphomas that express $E47^{S524A}$ exhibited malignancy beyond the thymus. Virtual all recipient mice transplanted with cells carrying control virus showed malignancy in the spleen. Thus, forced expression of E47 substantially interferes with the development of lymphoma in PTEN-deficient mice.

Previous experiments have established the FOXO proteins directly act on Id4 expression in the thymus. These data raise the possibility that FOXO- and PTEN-ablated mice develop thymic lymphomas because of an increase in Id4 expression. However, here we show that the absence of Id4 does not significantly interfere with the development of lymphoma in PTEN-null mutant mice. Thus, Id4 does not appear to be a significant target of FOXO activity. This then raises the question as to how forced E47 expression blocks cell growth in PTEN-ablated mice. It is conceivable that PTEN acts to maintain high levels of E-protein activity. It will be important to determine whether E-protein abundance are modulated in PTEN-ablated mice to address this question in detail.

Experimental Procedures

Generation of 293 cell line expressing E47

In order to generate a cell lines expressing an inducible form of E47, we generated an ecdysone-inducible mammalian expression system (Invitrogen). Specifically, FLAG and HIS peptides were merged with the E47 N-terminus of E47 and inserted into an ecdysome inducible promoter construct (Figure 1). One clone was isolated that showed substantial inducibility by E47 (E47-13 C2) using calcium-phosphate mediated transfection. Zeocin (400µg/ml) and Geneticin (1mg/ml) were used as selectable markers.

Western Blotting

Nuclear lysates were analyzed using a 4-12% Bis-tris gradient gel (Invitrogen). Gels were next transferred to PVDF membranes. The filters were blocked with 5% nonfat dry milk in 1xPBS with 0.1% tween-20) for 1 hour (room temperature) or overnight (4^oC). Subsequently, the filters were incubated with anti-E47 antibody 271 (BD Pharmingen) at a dilution of 1:5000 for 1 hour (room temperature) or overnight at 4^oC. The blots were washed with 1xPBS with 0.1% tween-20 for three consecutive times for a period of five minutes and incubated at room temperature in the presence of goat anti-mouse-HRP secondary antibody (Santa Cruz Biotechnology) using a dilution of 1:5000 for 1 hour. The filters were next washed with 1xPBS containing 0.1% Tween-20 3 X 5' and analyzed by phosphoimager.

Mass-spectrometry analysis

E47 was purified from nuclear lysates (Ponasterone A (5μM) induced in E47-13 C2 cells) and purified using EZview Red ANTI-FLAG M2 Affinity GEL (Sigma) and eluted twice with 100μg/ml 3X FLAG peptide. The two eluted fractions were combined and subjected to a second purification step using TALON Metal Affinity Resins (Upstate). 200mM Imidazole (plus 300mM NaCl and 50mM Sodium Phosphate) was used to elute the tagged proteins. The eluted proteins were reduced and alkylated using 2 mM Tris(2-carboxyethyl) phosphine (Fisher, AC36383) at 37°C for 30 minutes and 5 mM iodoacetamide (Fisher, AC12227) at 37°C in dark for 30 minutes, respectively. The proteins were digested with 1 μg trypsin (Roche, 03 708 969 001) at 37°C overnight.

Automated 2D nanoflow LC-MS/MS analysis was performed using LTQ tandem mass spectrometer (Thermo Electron Corporation, San Jose, CA) employing automated data-dependent acquisition. Each MS scan was followed by 4 MS/MS scans of the most intense ions from the parent MS scan. A dynamic exclusion of 1 minute was used to improve the duty cycle of MS/MS scans. About 150,000 MS/MS spectra were collected for each sample.

Raw data were extracted and searched using Spectrum Mill (Agilnet, version A.03.02.060b). MS/MS spectra with a sequence tag length of 1 or less were considered as poor spectra and discarded. The rest of the MS/MS spectra were searched against the IPI (International Protein Index) database limited to mouse taxonomy. A concatenated forward-reverse dataset is used to calculate the in-situ identification false positive rates (FDR). The enzyme parameter was limited to full tryptic peptides with a maximum mis-cleavage of 1. All other search parameters were set to SpectrumMill's default settings (carbamidomethylation of cysteines, +/- 2.5 Da for precursor ions, +/- 0.7 Da for

fragment ions, and a minimum matched peak intensity of 50%). None of the identified protein groups are from the reverse database. Thus the false positive rate of our filtering criteria is below 0.5% at the protein level.

Acknowledgments

We would like to acknowledge Kristina Beck for creation of the $E47^{S528A}$ mouse strain depicted herein. We also wish to acknowledge Lilyan Wright, who initially performed to the experiments transducing bone marrow leading to the suppression of thymic lymphoma. Her contributions in this chapter include Figures 3.2 and 3.3.

Additionally, Zhouxin Shen and Steve Briggs were instrumental in performing proteomics analysis leading to discovery of proteins associating with E47, and discovery of post-translational modification sites of E47.

Chapter 4, in part, has been submitted for publication of the material as it may appear in Molecular and Cell Biology, 2011, Teachenor, Robert; Beck, Kristina; Wright, Lilyan Y.T.; Shen, Zhouxin; Briggs, Steve; and Murre, Cornelis; 2011. The dissertation author was the primary investigator and author of this paper.

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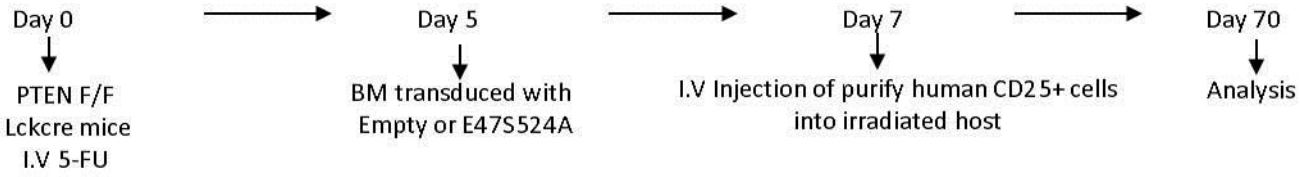


Figure 4.1 Suppression of increased thymic and splenic hypercellularity by overexpression of E47^{S528A}. Irradiated recipient mice were transplanted with PTEN$^{F/F}$ Lck-Cre^{+} bone marrow transduced with empty retrovirus showed increased cellularity in both the thymus and spleen. Mice reconstituted with PTEN$^{F/F}$ Lck-Cre^{+} bone marrow transduced with E47^{S528A} exhibited reduced hypercellularity in the thymus and the spleen.

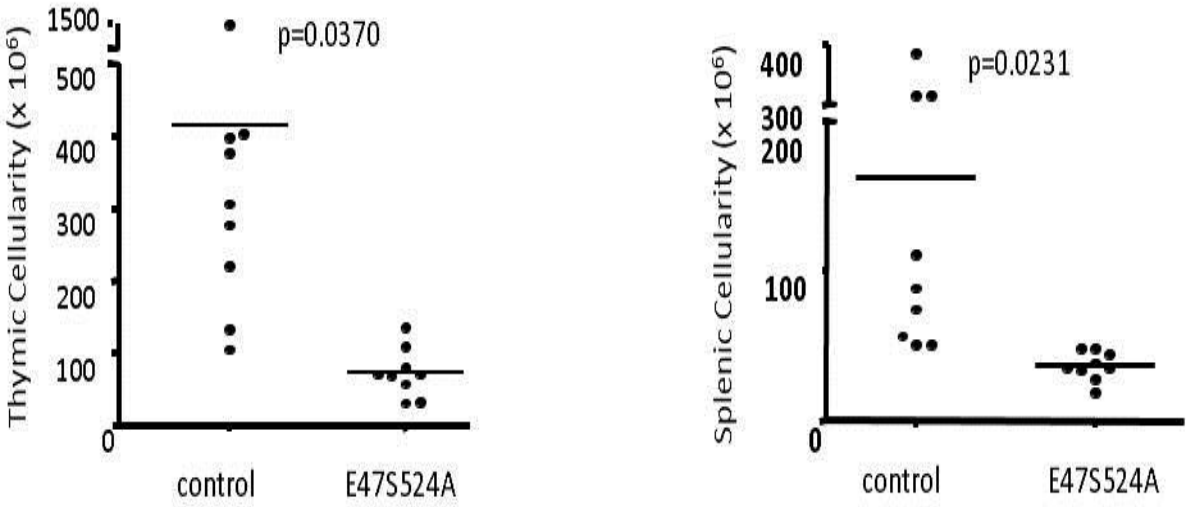


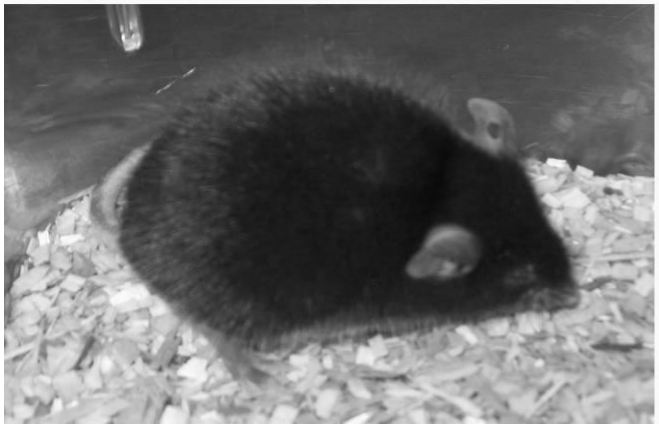
Figure 4.2 Suppression of increased thymic and splenic hypercellularity by overexpression of E47^{S528A}. Irradiated recipient mice were transplanted with PTEN$^{F/F}$ Lck-Cre^{+} bone marrow transduced with empty retrovirus showed increased cellularity in both the thymus and spleen. Mice reconstituted with PTEN$^{F/F}$ Lck-Cre^{+} bone marrow transduced with E47^{S528A} exhibited reduced hypercellularity in the thymus and the spleen.

Control vector

mouse number	histology diagnose	
	thymic lymphoma	spleen
#1	+	+
#2	+	transit
#3	Died at 10 weeks	-
#4	+	-
#5	+	+
#6	+	-
#7	+	+
#8	+	+
#9	+	transit
#10	Died at 11 weeks	N/A
#11	Died at 11 weeks	N/A

E47S524A

mouse number	histology diagnose	
	thymic lymphoma	spleen
#12	+	-
#13	+	-
#14	-	-
#15	-	-
#16	+	-
#17	+	-
#18	-	-
#19	-	-
#20	N/A	N/A
#21	N/A	N/A



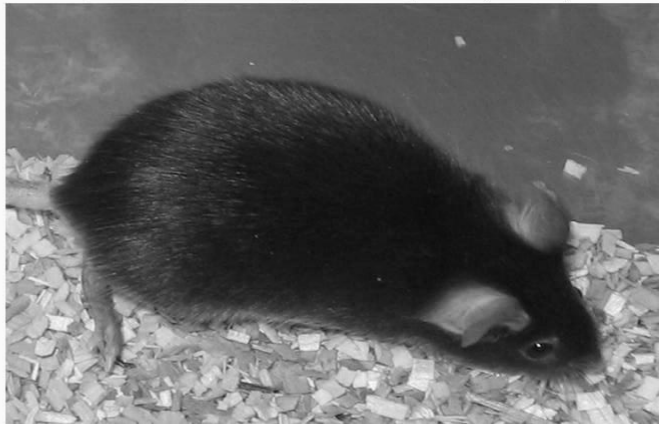


Figure 4.3 In vivo suppression of thymic lymphoma by overexpression of E47^{S528A}. Mice receiving PTEN$^{F/F}$ Lck-Cre^{+} bone marrow transduced with empty retrovirus showed poor grooming, hunched posture, listless behavior, and succumbed to thymic lymphoma. Mice receiving PTEN$^{F/F}$ Lck-Cre^{+} bone marrow transduced with E47^{S528A} exhibited significantly reduced incidence of thymic lymphoma.

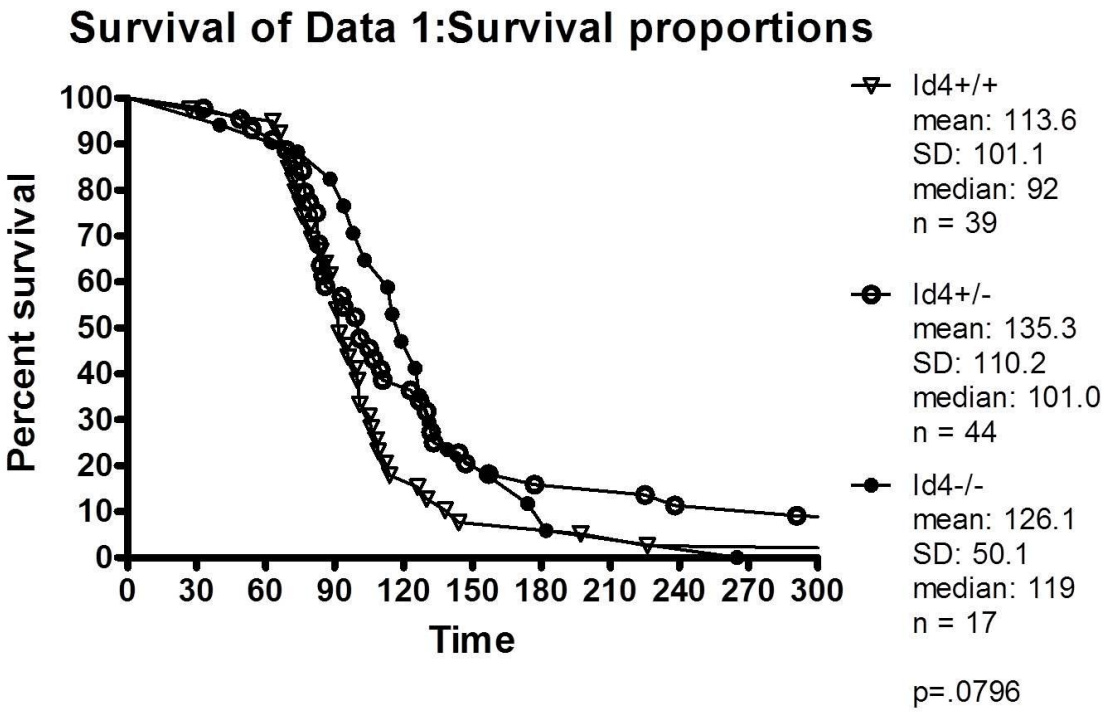


Figure 4.4 PTEN$^{F/F}$ Lck-Cre^{+} ID4+/+, PTEN$^{F/F}$ Lck-Cre^{+} ID4+/-; PTEN$^{F/F}$ Lck-Cre^{+} Id4-/- . Normally these mice succumb to thymic lymphoma at ~113 days of age. Loss of Id4 leads to increases in lifespan of PTEN$^{F/F}$ Lck-Cre^{+} mice, averaging ~135 days in mice hemizygous for Id4, and ~126 days for mice lacking Id4.

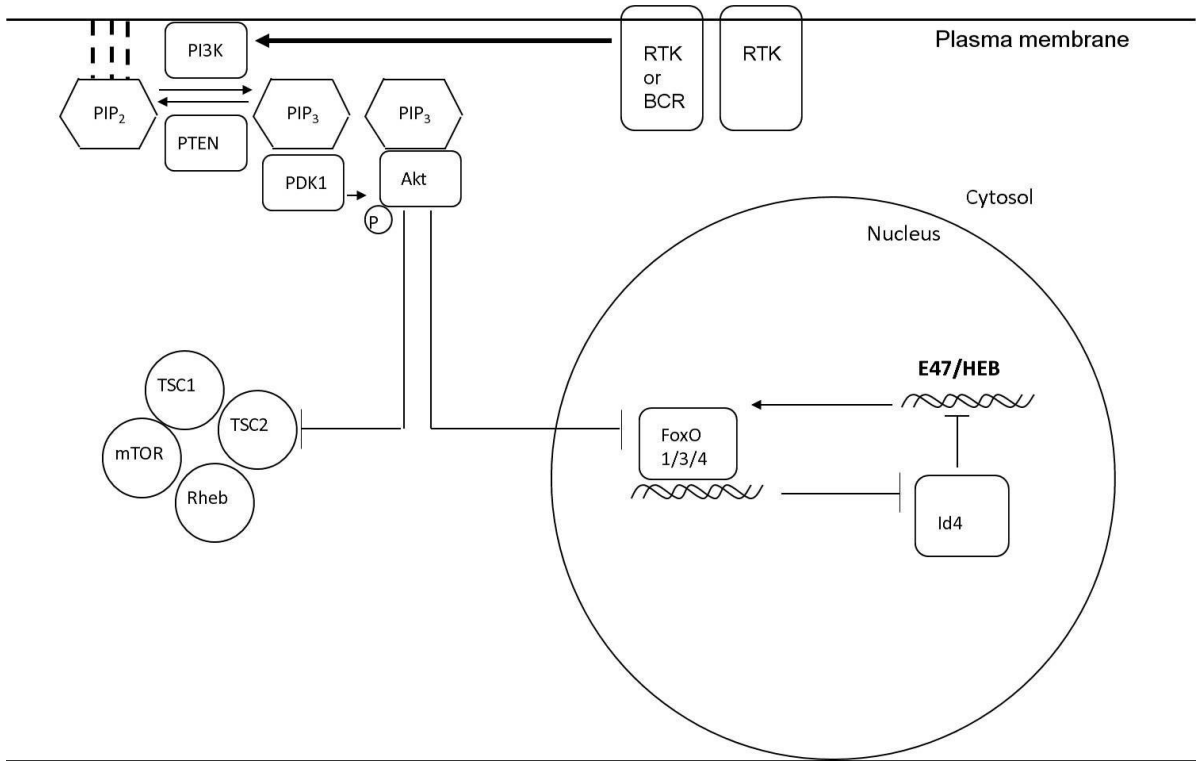


Figure 4.5 PI3K/Akt signaling pathway. When activated by growth factors, PI3K phosphorylates PIP_2, turning it into PIP_3, which subsequently acts as a docking site for PDK1 and Akt. PDK1 then activates Akt by phosphorylation, allowing it to diffuse into the cytosol and nucleus. Akt phosphorylates its downstream effectors, including the FOXO class of transcription factors. FOXO1,3, and 4 then are exported away from the nucleus to undergo proteolysis. In the absence of AKT signaling FOXO proteins suppress Id4 expression, whereas upon AKT signaling FOXO proteins dissociate from the DNA permitting the aberrant activation of Id4 expression.

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V.

General Discussion

Previous studies have demonstrated critical roles for the E2A proteins in multiple developmental pathways. For example, the E2A proteins act throughout B cell development to enforce developmental checkpoints and promote developmental progression (Murre, 2005). Similarly, during T cell development the E2A proteins act in concert with HEB to promote early T cell development and enforce the pre-TCR and TCR checkpoints (Engel et al., 2001; Engel et al., 2004). In both pathways they activate or repress down-stream target gene expression (Murre, 2005). In developing B cells the E2A proteins function to induce the expression of RAG1, RAG2, IL7Rα, EBF, PLCγ, λ5, V-preB, Igα, Pax5, and FoxO1 (Lin et al., 2010). Furthermore, the E2A proteins also have been implicated in the control of cell cycle progression, cell survival and tumor suppression (Engel et al., 2004; O'Neil et al., 2004).

The activity of the E2A proteins is modulated at different levels. They either act as homodimers or as heterodimers. The E2A proteins belong to a family of transcription factors, named helix-loop-helix (HLH) proteins (Murre, 2005). The HLH proteins can be segregated into different classes, based on their biochemical properties and expression patterns. Prominent among these classes is the Class I bHLH proteins, also named E-proteins. The E2A proteins belong to this latter subset of HLH proteins. The E2A proteins interact with E-box sites (GCACGTGG) that are spread across the genome. In B-lineage cells they bind DNA as homodimers. However, in developing thymocytes they readily form heterodimers with another E-protein termed HEB. The HLH domain mediates dimerization, whereas the DNA binding activity is controlled by a basic region located immediately N-terminal of the HLH region. The DNA binding activities of E-proteins are regulated by the Id gene products, named Id1-4. They lack the basic region

but encode for an HLH dimerization domain (Benezra et al., 1990). Id proteins readily form heterodimers with the E2A proteins to suppress their DNA binding activity. Here it is shown that the Id4 protein plays a role in suppressing the development of PTEN-deficient lymphomas.

Initially studies have suggested that the E2A proteins are dephosphorylated to permit the formation of homodimers (Shen et al., 1995). Here we have extended these studies and examined in detail the role of post-translational modifications across the E2A protein in B cell development. Phosphorylated E2A residues as identified by mass spectrometry were analyzed for their roles in B cell development. Specifically, we have examined the function of (1) phosphorylated residues located across a putative AKT substrate site, (2) two phosphorylated tyrosine residues and (3) a cluster of three phosphorylated serine residues in the E2A transactivation domains. In order to address the function of these residues, mutant mice were generated that carry mutation in these residues. We then analyzed the mutant mice for abnormalities in B cell development.

Of particular interest is the role of the putative AKT substrate site in B cell development. Mutant mice that carry mutations in E2A or components of the PI3K pathway have shown very similar phenotypes. Specifically, the E2A proteins and the PI3K pathway play critical roles during developmental progression of immature-B cells (Quong et al., 2004). E47 levels are high in immature-B cells in order to induce the expression of RAG and to promote Ig light chain gene rearrangement and subsequently receptor revision. However, E2A protein levels decline in the absence of auto-reactivity, permitting the developmental maturation towards the mature-B cell stage (Quong et al. 2004). Similarly, previous observations have demonstrated that in the absence of auto-

reactivity, PI3K p110d subunit is essential to decrease RAG expression (Llorian et al., 2007). Thus it seems conceivable that the E2A proteins are potential targets of PI3K signaling in mature-B lineage cells. This indeed seems reasonable since the E2A proteins are phosphorylated at a highly conserved putative AKT substrate residue. However, here we demonstrate that mutation of the putative AKT substrate recognition site does not significantly affect B cell development at any stage. We note that this does not imply that phosphorylation of the AKT substrate residue does not play a role in the developmental transition of the immature- to the mature-B cell stage. It is quite conceivable that modulation of E2A activity by the PI3K-AKT pathway acts in concert with the modulation of FOXO activity. Thus, it will be of important to determine whether FOXO and E2A act in concert to modulate developmental progression through the immature-B cell checkpoint.

As described above, mutations in the triple serine residues show a modest impact on B cell development. The N-terminal portion of the E2A proteins contains the transactivation domains. Two domains are present in E-proteins that mediate transactivation, named the AD1 and AD2 (Massari et al., 1999; Massari et al., 1999). The AD1 region folds into a helical conformation upon interacting with co-activators or co-repressors (Massari et al., 1999). The AD1 domain is characterized by a series of highly conserved residues, named the LFDS motif. The LFDS motif interacts with CBP/p300 to activate downstream target gene expression (Murre, 2005). Interestingly, it also acts as a transcriptional repressor region upon interacting with members of the ETO proteins (Zhang et al., 2004). ETO proteins act to recruit the co-repressor N-COR and members of the histone deacetylase family. The AD2 domain consists of a putative loop-

helix structure that is present in all E-proteins (Quong et al. 1993). In this study we show that mutation of a series of clustered serine residues (S135; S140; S142) that are phosphorylated modestly perturb B-lineage development. These mutations are located immediately C-terminal of the first activation domain of E2A (Massari et al., 1996; Massari et al., 1999; Massari and Murre, 2000). This domain contains the LDFS motif, previously shown to interact with the SAGA histone acetyltransferase complex, or with CBP/p300, alternatively (Massari et al., 1999). We speculate that this cluster of highly conserved residues modulates down-stream target gene expression by regulating the ability of E2A to interact with the co-activator complex p300 or members of the SAGA complex.

The most dramatic phenotypes were observed in mice that carry the YYFF mutations. The two tyrosine residues (Y194 and Y201) are not located in close proximity to the transactivation domains but rather seemed to be positioned between the two transactivation domains. How do these two tyrosine residues modulate E2A activity and consequently B cell development? Although still to be proven we suggest that Y194 and Y201 act to modulate the induction of transcription mediated by E2A. Structural analysis of the E2A transactivation domains will be required in order to gain insight into how the phosphorylation of these highly conserved residues modulate down-stream target gene expression.

As aforementioned the AD1 domain acts to recruit members of the SAGA complex as well as CBP/p300 (Massari et al., 2000). Furthermore, it has been demonstrated that E2A binding is closely associated with an increase in H3K4me1 (Lin et al., 2011). It will be particularly interesting to determine whether phosphorylation of

E2A residues and histone acetylation and methylation are directly linked to modulate down-stream target gene expression.

Finally, we note that the majority of the mutations described here show only a modest defect. It seems likely that they act in a redundant fashion. To address this problem, it will be necessary for future investigators to generate mice strains that carry the entire spectrum of mutations (SSSAAA, YYFF, and S528A) in a single mouse strain.

Our data also provides insight into the putative links involving E2A and the PI3K pathway in T cell lymphomagenesis. Both PTEN- and E2A-deficient mice enforce the pre-TCR checkpoint and have been shown to rapidly develop T-cell lymphoma (Bain et al., 1994; O'Neil et al., 2004; Hagenbeek et al., 2004). These previous observations raised the possibility that the PI3K pathway and E2A activity are directly linked to promote the development of T cell lymphoma. In order to address this question we forced the expression of E47 in PTEN-deficient mice. Strikingly, we found that loss-of-PTEN-mediated lymphomagenesis was suppressed by the forced expression of E47 (E47S524A). We note, however, that the development of lymphoma was not completely blocked in the presence of forced E47 expression. In fact, several of the PTEN-deficient mice transplanted with bone marrow cells that overexpressed E47 showed modest infiltration of transformed cells two months post injection. On the other hand, none of the mice transplanted with PTEN-deficient bone marrow cells expressing E47 showed spread beyond the thymus. Thus, forced E47 expression acts to suppress, in part, lymphomagenesis in PTEN-deficient thymocytes.

In previous studies it was established that the FOXO proteins interact directly with regulatory elements located in the Id4 promoter region. Furthermore, it was shown

that FOXO-1, 3, 4 triple deficient lymphomas readily develop T cell lymphoma (Paik et al., 2007). Interestingly, Id4 expression was substantially elevated in FOXO-1, 3, 4 triple-deficient mice (Paik et al., 2007). However, here we show that the absence of Id4 only modestly interferes with the development of lymphoma in PTEN-deficient mice. Thus, Id4 does not appear to be a critical target in loss-of-PTEN mediated lymphomagenesis. We are now faced with the question as to how forced E47-expression interferes with the development of lymphoma. We propose that the increase in Id4 expression in FOXO-1, 3, 4 contributes to the development of lymphoma, but that additional targets, possibly E2A and/or HEB act in concert with the increase in Id4 expression to promote the development of lymphoma in FOXO 1, 3, 4 triple deficient mice. Finally, we note that additional factors may also contribute to the development of loss-of-PTEN mediated lymphoma.

In conclusion, the studies described here show that post-translational modifications of the E2A proteins play a modest but significant role in promoting the developmental progression of B-lineage cells. In addition, we have shown that the E- and Id-protein machinery play a critical role in the development of PTEN-ablated T cell lymphomas. It will be particularly important to determine how post-translational modifications affect the transcriptional activity of E2A at a mechanistic level. We propose that phosphorylation of these residues permit the recruitment of histone acetyl transferases or enzymes involved in the methylation of lysine and arginine residues. Further studies should provide insight into these important questions.

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