



A recurrent dominant negative E47 mutation causes agammaglobulinemia and BCR⁻ B cells

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Approximately 90% of patients with isolated agammaglobulinemia and failure of B cell development have mutations in genes required for signaling through the pre-B cell and B cell receptors. The nature of the gene defect in the majority of remaining patients is unknown. We recently identified 4 patients with agammaglobulinemia and markedly decreased numbers of peripheral B cells. The B cells that could be detected had an unusual phenotype characterized by the increased expression of CD19 but the absence of a B cell receptor. Genetic studies demonstrated that all 4 patients had the exact same de novo mutation in the broadly expressed transcription factor E47. The mutant protein (E555K) was stable in patient-derived EBV-transformed cell lines and cell lines transfected with expression vectors. E555K in the transfected cells localized normally to the nucleus and resulted in a dominant negative effect when bound to DNA as a homodimer with wild-type E47. Mutant E47 did permit DNA binding by a tissue-specific heterodimeric DNA-binding partner, myogenic differentiation 1 (MYOD). These findings document a mutational hot-spot in E47 and represent an autosomal dominant form of agammaglobulinemia. Further, they indicate that E47 plays a critical role in enforcing the block in development of B cell precursors that lack functional antigen receptors.

Introduction

Approximately 85% of patients with early onset of infections, panhypogammaglobulinemia, and less than 2% CD19⁺ B cells in the peripheral circulation have X-linked agammaglobulinemia (1). This disorder is caused by mutations in Bruton tyrosine kinase (BTK) (2), a cytoplasmic tyrosine kinase that is activated by cross-linking of the pre-B cell and B cell antigen receptors (BCRs) (3). An additional 5%–7% of patients have rare autosomal recessive defects in components of the pre-BCR or BCR or in the downstream scaffold molecule B cell linker protein (BLNK) (4, 5). These genetic disorders all result in a block in B cell differentiation at the pro-B cell to pre-B cell transition, the stage at which the pre-B cell receptor is first expressed.

We have recently described a group of 4 unrelated patients, 2 males and 2 females, with agammaglobulinemia and a very small number of B cells characterized by the lack of a BCR, but increased expression of CD19 (6). Bone marrow studies from these patients demonstrated a profound reduction in the number of CD19⁺ cells and a block in B cell differentiation at the common lymphoid precursor to pro-B cell stage of differentiation, a stage earlier than that seen in patients with BTK deficiency or mutations in components of the BCR signaling pathway.

None of the 4 patients had a family history of immunodeficiency (Figure 1A) or belonged to isolated populations or consanguineous families; therefore, we hypothesized that these

patients might have de novo mutations in a gene required for B cell development.

Results and Discussion

Whole-exome sequencing was used to analyze one of the BCR⁻ patients and his parents. Alterations that were found in the patient but not in his parents were further evaluated by Sanger sequencing. Only one gene was confirmed to have a de novo mutation in the coding regions from the DNA of the patient. Buccal swab as well as blood DNA from the patient demonstrated the mutation, indicating that the mutation was germline in origin, rather than somatic. The mutant gene, *TCF3* (also known as *E2A*), is known to be required for B cell development in mice (7–9).

TCF3 encodes the 2 broadly expressed founding members of the basic helix-loop-helix (bHLH) family of transcription factors, E12 and E47 (10). E12 and E47 are identical except for their bHLH domains, which are provided by alternatively spliced exons (Figure 1B and ref. 11), which encode both the DNA-binding domain, in the basic region, and the dimerization domain in the HLH region (12). Both E12 and E47 bind the consensus sequence CANNTG as a homodimer or heterodimer with other bHLH proteins, including tissue-specific master genes, such as myogenic differentiation (*MyoD*) (13).

The mutation found in the patient, a G-to-A substitution at nucleotide 1663 in the cDNA, occurred within the exon specific to E47, and resulted in the replacement of the wild-type glutamic acid with lysine at codon 555 (E555K) within the DNA-binding domain (Figure 1C). The glutamic acid at this site is highly

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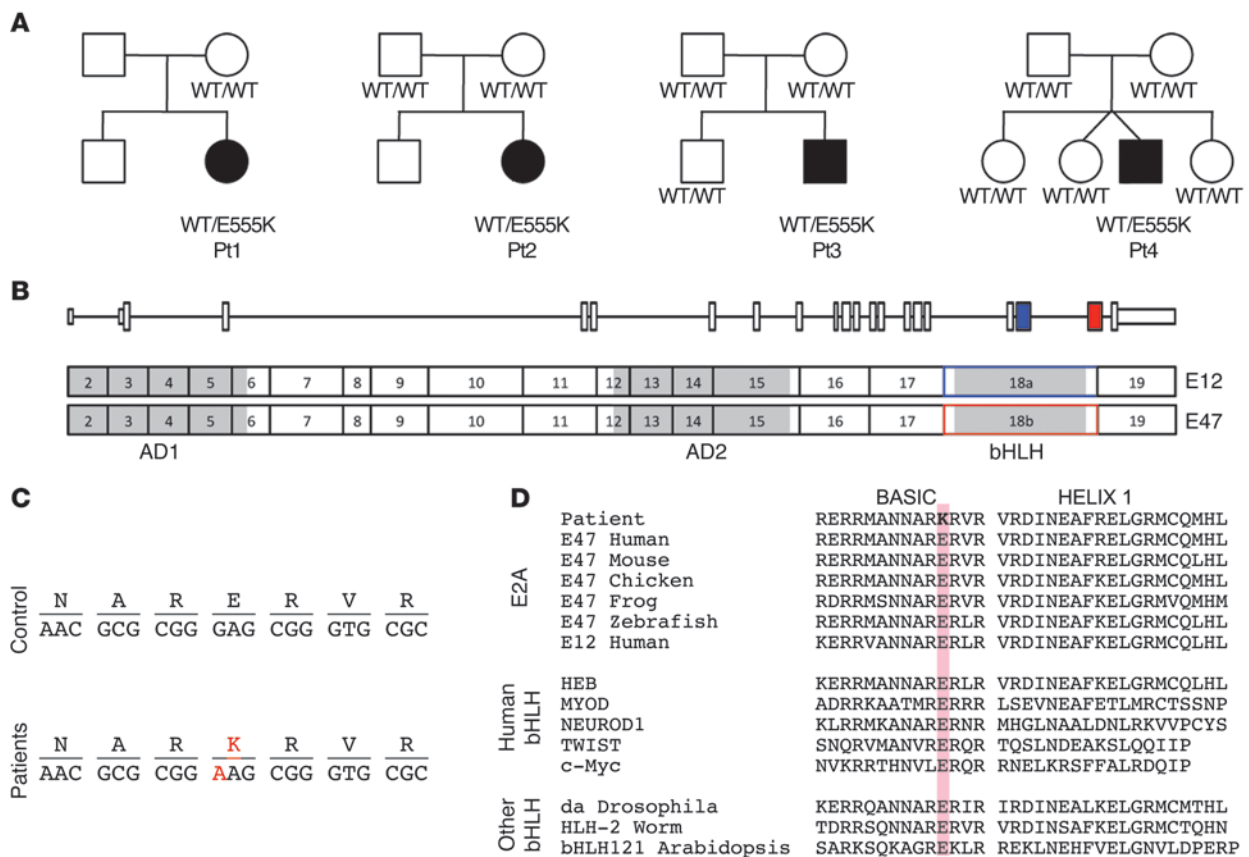


Figure 1

The E555K mutation in E47. (A) The pedigrees of the 4 patients are shown. Squares denote males, circles denote females, and black symbols indicate affected patients. (B) Schematic diagrams of the E2A intron/exon structure (top) and the protein structure (bottom) are depicted. The exons and domains specific for E12 and E47 are shown in blue and red, respectively. The functional domains of the proteins, the activation domains 1 and 2 (AD1 and AD2), and the bHLH domain are shaded in gray; exons are numbered. (C) The specific base pair and amino acid alterations seen in the patients are shown. (D) The conservation of the glutamic acid at codon 555 is demonstrated.

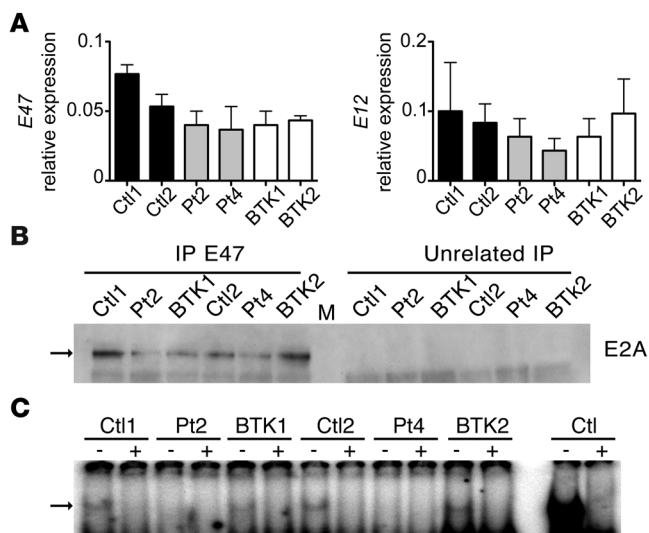
conserved in all bHLH family members (Figure 1D) and is known to be critical for binding the E2A consensus site (14). Surprisingly, the same base pair alteration was seen in genomic DNA from all 3 of the other BCR⁻ patients, but was not seen in an additional 40 patients with B cell defects of unknown etiology without the BCR⁻ phenotype. The mutation was not found in 450 individuals from an in-house database or 1052 controls from the CEPH panel. DNA was available and analyzed from 7/8 parents and 4/6 siblings of the 4 patients and none were found to carry the same mutation, suggesting that the mutation was a de novo event in all 4 patients. The identification of an identical de novo mutation in all 4 patients was unexpected, but consistent with recent studies that indicate that de novo mutations do not occur at random. Some sites, including essential genes, are more vulnerable than others (15, 16).

The functional consequences of the E555K mutation were evaluated in EBV-transformed B cell lines from 2 of the patients. Quantitative PCR showed that the amounts of E12 and E47 transcripts in the patient cell lines were comparable to those found in EBV cell lines from healthy controls or patients with mutations in BTK (Figure 2A). To determine whether the mutation in E47 altered protein stability, total cell lysates from the EBV lines were immunoprecipitated with

a monoclonal antibody to E47 and developed with a polyclonal antibody that reacted to both E12 and E47 (Figure 2B). Although the amount of E47 in the EBV lines was variable, the patient lines were similar to the control lines. The ability of the nuclear extracts from the EBV lines to bind to the classic DNA target site for E proteins, the murine μ E5 enhancer, was analyzed by EMSA. A faint signal could be seen using the nuclear extracts from the control lines but not the lines from the E555K patients. This signal was not seen when the extracts were preincubated with an anti-E47 antibody (Figure 2C).

To further examine the consequences of the E555K mutation in E47, HEK-293 cells were transfected with CMV driven expression vectors encoding wild-type E12 or E47, or E555K mutant E47. Western blots demonstrated that mutant E47 could localize normally to the nucleus, that the monoclonal anti-E47 antibody was specific for E47, and that this antibody recognized the mutant protein as well as the wild-type E47 (Figure 3A).

Nuclear extracts from HEK-293 cells transfected with an empty vector or expression vectors producing wild-type E12 or E47 or E555K E47 were incubated with the μ E5 probe in EMSAs. As shown in Figure 3B, wild-type E12 and E47 provided strong signals, but the mutant E47 gave a signal that was consistently less than 5% of that seen for wild-type E47. EMSAs performed using

**Figure 2**

The stability of the mutant transcript and protein. **(A)** Quantitative RT-PCR was used to evaluate the amount of E47 (left) and E12 (right) transcripts in EBV-transformed cell lines from controls (black bars), patients with the E555K mutation in E47 (gray bars), or patients with mutations in BTK (white bars). **(B)** Total cell lysates from 5×10^7 EBV-transformed cells were immunoprecipitated with monoclonal antibody to E47 or an unrelated isotype-matched antibody and then developed with an antibody that recognizes both isoforms of E2A. Arrow indicates the E47-specific band. **(C)** Nuclear extracts from the same EBV lines were left untreated or were preincubated with anti-E47 and then allowed to bind the μ E5 immunoglobulin enhancer in EMSAs. Nuclear extracts from HEK-293 cells transfected with wild-type E47 acted as a positive control. The specificity of the protein/DNA complex was demonstrated by supershifting with an antibody to E47.

the same μ E5 probe and nuclear extracts from HEK-293 cells transfected with wild-type or mutant E47 vectors, or variable amounts of each vector, demonstrated that addition of E555K E47 to the wild-type protein markedly decreased the signal given by the wild-type protein. These results indicate that E555K E47 has a strong dominant negative effect when binding to a probe as a homodimer with wild-type E47 (Figure 3C). To mimic a heterozygous condition, the μ E5 probe was incubated with nuclear extracts from HEK-293 cells transfected with 500 ng of wild-type or mutant E47 or 250 ng of each. The intensity of the signal achieved with an extract simulating the heterozygous state was 13% of that seen with the wild-type protein (Figure 3C). In a similar fashion, when HEK-293 cells were transfected with a luciferase reporter construct driven by the μ E5- μ E2 sequence plus expression vectors for the wild-type and/or mutant E47, the combined vectors gave a signal that was 10% of that seen with the wild-type E47 (Figure 3C).

The ability of E555K to bind DNA as a heterodimer with MyoD was evaluated by EMSAs using a probe from creatinine kinase regulatory regions. This tissue-specific bHLH transcription factor homodimerizes poorly and requires an E protein as a heterodimeric DNA-binding partner (11). Although E555K E47 did not bind to the μ E5 or creatinine kinase probes as a homodimer, it did allow MyoD to bind to the creatinine kinase probe (Figure 3D). Preincubation of the nuclear extracts with antibody to E47 or MyoD resulted in a supershift of the MyoD-DNA complex (Figure 3D). The ability of mutant E47 to permit DNA binding by this tissue-specific bHLH protein was not specific to the E555K mutation, but was also seen with an in vitro-created mutation (R556K) that was previously shown to inhibit DNA binding by wild-type E47 (12).

Mice that are null for E2A, lacking both E12 and E47, have poor viability in the perinatal period and demonstrate a complete block in B cell development at the earliest stages of differentiation (7, 8). The mice that survive have abnormalities in T cell development and a high incidence of T cell lymphomas (17). Mice that are null for E47, but not E12, have normal viability but have less than 1% B lineage cells in the bone marrow and spleen (18). However, mice that lack E12, but not E47, have normal numbers of B cells (18). Mice that are heterozygous for null mutations in E2A, E47, or E12 have normal numbers of B220⁺ B cells. It is possible that deleterious mutations in exons common to E12 and E47 result in an embryonic

lethal phenotype in humans, whereas mutations that are specific to the E12 exon may not have an easily recognized phenotype.

Somatically acquired activating mutations in the bHLH domain of E47 have recently been reported in patients with Burkitt lymphoma; one of these mutations, E555Q, occurred at the same base pair affected by the E555K mutation (19). In contrast, heterozygous loss of the entire E2A locus is common in Sézary syndrome (20), a subtype of T cell lymphoma. None of the patients included in this study (10 to 40 years of age) have had lymphomas or infections that are typical of T cell defects. Detailed analysis of the T cell immunophenotype and TCR V β repertoire in 2 of the patients was similar to that seen in patients with mutations in BTK (ref. 21 and Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI71927DS1).

The E555K mutation in E47 has effects that differ from those seen in all of the E2A-related knockout mice because it results in the production of a stable protein that is able to dimerize with other bHLH proteins. This allows enhanced DNA binding of some bHLH family members, such as MyoD, that homodimerize poorly and cannot bind DNA alone (13). The E555K mutant E47 would also be expected to bind to and sequester members of the Id family, a group of HLH transcription factors that lack a DNA-binding domain and therefore inhibit DNA binding by E family members (22). It is also possible that the E555K mutant E47 binds to DNA target sequences that differ from the canonical E protein consensus sequence. This might result in enhanced or reduced production of proteins that are not usually controlled by E47.

The B cells lacking a BCR in the patients with the E555K mutation suggest that there is a threshold for E47 function. A small amount of E47 allows some developmental progression of B lineage cells, but more E47 is required to enforce the block in differentiation of cells that do not have a functional pre-BCR. This is similar to the effects of decreased or absent E2A function in T cell development. *Rag*^{-/-} mice that have decreased or absent E2A develop some CD4⁺CD8⁺ cells, cells that are not usually seen in *Rag*-deficient mice because that stage of development requires a functional pre-TCR (23, 24). However, these cells do not mature further and do not exit the thymus. B cells lacking a BCR have not been previously described in the humans, and the presence of these cells in the patients with the E555K mutation indicates that the pro-B cell

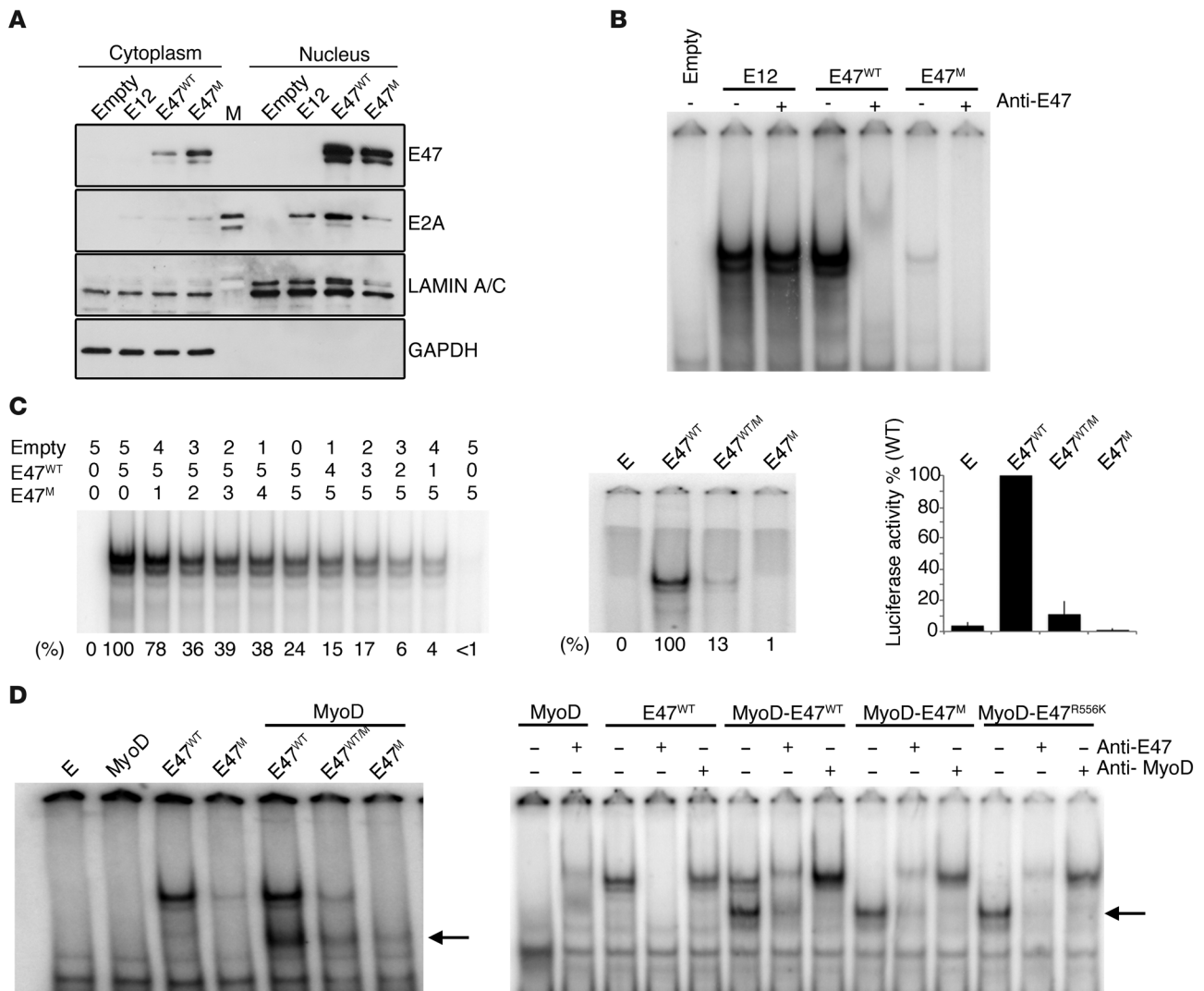


Figure 3 Functional consequences of the E555K mutation. (A) Cytoplasmic and nuclear extracts from HEK-293 cells transfected with an empty vector, or expression vectors for wild-type E12, E47, or E555K mutant E47 were evaluated by Western blot using an E47-specific antibody, an antibody that recognizes both E12 and E47, or antibodies to known cytoplasmic or nuclear proteins. (B) EMSAs were performed using a ³²P-labeled μ E5 probe and nuclear extracts from HEK-293 cells transfected with vectors producing wild-type E12, E47, or E555K mutant E47. The wild-type and mutant E47 bands, but not the E12 bands, were supershifted with antibody to E47. (C, left) Nuclear extracts from cells transfected with varying amounts (0 to 500 ng as indicated at the top of each lane) of wild-type and/or E555K mutant E47 were incubated with the μ E5 probe. Percentage binding by each extract is shown below each lane. The effect of the E555K mutation in a heterozygous patient is modeled in C (center and right). HEK-293 cells were transfected with 250 ng of the wild-type or E555K mutant E47 or with 125 ng of each vector. Nuclear extracts from these cells were allowed to bind to the μ E5 probe and the percentage binding was determined by phosphorimager (center). A luciferase immunoglobulin enhancer reporter construct and 250 ng of the wild-type E47, E555K mutant E47, or 125 ng of each vector was transfected into HEK-293 cells and the luciferase was measured. (D) Nuclear extracts containing MyoD, wild-type E47, mutant E47, or combinations of these proteins were allowed to bind to a creatinine kinase probe (left). Nuclear extracts were preincubated with antibody to E47 or MyoD to supershift the DNA-protein complexes (right). The arrows indicate the MyoD/E47-specific bands.

to pre-B cell transition is controlled not only by signaling through the pre-BCR but also by quality control mechanisms that prevent cells that lack a pre-BCR from further development. Our studies show that E47 plays a critical role in this quality control.

Methods

Whole-exome sequencing was performed as previously described (25), with additional details in the Supplemental Methods. Sanger sequencing was

used to validate the mutation in the BCR⁻ patients. Expression vectors were produced by cloning E47 cDNA into a CMV-driven expression vector. Site-directed mutagenesis was used to create the mutant cDNA. The probes for EMSA were derived from the regulatory regions of murine immunoglobulin heavy chain enhancer (μ E5 GATCCCAGAACACCTGCAGCAG) and murine creatinine kinase (GTCACCCCAACACCTGCTGCCTA).

Statistics. The bar graphs shown in Figures 2 and 3 indicate the mean \pm SEM of 3–5 experiments.



Study approval. These studies were conducted as part of a research protocol approved by St. Jude Children's Research Hospital IRB to identify and characterize genetic defects of the immune system. Written informed consent was obtained from all the patients or their parents.

Full methods are available in the Supplemental Methods.

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1. Conley ME, Mathias D, Treadaway J, Minegishi Y, Rohrer J. Mutations in *brk* in patients with presumed X-linked agammaglobulinemia. *Am J Hum Genet.* 1998;62(5):1034–1043.
2. Tsukada A, et al. Deficient expression of a B cell cytoplasmic tyrosine kinase in human X-linked agammaglobulinemia. *Cell.* 1993;72(2):279–90.
3. de Weers M, et al. B-cell antigen receptor stimulation activates the human Bruton's tyrosine kinase, which is deficient in X-linked agammaglobulinemia. *J Biol Chem.* 1994;269(39):23857–23860.
4. Conley ME, et al. Primary B cell immunodeficiencies: comparisons and contrasts. *Annu Rev Immunol.* 2009;27:199–227.
5. Minegishi Y, et al. An essential role for BLNK in human B cell development. *Science.* 1999; 286(5446):1954–1957.
6. Dobbs AK, Bosompem A, Coustan-Smith E, Tyerman G, Saulsbury FT, Conley ME. Agammaglobulinemia associated with BCR- B cells and enhanced expression of CD19. *Blood.* 2011;118(7):1828–1837.
7. Bain G, et al. E2A proteins are required for proper B cell development and initiation of immunoglobulin gene rearrangements. *Cell.* 1994;79(5):885–892.
8. Zhuang Y, Soriano P, Weintraub H. The helix-loop-helix gene E2A is required for B cell formation. *Cell.* 1994;79(5):875–884.
9. Sun XH. Constitutive expression of the Id1 gene impairs mouse B cell development. *Cell.* 1994; 79(5):893–900.
10. Murre C, McCaw PS, Baltimore D. A new DNA binding and dimerization motif in immunoglobulin enhancer binding, daughterless, MyoD, and myc proteins. *Cell.* 1989;56(5):777–783.
11. Sun XH, Baltimore D. An inhibitory domain of E12 transcription factor prevents DNA binding in E12 homodimers but not in E12 heterodimers. *Cell.* 1991;64(2):459–470.
12. Voronova A, Baltimore D. Mutations that disrupt DNA binding and dimer formation in the E47 helix-loop-helix protein map to distinct domains. *Proc Natl Acad Sci U S A.* 1990;87(12):4722–4726.
13. Wendt H, Thomas RM, Ellenberger T. DNA-mediated folding and assembly of MyoD-E47 heterodimers. *J Biol Chem.* 1998;273(10):5735–5743.
14. Maerkl SJ, Quake SR. Experimental determination of the evolvability of a transcription factor. *Proc Natl Acad Sci U S A.* 2009;106(44):18650–18655.
15. Michaelson JJ, et al. Whole-genome sequencing in autism identifies hot spots for de novo germline mutation. *Cell.* 2012;151(7):1431–1442.
16. Riviere JB, et al. De novo germline and postzygotic mutations in AKT3, PIK3R2 and PIK3CA cause a spectrum of related megalencephaly syndromes. *Nat Genet.* 2012;44(8):934–940.
17. Bain G, et al. E2A deficiency leads to abnormalities in alphabeta T-cell development and to rapid development of T-cell lymphomas. *Mol Cell Biol.* 1997;17(8):4782–4791.
18. Beck K, Peak MM, Ota T, Nemazee D, Murre C. Distinct roles for E12 and E47 in B cell specification and the sequential rearrangement of immunoglobulin light chain loci. *J Exp Med.* 2009; 206(10):2271–2284.
19. Schmitz R, et al. Burkitt lymphoma pathogenesis and therapeutic targets from structural and functional genomics. *Nature.* 2012;490(7418):116–120.
20. Steininger A, et al. Genomic loss of the putative tumor suppressor gene E2A in human lymphoma. *J Exp Med.* 2011;208(8):1585–1593.
21. Martini H, et al. Importance of B cell co-stimulation in CD4(+) T cell differentiation: X-linked agammaglobulinemia, a human model. *Clin Exp Immunol.* 2011;164(3):381–387.
22. Ruzinova MB, Benezra R. Id proteins in development, cell cycle and cancer. *Trends Cell Biol.* 2003; 13(8):410–418.
23. Kim D, et al. Helix-loop-helix proteins regulate pre-TCR and TCR signaling through modulation of Rel/NF- κ B activities. *Immunity.* 2002;16(1):9–21.
24. Engel I, Johns C, Bain G, Rivera RR, Murre C. Early thymocyte development is regulated by modulation of E2A protein activity. *J Exp Med.* 2001; 194(6):733–745.
25. Conley ME, et al. Agammaglobulinemia and absent B lineage cells in a patient lacking the p85 α subunit of PI3K. *J Exp Med.* 2012;209(3):463–470.