Context-Dependent Gene Expression Programs Promote Lymphocyte Development and Function and Suppress Transformation

Amy Demicco

University of Pennsylvania, ademicco8@gmail.com

Follow this and additional works at: https://repository.upenn.edu/edissertations

Part of the Allergy and Immunology Commons, Cell Biology Commons, Immunology and Infectious Disease Commons, Medical Immunology Commons, and the Molecular Biology Commons

Recommended Citation


https://repository.upenn.edu/edissertations/1682

This paper is posted at ScholarlyCommons. https://repository.upenn.edu/edissertations/1682

For more information, please contact repository@pobox.upenn.edu.
Context-Dependent Gene Expression Programs Promote Lymphocyte Development and Function and Suppress Transformation

Abstract
Coordinated orchestration of gene expression programs at the transcriptional, post-transcriptional, and post-translational levels is essential for development and function of all cells, including lymphocytes. Normal tissue function also demands that the genome be faithfully passed from mother to daughter cell during the many rounds of cell division required to generate a mammalian organism. Genome integrity is maintained in part by integration of DNA damage signaling with cell cycle control. These mechanisms are especially critical for lymphocytes following V(D)J recombination, since V(D)J recombination involves genetic cutting and pasting of germline gene segments to form antigen receptors (AgRs). Using conditional deletion of the p53 tumor suppressor in mice, I found that p53 promotes genome stability in developmental stage-specific ways. Inactivation of p53 beginning in hematopoietic stem cells yields thymic tumors with aneuploidy; whereas deletion of p53 at the beginning of thymocyte development results in tumors bearing T cell AgR translocations. I also show that downregulation of the G1 phase cyclin D3 occurs in immature B and T cells in response to exogenously-induced DNA breaks through lineage-specific mechanisms. Further, this downregulation of D3 may be important for delaying S phase entry in response to DNA breaks, providing an additional mechanism to promote genome stability during lymphocyte development. Finally, I discovered novel roles of the HuR RNA-binding protein in regulating B cell function. Specifically, HuR is largely dispensable for B cell development and in vitro B cell function; however, it is crucial for the in vivo T cell-dependent immune response in mice, likely by facilitating the ability of B cells to interact with other immune cells in the follicular milieu. This work provides new insight into the lineage- and developmental stage-specific ways in which complex gene expression programs contribute to the normal development and function of B and T lymphocytes, while suppressing malignant transformation.

Degree Type
Dissertation

Degree Name
Doctor of Philosophy (PhD)

Graduate Group
Cell & Molecular Biology

First Advisor
Craig H. Bassing

Keywords
cell cycle checkpoints, cyclin D3, genome stability, HuR, lymphocyte development, p53

Subject Categories
Allergy and Immunology | Cell Biology | Immunology and Infectious Disease | Medical Immunology | Molecular Biology

This dissertation is available at ScholarlyCommons: https://repository.upenn.edu/edissertations/1682
CONTEXT-DEPENDENT GENE EXPRESSION PROGRAMS PROMOTE LYMPHOCYTE DEVELOPMENT AND FUNCTION AND SUPPRESS TRANSFORMATION

Amy DeMicco

A DISSERTATION

in

Cell and Molecular Biology

Presented to the Faculties of the University of Pennsylvania

in

Partial Fulfillment of the Requirements for the

Degree of Doctor of Philosophy

2015

Supervisor of Dissertation

___________________________
Craig H. Bassing, Ph.D.
Associate Professor of Pathology and Laboratory Medicine

Graduate Group Chairperson

___________________________
Daniel S. Kessler
Associate Professor of Cell and Developmental Biology

Dissertation Committee

Kristen Lynch, Ph.D. (chair), Professor of Biochemistry and Biophysics
Michael Atchison, Ph.D., Professor of Biochemistry
David Roth, M.D., Ph.D., Simon Flexner Professor of Pathology and Laboratory Medicine
Matthew Weitzman, Ph.D., Associate Professor of Pathology and Laboratory Medicine
CONTEXT- DEPENDENT GENE EXPRESSION PROGRAMS PROMOTE LYMPHOCYTE DEVELOPMENT AND FUNCTION AND SUPPRESS TRANSFORMATION

COPYRIGHT

2015

Amy C. DeMicco

This work is licensed under the Creative Commons Attribution-NonCommercial-ShareAlike 3.0 License

To view a copy of this license, visit

http://creativecommons.org/licenses/by-nc-sa/2.0/
ACKNOWLEDGMENTS

Many thanks go out to my advisor, Craig Bassing, for his unflagging support during my thesis work. I gratefully acknowledge the time, effort, and advice of my thesis committee members, Kristen Lynch (chair), Michael Atchison, David Roth, and Matthew Weitzman, without whom this thesis would not have been possible. I am also appreciative of several current and former Bassing lab members, including Katherine, Natalie, Julie, Meg, and Sarah for their camaraderie during my years in the lab. Claire O’leary offered much scientific advice, particularly on analysis of the proteomics dataset described in the appendix. Tyler Reich, formerly of the Bassing lab, contributed data characterizing the LckCre HuRflox/flox mice. I especially want to acknowledge that several of the experiments described in Chapter IV were performed in whole or part by Martin Naradikian from the laboratory of Michael Cancro. To this end, I also want to thank Mike and Martin for providing valuable advice and reagents to aid in the completion of work that is very much outside of the normal expertise of the Bassing lab. This work was financially supported by the Cell and Molecular Biology training grant (GM 072290), NCI F31 pre-doctoral Fellowship (CA177092), and the Patel Family Scholar Award of the Abramson Cancer Center.
ABSTRACT

CONTEXT-DEPENDENT GENE EXPRESSION PROGRAMS PROMOTE LYMPHOCYTE DEVELOPMENT AND FUNCTION AND SUPPRESS TRANSFORMATION

Amy DeMicco
Craig H. Bassing

Coordinated orchestration of gene expression programs at the transcriptional, post-transcriptional, and post-translational levels is essential for development and function of all cells, including lymphocytes. Normal tissue function also demands that the genome be faithfully passed from mother to daughter cell during the many rounds of cell division required to generate a mammalian organism. Genome integrity is maintained in part by integration of DNA damage signaling with cell cycle control. These mechanisms are especially critical for lymphocytes following V(D)J recombination, since VDJ recombination involves genetic cutting and pasting of germline gene segments to form antigen receptors (AgRs). Using conditional deletion of the p53 tumor suppressor in mice, I found that p53 promotes genome stability in developmental stage-specific ways. Inactivation of p53 beginning in hematopoietic stem cells yields thymic tumors with aneuploidy; whereas deletion of p53 at the beginning of thymocyte development results in tumors bearing T cell AgR translocations. I also show that downregulation of the G1 phase cyclin D3 occurs in immature B and T cells in response to exogenously-induced DNA breaks through lineage-specific mechanisms. Further, this downregulation of D3 may be important for delaying S phase entry in response to DNA breaks, providing an additional mechanism to promote genome stability during lymphocyte development. Finally, I discovered novel roles of the HuR RNA-binding protein in regulating B cell
function. Specifically, HuR is largely dispensable for B cell development and in vitro B cell function; however, it is crucial for the in vivo T cell-dependent immune response in mice, likely by facilitating the ability of B cells to interact with other immune cells in the follicular milieu. This work provides new insight into the lineage- and developmental stage-specific ways in which complex gene expression programs contribute to the normal development and function of B and T lymphocytes, while suppressing malignant transformation.
# TABLE OF CONTENTS

**ACKNOWLEDGMENTS** .................................................................................................................. VIII

**LIST OF TABLES** .......................................................................................................................... VIII

**LIST OF ILLUSTRATIONS** ........................................................................................................ IX

**CHAPTER I: INTRODUCTION** ...................................................................................................... 1

**CHAPTER II: SOMATIC INACTIVATION OF TP53 IN HEMATOPOIETIC STEM CELLS OR THYMOCYTES PREDISPOSES MICE TO THYMIC LYMPHOMAS WITH CLONAL TRANSLOCATIONS** .............................................................................. 15

**ABSTRACT** ................................................................................................................................... 15

**INTRODUCTION** .......................................................................................................................... 16

**RESULTS** ...................................................................................................................................... 20

**DISCUSSION** ............................................................................................................................... 28

**FIGURES** ...................................................................................................................................... 31

**CHAPTER III: LINEAGE- AND DEVELOPMENTAL STAGE-SPECIFIC MECHANISMS REGULATE CYCLIN D3 EXPRESSION IN RESPONSE TO IONIZING RADIATION** ................................................................................. 44

**ABSTRACT** ................................................................................................................................... 44

**INTRODUCTION** .......................................................................................................................... 45

**RESULTS** ...................................................................................................................................... 49

**DISCUSSION** ............................................................................................................................... 58

**FIGURES** ...................................................................................................................................... 62

**CHAPTER IV: B CELL-INTRINSIC EXPRESSION OF THE RNA-BINDING PROTEIN HUR IS REQUIRED FOR THE T CELL-DEPENDENT IMMUNE RESPONSE IN VIVO** ......................................................................................... 70
LIST OF TABLES

Chapter II

Table 1. Summary of VP tumor cohort. 35
Table 2. Summary of LP tumor cohort. 36

Methods

Table 3. Primer sequences. 124
Table 4. Antibodies used. 125

Appendix

Table A-1. List of proteins potentially misexpressed in HuR-deficient B cells after in vitro stimulation. 128
LIST OF ILLUSTRATIONS

Chapter I

Figure I-1. Cell cycle progression. 1
Figure I-2. p53 tumor suppressor: inputs and outputs. 4
Figure I-3. V(D)J recombination and G1/S checkpoint control. 6
Figure I-4. T cell-dependent B cell activation. 10

Chapter II

Figure 1. Mice with conditional inactivation of p53 initiating in HSCs or DN thymocytes reproducibly succumb to thymic lymphomas. 31
Figure 2. Mice with conditional inactivation of p53 initiating in HSCs or DN thymocytes develop clonal thymic lymphomas. 32
Figure 3. Mice with conditional inactivation of p53 initiating in HSCs also develop clonal B lineage lymphomas. 33
Figure 4. Mice with conditional inactivation of p53 initiating in HSCs or DN thymocytes develop lymphomas with oncogenic antigen receptor locus translocations. 34
Supplementary figure 1. Cre-mediated deletion of floxed Tp53 exons is robust and limited to expected tissues. 37
Supplementary figure 2. Lymphocyte development is normal in young LP and VP mice. 38
Supplementary figure 3. Flow cytometry analysis of VP and LP thymic lymphomas 40
Supplementary figure 4. Spectral karyotyping analysis of VP and LP lymphomas. 42
Chapter III

Figure 1. Irradiation of primary mouse pre-B cells induces loss of D3 protein in an ATM-, p53-, and Dicer-dependent manner, but does not alter D3 protein stability.

Figure 2. Irradiation of mouse pre-B cells induces an ATM-dependent loss of D3 transcription.

Figure 3. Overexpression of non-regulated D3 causes an increased number of pre-B cells to fail cell cycle arrest after IR.

Figure 4. Mature B cells undergoing CSR in vitro do not downregulate D3 upon IR treatment.

Figure 5. Thymocytes from irradiated mice show reduced D3 protein but not mRNA.

Figure 6. Thymocyte-specific deletion of HuR impairs DN to DP development and reduces D3 expression.

Figure 7. Model of D3 regulation in response to IR-induced breaks in developing lymphocytes and extension to physiologic DSB levels.

Chapter IV

Figure 1. B lineage-specific deletion of HuR leads to decreased numbers of immature and mature B cells.

Figure 2. HuR protects immature B cells from p53-dependent elimination.

Figure 3. In vitro stimulated HuRΔ/Δ B cells exhibit a mild proliferation defect, enhanced survival, and normal IgH isotype switching.

Figure 4. HuR is required for antibody production and numbers of peritoneal B1 cells but dispensable for in vitro functions.

Figure 5. HuR is required in vivo for generation of GC B cells and high-affinity antibodies.
Figure 6. HuR is required for in vivo formation of germinal centers in context. 101

Supplemental figure 1. Characterization of HuRA/Δ and Mb1Cre+ mice. 103

Supplemental figure 2. Characterization of HuRA/Δ and Mb1Cre+ B cell function. 104

Appendix:

Figure A-1. Proteins identified by proteomic analysis of WT and HuRA/Δ B cells stimulated in vitro for 48h with α-IgM and α-CD40. 127

Figure A-2. HuR-insufficient cells have increased expression of cyclin D3 but HuR-deficient D3-deficient B cells have a pro- to pre-B developmental block. 129
Chapter I: Introduction

Maintenance of genome stability during proliferation

The error-free transmission of genetic material from mother cell to daughter cell is critical for cell survival as well as for organismal survival. Although mutations can and do occur in quiescent cells, the DNA is particularly vulnerable during its replication and distribution between dividing cells. Thus, the cell cycle machinery is intimately coordinated with the DNA damage response (DDR). Each round of cell division begins in G1 phase when internal or external pro-proliferative signals cause upregulation of one of three D-type cyclins (reviewed in (Malumbres and Barbacid 2009, Musgrove et al. 2011)). D-cyclins pair with one of their kinase partners, cyclin-dependent kinase 4 or 6 (CDK4/6). Active D-CDK4/6 complexes phosphorylate many target proteins, leading to the inactivation of Rb family proteins and inducing the expression of cyclin E. Cyclin E-CDK2 complexes phosphorylate targets to initiate the replication of DNA, which takes place in S phase. S phase is followed by G2, where the cell readies itself for M phase, in which mitosis and cytokinesis occur and cell division is complete.

Figure I-1. Mitogenic signals upregulate one of the three D-type cyclins (D1, D2, D3). D-cyclins bind to either CDK4 or CDK6, and together these factors promote entry into S phase, where cyclin E-CDK2 complexes take over and promote DNA replication. The second gap phase precedes M phases, where mitosis and cell division mark the end of a round of proliferation.
Once the cell reaches late G1, at a point termed the “restriction point”, the cell is committed to a full round of proliferation, and will carry out the necessary processes in chronological order with no external stimuli required (Pardee 1989). However, this sequence of events can be interrupted or terminated at distinct points when problems arise. The ability of cells to respond to problems with the DNA during the cell cycle is crucial for preserving the correct DNA sequence, as evidenced by the frequent mutation of checkpoint factors in many types of human tumors (Kastan and Bartek 2004, Squatrito et al. 2010).

DNA is vulnerable to breakage and misrepair during DNA replication. Attempting to replicate broken DNA increases the likelihood of large-scale genetic amplification, loss, or translocation, in which pieces of distinct chromosomes become aberrantly joined (Alt et al. 2013). Thus, the G1/S checkpoint prevents the initiation of replication in response to DSBs sensed during G1 phase. DSBs are first bound by the MRN complex, consisting of the proteins Mre11, Rad50, and Nbs1 (Lee and Paull 2005). The MRN complex recruits and activates ATM, which in turn phosphorylates the histone H2A variant H2AX. Phosphorylated H2AX (γ-H2AX) augments the DSB signal and recruits other DDR factors such as MDC1 (Stucki et al. 2005, Savic et al. 2009). ATM phosphorylates many additional targets at S/TQ motifs, including Chk2 and p53 (Banin et al. 1998, Matsuoka et al. 2000, Matsuoka et al. 2007). Chk2 also phosphorylates p53, further contributing to stabilization of the p53 protein and promoting its activity (Hirao et al. 2000). Activated p53 transcriptionally and post-transcriptionally activates gene targets that promote cell cycle arrest (Beckerman and Prives 2010, Marcel et al. 2015).

Within S phase, replication stress occurs when the helicase that unwinds double stranded DNA becomes uncoupled from the polymerase following behind it. Replication
stress can be caused by factors including inadequate supply of nucleotides, structural
obstacles in the DNA that stall the polymerase, DNA lesions, and co-occurring gene
transcription (Mazouzi et al. 2014). When the helicase and polymerase become
uncoupled, the resulting long stretch of single-stranded DNA is bound by RPA proteins,
which signal the recruitment and activation of response factors, including the kinase ATR
(Zou and Elledge 2003, Mazouzi et al. 2014). Activated ATR phosphorylates and
activates Chk1 in order to induce an intra-S phase checkpoint, characterized by
inhibition of new origin firing (Toledo et al. 2013). If replication stress is severe or
persistent, the replication fork may collapse, generating a DSB, which then recruits ATM
and the related kinase DNA-PK (Liu et al. 2012, Toledo et al. 2013). Collapsed
replication forks are often able to appropriately re-start (Petermann and Helleday 2010,
Mazouzi et al. 2014); however, failure to initiate the intra-S phase checkpoint promotes
genomic instability (Lopez-Contreras and Fernandez-Capetillo 2010).

Normal cellular function depends on correct gene dosage. Gene dosage can be
disturbed by small mutations and deletions as well as whole chromosomal gains and
losses. Aberrant chromosome numbers, known as aneuploidy, results from defective
chromosome segregation within M phase (Veitia and Potier 2015). Normal mitosis
involves a highly ordered set of processes beginning with the condensation of each
chromosome, pairing and alignment of sister chromatids at the midzone between the two
centrosomes (Nath et al. 2015). The spindle assembly checkpoint (SAC) pauses mitosis
until each chromosome is successfully captured by microtubules. Once the SAC is
alleviated by proper chromosome capture and alignment, chromosomes are pulled to
opposite poles, and then cytokinesis occurs to complete cell division. Aneuploidy often
results from a faulty SAC, allowing chromosome separation and cell division before
chromosomes are properly positioned and captured (Holland and Cleveland 2012). Aneuploidy is common in human tumors (Mitelman 2015).

Because the average cell experiences many breaks and nucleotide lesions per day (Lodish 2004), even the existence of redundant and multi-faceted checkpoint and repair pathways is insufficient to prevent errors. Therefore, mechanisms for sensing and eliminating cells with defective DNA are critical for tissue fitness and suppression of tumorigenesis. Apoptosis is the process by which damaged cells initiate their own death and undergo orderly disassembly of DNA and cellular components. Perhaps the best-studied inducer of apoptosis is the tumor suppressor p53 (Meek 2009). In cases of severely damaged DNA, activated p53 induces pro-apoptotic factors like Bax, which are able to turn on caspases to degrade DNA and other cellular material (Meek 2009).

Consistent with its role in promoting genome stability, p53 is the most prevalently mutated tumor suppressor gene across all human tumors, and its inactivation is associated with increased genome instability (Cheung et al. 2009, Dicker et al. 2009). However, in some contexts, the activation of p53 does not lead to apoptosis (Figure I-2). For example, oncogene activation most often leads to permanent cell-cycle arrest known as senescence (Astle

\[ \text{Figure I-2. The p53 tumor suppressor facilitates the inactivation or elimination of defective cells by responding to many cellular stresses and inducing gene expression changes that lead to an appropriate response.} \]
et al. 2012, Cisowski et al. 2015), and transient p53 activation in response to DSBs induces transcription of CDK-inhibitors like p21 (Beckerman and Prives 2010). Additional evidence suggests that the cellular context within which p53 inactivation occurs influences the phenotype of consequent tumors (Rowh et al. 2011, DeMicco et al. 2013). No matter the specific biologic outcome, the role of p53 is to minimize genome instability and prevent the propagation of defective cells.

Coordination of programmed DNA breaks, proliferation, and differentiation during lymphocyte development

B and T lymphocytes provide a useful model in which to study the integration of DNA damage and cell cycle control. Adaptive immunity in mice and humans requires the development and function of B and T lymphocytes, each with a unique and specific antigen receptor (AgR). The diversity of B and T cell receptors (BCRs and TCRs) is accomplished by genetic cutting and pasting of germline encoded variable (V), diversity (D), and joining (J) segments through the process of V(D)J recombination (reviewed in Bassing et al. 2002, Alt et al. 2013)). B cell receptors are composed of an immunoglobulin heavy chain (IgH) protein paired with either an Ig kappa or Ig lambda light chain. The majority of T cell receptors are comprised of a TCRα and TCRβ chain; however, some portion of T cells carry TCRγ and TCRδ receptors (von Boehmer 2004, Xiong and Raulet 2007). Each of these proteins contains a variable exon that is assembled by V(D)J recombination, as well as a constant region. V(D)J recombination begins when a complex containing Rag1 and Rag2 proteins recognizes recombination signal sequences within antigen receptor loci upstream of two segments to be
recombined and nicks the DNA at each (McBlane et al. 1995). Transesterification results in two DSBs with one side of each break containing a closed hairpin structure (McBlane et al. 1995). The Artemis nuclease opens the hairpin (Ma et al. 2002), and the non-homologous (NHEJ) machinery repairs the broken DNA, resulting in an extra-chromosomal signal join and the protein-encoding coding join. Further diversity in the coding join is accomplished by stochastic non-templated addition of nucleotides at junctions by the enzyme terminal deoxynucleotide transferase (Benedict et al. 2000).

Proliferation of lymphocytes at specific times within their lifespan is critical for generation of normal numbers of naïve B and T cells. However, many of these periods of proliferation closely follow programmed DSBs in developmental time (Bednarski and Sleckman 2012). One way that lymphocytes limit the incorrect repair of programmed

![Figure I-3](image_url)

**Figure I-3.** V(D)J recombination, initiated in pro-B cells and DN thymocytes by the RAG complex, can result in a recombined V(D)J join that is expressed on the cell surface. The resulting pre-BCR or pre-TCR signaling upregulates cyclin D3, leading to a period of rapid proliferation. In some cells, secondary DNA breaks, at the other antigen receptor allele or elsewhere in the genome, initiate an ATM- and p53-dependent G1/S checkpoint. This checkpoint ensures that broken DNA is maintained in G1, thus promoting genome stability and cell survival.
DSBs is by restricting V(D)J recombination to G1 phase. This is achieved in part by G1-specific expression of Rag2 protein (Li et al. 1996). In addition, breaks initiated by Rag activate a set of DDR and checkpoint genes that largely overlaps with those induced by exogenous breaks (Bredemeyer et al. 2008), consistent with the idea that canonical checkpoint pathways are also important for controlling genome stability during V(D)J recombination (Figure I-3).

Lymphocyte development can fail at many stages. For example, due to the stoachastic addition or deletion of nucleotides in the V(D)J join (Benedict et al. 2000, Boboila et al. 2012), the recombined sequence has a one in three chance of being in-frame as relates to translation. Although cells with out of frame rearrangements usually attempt further rearrangements (Schatz and Ji 2011), some cells ultimately fail to generate a join capable of encoding protein. Additionally, Rag-induced breaks are mis-repaired at some frequency, leading to copy number variations and/or translocations (Nussenzweig and Nussenzweig 2010). Further, some rearrangements produce receptors that react to self-antigens, and thus should be eliminated from the repertoire. Since millions of immature lymphocytes are generated in the mouse per day (Opstelten and Osmond 1983), even occasional escape of defective cells could cause tumors or autoimmunity.

Quality control for AgRs on developing B and T lymphocytes occurs at the pre-BCR and β-selection checkpoints, respectively (Morris and Allen 2012, Rickert 2013). Pro-B cells undergo stepwise rearrangement of the IgH locus, in which D segments are joined to J segments before the V segment is recombined with the D-J join. If the coding join yields in an in-frame sequence capable of generating a functional IgH chain, this chain will pair with λ5 and VpreB proteins to form a pre-BCR on the cell surface (Rickert
Pre-BCR signaling inhibits further recombination at the IgH locus (Grawunder et al. 1995), and upregulates pro-survival factors, largely through NFκB-dependent pathways (Saijo et al. 2003), ensuring that only B cells with a functional IgH chain can proceed through development. Pre-BCR signaling also upregulates cyclin D3, driving cells through several rounds of division (Cooper et al. 2006). The pre-BCR checkpoint may also reduce autoimmune specificities by promoting allelic exclusion (Rickert 2013). Pre-B cells that pass this checkpoint then re-arrange an Ig light chain, attempting Igκ first followed by Igλ (Clark et al. 2014). Generation of an IgL that is able to pair with the cell’s unique IgH chain permits further survival and differentiation (Bednarski and Sleckman 2012). Immature B cells expressing a complete BCR leave the bone marrow for secondary lymphoid organs, where they complete their maturation into mature naïve B cells (Allman et al. 1993).

Analogous to the pre-BCR checkpoint, α/β T cells undergo positive and negative selection during their development in the thymus (von Boehmer 2004, Morris and Allen 2012). Double negative (DN) thymocytes characterized by expression of neither CD4 nor CD8 undergo TCRβ recombination. TCRβ pairs with pre-Tα to form the pre-TCR (Guidos 2006). TCRs are selected such that they can recognize self-major histocompatibility (MHC) peptides with low affinity, thereby eliminating cells that cannot “see” MHC, but also eliminating strongly auto-reactive T cells (Morris and Allen 2012). Cells that pass this checkpoint differentiate into CD4+CD8+ (double positive, DP) thymocytes, which then recombine the TCRα locus (Bassing et al. 2002). DP cells that successfully generate a mature TCR differentiate into either CD4+ or CD8+ (single positive, SP) T cells. Newly minted mature T cells exit the thymus and migrate to secondary lymphoid organs, where they await activation.
B cell-mediated immune responses

Mature B cells are broadly characterized as B1 or B2 cells. The majority of B1 cells reside in the peritoneal cavity where they secrete antibodies to repetitive pathogenic antigens (Zhang 2013). B2 B cells predominate in the spleen and LN where they participate in pathogen-specific adaptive immune responses (Garraud et al. 2012). B2 B cells can be further classified as either marginal zone (MZ) or follicular (Fo) B cells.

Within the spleen, MZ B cells are positioned between the red pulp, containing dendritic cells, macrophages, and other cell types, and the lymphocyte-rich white pulp, (Pillai and Cariappa 2009). MZ B cells bridge the gap between innate- and adaptive- immune responses, more often encoding polyvalent BCRs that recognize antigenic patterns (Cerutti et al. 2013). MZ B cells mount rapid, relatively low-affinity antibody responses, in a largely T cell-independent manner (Cerutti et al. 2013). In contrast, Fo B cells, so named because they occupy B cell follicles within the splenic white pulp, are most important in the response to T cell-dependent antigen encounters (Victora and Nussenzweig 2012).

Fo B cells constantly circulate through the spleen, waiting to encounter the cognate antigen that fits their unique BCR. B cells most often receive antigens via professional antigen-presenting cells such as dendritic cells (DCs). Antigen binding to the BCR activates the B cell, resulting in broad changes in gene expression and cell activity (Klein and Dalla-Favera 2008). To undergo a T cell-dependent response, these activated B cells must encounter and engage an activated CD4+ T cell that shares the same antigen specificity. The T cell will have been separately primed by its own
encounter with the cognate antigen, usually also from a DC (Vinuesa and Cyster 2011).

When these B-T cell encounters occur productively at the outer edge of the B cell follicle,

![Diagram](image)

**Figure I-4.** B cell activation in the context of a T cell-dependent immune response requires B cells to interact with other immune cell types, send and receive correct signals, and migrate within lymphoid organs. Both cells undergo further activation and differentiation and move into the follicle to initiate a germinal center (Pereira et al. 2010, Victora and Nussenzweig 2012). Germinal centers are transient structures consisting predominantly of B cells undergoing rapid proliferation, class switch recombination (CSR) and somatic hypermutation (SHM). Germinal centers are thus required for long-lasting memory of high-affinity, class switched antibody responses.

The GC can be histologically divided into the light zone (LZ) and the dark zone (DZ) (Victora and Nussenzweig 2012). DZ B cells are highly proliferative. In contrast, CSR and SHM take place in the LZ. The IgH locus is unique among AgR loci in that it contains several constant regions, which can be utilized following CSR. Activated B cells express the activation-induced deamination (AID) enzyme, which deaminates cytosine residues within regulatory regions upstream of IgH constant regions (Muramatsu et al. 2000, Honjo et al. 2002). Processing of these deaminated residues results in a DSB, which can then be repaired by NHEJ to excise intervening constant regions. Use of
different constant regions imbues the resulting BCR and antibody with distinct properties that are important for the humoral immune response on an organismal level (Longerich et al. 2006). The AID enzyme also initiates the process of SHM (Muramatsu et al. 2000, Honjo et al. 2002). SHM introduces point mutations into the antigen-specific variable region of the IgH gene causing variations in affinity of the receptor for antigen. Many of these mutated BCRs have poor affinity for antigen and ultimately die; however, clones with improved antigen affinity survive and may differentiate into antibody secreting cells called plasma cells or long-lived memory B cells (Victora and Nussenzweig 2012). Further, the mutagenic activity of AID in the GC reaction makes mature B cells vulnerable not only to IgH translocations, but also to point mutations incurred during SHM (Alt et al. 2013).

Although the majority of cells in the germinal center are B cells, other cell types play important roles. T follicular helper (Tfh) cells are a specialized subset of CD4+ T cells and are required for the initiation and maintenance of GCs (Victora and Nussenzweig 2012, Cubas et al. 2013). Tfh cells promote the GC response in part by secretion of IL-6 and IL-21 (Eto et al. 2011). T cells also present the ligand for the CD40 receptor expressed on B cells, thereby providing critical survival and differentiation signals to GC B cells (Foy et al. 1994, Linterman and Vinuesa 2010). Conversely, differentiation and maintenance of mature GC Tfh cells requires B cells to express antigen-loaded MHCII molecules (Goenka et al. 2011, Baumjohann et al. 2013), the inducible co-stimulator T cell-ligand (ICOSL) (Nurieva et al. 2008), and likely other co-stimulatory molecules (Akiba et al. 1999, Groth et al. 2002). Thus, GC B cells and GC Tfh cells are uniquely co-dependent.
Regulation of gene expression at multiple levels throughout lymphocyte development and activation

Throughout lymphocyte development and function gene expression changes are tightly regulated, and many overlapping layers of regulatory mechanisms precisely tune the biologic response. Gene expression can be regulated at the levels of transcription, splicing, mRNA decay, mRNA translation, protein turnover, and protein activity. Many examples of each of these mechanisms are observed in lymphocyte-specific phenomena and play critical roles in the development and function of lymphocytes.

At the level of transcription, combinatorial binding of transcription factors activates or represses transcription at promoter and enhancer elements upstream of coding sequences. In turn, these factors are influenced by epigenetic marks placed on chromatin encasing the genes themselves, as well as by the three-dimensional architecture of the chromatin within the nucleus. V(D)J recombination provides ample evidence of this type of gene regulation. Recombination of the correct AgR loci in each lineage, as well as the ordering of recombination within each locus is tightly regulated, largely at the level of transcription and chromatin accessibility (reviewed in (Schatz and Ji 2011)).

Beyond transcription, alternative splicing generates multiple transcripts emanating from the same gene. The transcription factor XBP1 is required for differentiation of GC B cells into plasma cells (Reimold et al. 2001). However, XBP1 is only activated upon alternative splicing of the message by the upstream nuclease IRE1 (Calfon et al. 2002, Iwakoshi et al. 2003). Similarly, alternative splicing of the CD45
mRNA in human T cells results in expression of different protein isoforms, which in turn influence TCR signaling (McKenney et al. 1995, Tong et al. 2005).

Once a mature mRNA leaves the nucleus, it can be stabilized or de-stabilized by miRNAs or RNA-binding proteins (RBPs), and also can also be variably translated into protein. RBPs are now appreciated to play a prominent role in post-transcriptional gene regulation in lymphocytes (Turner and Hodson 2012). Many cytokine mRNAs contain 3’UTR elements that are targets of RBPs. For example, the HuR RBP stabilizes cytokine mRNAs, including those encoding TNFα and IL-4 (Dean et al. 2001, Atasoy et al. 2003, Yarovinsky et al. 2006). HuR also regulates the stability of cyclin D3 mRNA in response to nutrient stress in human T cells (Rodriguez et al. 2010), thereby regulating cell cycle progression.

Proteins are often post-translationally modified, affecting their stability, subcellular localization, and/or activity. For example, nuclear versus cytoplasmic localization of Rag proteins may contribute to regulation of Rag activity. Genotoxic stress causes inducible ATM-dependent Rag2 export from the nucleus (Rodgers et al. 2015). Rag2 is also de-stabilized by CDK-dependent phosphorylation at the Rag2 C-terminus, ensuring that the Rag complex is only active during G1 phase (Li et al. 1996). Cell cycle control is also regulated in part by targeted protein degradation. Cyclin D3 is proteolytically degraded following its phosphorylation by GSK3β in response to cyclic AMP signaling in mature B cells (Naderi et al. 2004). Localization and activity of the HuR RBP is regulated by phosphorylation of several sites by upstream regulators including Chk2 and Cdk1 (Abdelmohsen et al. 2007, Mazan-Mamczarz et al. 2011).
In summary, lymphocyte development and function require carefully coordinated gene expression programs that promote normal lymphocyte development and function, while suppressing malignant transformation. Further, as shown in Chapters II-IV, these processes are carried out in ways that are often specific to the developmental stage and lineage of the cell in question. These findings shed new light on mechanisms controlling diverse cellular processes that in sum maintain genome stability and promote adaptive immunity.
Chapter II: Somatic inactivation of Tp53 in hematopoietic stem cells or thymocytes predisposes mice to thymic lymphomas with clonal translocations

**ABSTRACT**

TP53 protects cells from transformation by responding to stresses including aneuploidy and DNA double-strand breaks (DSBs). TP53 induces apoptosis of lymphocytes with persistent DSBs at antigen receptor loci and other genomic loci to prevent these lesions from generating oncogenic translocations. Despite this critical function of TP53, germline Tp53−/− mice succumb to immature T-cell (thymic) lymphomas that exhibit aneuploidy and lack clonal translocations. However, Tp53−/− mice occasionally develop B lineage lymphomas and Tp53 deletion in pro-B cells causes lymphomas with oncogenic immunoglobulin (Ig) locus translocations. In addition, human lymphoid cancers with somatic TP53 inactivation often harbor oncogenic Ig or T-cell receptor (TCR) locus translocations. To determine whether somatic Tp53 inactivation unmasks translocations or alters the frequency of B lineage tumors in mice, we generated and analyzed mice with conditional Tp53 deletion initiating in hematopoietic stem cells (HSCs) or in lineage-committed thymocytes. Median tumor-free survival of each strain was similar to the lifespan of Tp53−/− mice. Mice with HSC deletion of Tp53 predominantly succumbed to thymic lymphomas with clonal translocations not involving Tcr loci; however, these mice occasionally developed mature B-cell lymphomas that harbored clonal Ig translocations. Deletion of Tp53 in thymocytes caused thymic lymphomas with aneuploidy and/or clonal translocations, including oncogenic Tcr locus translocations. These data demonstrate that the developmental stage of Tp53 inactivation affects karyotypes of lymphoid
malignancies in mice where somatic deletion of Tp53 initiating in thymocytes is sufficient to cause thymic lymphomas with oncogenic translocations.

**INTRODUCTION**

The TP53 tumor suppressor maintains cellular homeostasis in response to a wide range of stresses including aneuploidy, DSBs, genomic instability, and oncogene activation (Meek 2009, Reinhardt and Schumacher 2012). These stresses stabilize and activate TP53, leading to changes in expression of target genes, including those involved in cell cycle control and apoptosis (Meek 2009, Reinhardt and Schumacher 2012). TP53 is the most frequently inactivated tumor suppressor gene, with mutation or deletion occurring in over 60% of all human cancers (Cheung et al. 2009, Meek 2009), indicating that TP53 prevents malignant transformation of multiple cell types. Although TP53 inactivation occurs less frequently in lymphoid malignancies than in solid tumors, TP53 loss is more common in aggressive lymphoma subtypes and correlates with increased tumor grade, treatment resistance, and poor patient survival (Bhatia et al. 1992, Stilgenbauer et al. 2002, Hof et al. 2011).

Lymphocyte development involves cellular proliferation and antigen receptor gene assembly. Bone marrow HSCs differentiate into early progenitor B cells that remain in the bone marrow or into early thymic progenitors that migrate to the thymus. These cells proliferate and differentiate into pro-B or pro-T cells, respectively, which induce expression of the RAG1/RAG2 (RAG) endonuclease (Chi et al. 2009, Ramirez et al. 2010). RAG catalyzes the assembly of TCR and Ig genes in G1 phase cells through induction of DSBs at variable (V), diversity (D), and joining (J) gene segments (Schatz and Ji 2011, Alt et al. 2013). Non-homologous end-joining (NHEJ) factors repair these
DSBs to generate V(D)J coding joins that encode the first exons of TCR and Ig genes (Lieber 2010, Alt et al. 2013). Assembly of TCRβ, TCRγ, and TCRδ genes occurs in CD4−CD8− “double-negative” (DN) pro-T cells (Krangel et al. 2004, Jung et al. 2006). Expression of functional TCRγ and TCRδ genes signals differentiation into mature γδ T cells (Xiong and Raulet 2007). In contrast, expression of functional TCRβ genes triggers proliferation as cells differentiate into CD4+CD8− “double positive” (DP) thymocytes (Bell and Bhandoola 2008). In DP thymocytes, TCRα gene assembly followed by αβ TCR selection permit differentiation into CD4+ or CD8+ “single positive” (SP) thymocytes that exit the thymus as naive αβ T cells (Krangel et al. 2004, von Boehmer and Melchers 2010). Assembly and expression of IgH genes in pro-B cells drives proliferation as cells differentiate into pre-B cells, which must recombine either Igκ or Igλ genes to differentiate into immature B cells that exit the bone marrow and migrate to the spleen as they mature (Jung et al. 2006, Nemazee 2006, Kuo and Schlissel 2009). In response to antigen, mature B cells proliferate and undergo IgH class switch recombination (CSR) through DSB intermediates (Longerich et al. 2006, Keim et al. 2013) (Bassing et al. 2003, Boboila et al. 2012). In addition to programmed DSBs in antigen receptor loci, lymphocytes experience spontaneous DSBs that arise from errors in DNA replication during periods of proliferation (Bassing and Alt 2004).

*TP53* inactivation occurs in human B and T lineage lymphomas containing aneuploidy as well as in those exhibiting genomic instability (Cheung et al. 2009, Dicker et al. 2009), suggesting that functions of TP53 in response to chromosome missegregation and DSBs are each important to suppress transformation of differentiating lymphocytes. Aberrant segregation of chromosomes during cellular division leads to TP53-dependent apoptosis of ensuing aneuploid daughter cells.
(Thompson and Compton 2010). Induction of DSBs stabilizes and activates TP53, which promotes temporary cell cycle arrest to provide cells time to repair these lesions or induce apoptosis if they cannot be repaired (Reinhardt and Schumacher 2012). Germline *Tp53* inactivation in mice leads to aneuploidy and genomic instability in differentiating and mature lymphocytes (Fukasawa et al. 1997), and enables pro-T and pro-B cells with un-repaired RAG-induced Tcrδ and IgH locus DSBs to survive, progress into S phase, and generate translocations (Nacht et al. 1996, Difilippantonio et al. 2002, Zhu et al. 2002, Gladdy et al. 2003, Dujka et al. 2010). Despite roles of TP53 in response to both chromosome missegregation and DSBs, most *Tp53*-/- mice succumb to aneuploid TCRβ+ thymic lymphomas that lack Tcr translocations, though a small fraction succumb to B cell lymphomas that have not been assayed for translocations (Donehower et al. 1992, Jacks et al. 1994, Liao et al. 1998, Ward et al. 1999, Celeste et al. 2003, Jacobs et al. 2011). However, mice with combined germline inactivation of Tp53 and NHEJ factors reproducibly succumb to pro-B cell lymphomas with RAG-dependent IgH translocations that amplify the c-Myc oncogene (Guidos et al. 1996, Difilippantonio et al. 2000, Zhu et al. 2002), and occasionally develop TCRβ- thymic lymphomas with Tcrδ translocations (Rooney et al. 2004). In addition, *Tp53*-/- mice with germline inactivation of the H2ax DSB repair factor predominantly succumb to TCRβ- thymic lymphomas with clonal translocations not involving Tcr loci, but occasionally develop TCRβ- lymphomas with Tcrα/δ translocations or pro-B cell lymphomas with *Igh;c-myc* translocations (Bassing et al. 2003, Celeste et al. 2003). Furthermore, on a genetic background with a block in αβ T cell development at the DN stage, germline *Tp53* deficiency causes TCRβ- thymic lymphomas with RAG-dependent translocations that may involve Tcrδ or IgH loci (Haines et al. 2006). Together, these studies showed
that TP53 functions in response to chromosome missegregation and DSBs are critical to prevent transformation of differentiating lymphocytes. However, they concluded that Tp53 only suppresses oncogenic translocations in cells with DSB repair or differentiation impaired.

Cancers develop through somatic acquisition and selection of mutations such as TP53 inactivation and other oncogenic lesions (Aparicio and Caldas 2013). We previously showed, that while germline $H2ax^{-/-} Tp53^{-/-}$ mice succumb to TCR$\beta^-$ thymic lymphomas with clonal translocations, conditional deletion of $H2ax$ and $Tp53$ in mouse DN thymocytes prolongs lifespan and leads to TCR$\beta^+$ thymic lymphomas (Bassing et al. 2003, Celeste et al. 2003, Yin et al. 2011). That somatic inactivation of H2ax and Tp53 causes more mature thymic lymphomas with longer latency compared to germline inactivation indicates that oncogenic lesions prior to T cell commitment drive transformation of thymocytes. We recently showed that conditional deletion of $Tp53$ in pro-B cells predisposes mice to B lineage lymphomas with oncogenic translocations, including $Igh;c-myc$ and other $Ig$ translocations (Rowh et al. 2011). The tumor-free survival of these mice is similar to that of $Tp53^{-/-}$ mice, suggesting that development of thymic lymphomas from aneuploidy prevents B cell lymphomas from oncogenic $Ig$ translocations in $Tp53^{-/-}$ mice. To determine whether somatic inactivation of $Tp53$ unmask...
and/or clonal translocations including \( Tcra/\delta \) locus translocations. Our data demonstrate that the developmental stage of \( Tp53 \) inactivation affects karyotypes of lymphoid cancers in mice where somatic deletion of \( Tp53 \) initiating in thymocytes is sufficient to cause thymic lymphomas with oncogenic translocations.

**RESULTS**

**Conditional deletion of \( Tp53 \) in HSCs or thymocytes predisposes mice to thymic lymphomas.**

To determine whether \( Tp53 \) inactivation initiating in HSCs or thymocytes predisposes mice to lymphoma, we established and characterized \( Vav-\text{cre}^{+/-}p53^{\text{floxed/floxed}} \) (VP) and \( Lck-\text{cre}^{+/-}p53^{\text{floxed/floxed}} \) (LP) mice. Vav-cre and Lck-cre induce deletion of "floxed" genes in HSCs and DN thymocytes, respectively (Lee et al. 2001, Georgiades et al. 2002). We detected nearly complete deletion of \( Tp53 \) in bone marrow cells and thymocytes of VP mice and in thymocytes of LP mice, but no \( Tp53 \) deletion in LP bone marrow cells (Supplementary figure 1), confirming the expected developmental timing of \( Tp53 \) inactivation in VP and LP mice. We also found grossly normal T and B cell development in VP and LP mice as compared to age-matched wild-type controls (Supplementary figure 2), consistent with the phenotype of \( Tp53^{-/-} \) mice (Lowe et al. 1993). We generated and aged cohorts of 22 VP and 20 LP mice to evaluate their spontaneous predisposition to cancer. We observed that cohort VP mice survived cancer-free between 91-365 days with median age of mortality of 144.5 days, whereas cohort LP mice survived cancer-free between 70-365 days with a median age of mortality of 119 days (Figure 1). The median ages of cancer-free survival of cohort VP and LP mice were not statistically significant (p=0.134) and were comparable to the survival of germline \( Tp53^{-/-} \) mice (Donehower et
al. 1992, Jacks et al. 1994). All VP and LP mice succumbed to lymphoma, except for one LP mouse that was euthanized due to development of a sarcoma and one mouse of each genotype that died without lymphoma (Table 1, 2). Most VP mice and LP mice succumbed to lymphomas that were only visible in the thymus (Table 1, 2). However, three VP mice (nos. 202, 228, and 623) and two LP mice (nos. 119 and 983) had lymphoma cells in their spleens, while one VP mouse (no. 602) succumbed to a disseminated lymphoma found in the thymus, spleen, and multiple lymph nodes (Table 1, 2). In addition, two VP mice (nos. 421 and 426) succumbed to lymphomas that were located in the spleen and multiple lymph nodes, but not visible in the thymus. We did not characterize cohorts of Vav-cre\textsuperscript{+/−}, Lck-cre\textsuperscript{+/−}, or p53\textsuperscript{floxflox} mice since none of these mice exhibits increased tumor predisposition (Jonkers et al. 2001, Lee et al. 2001, Georgiades et al. 2002). Consistent with this notion, none of the Vav-cre\textsuperscript{+/−}, Lck-cre\textsuperscript{+/−}, or p53\textsuperscript{floxflox} mice we used for breeding until one year of age developed cancer.

**Tp53 inactivation in HSCs or thymocytes causes clonal immature T cell lymphomas.**

To determine the lymphocyte lineages and developmental stages to which inactivation of Tp53 in HSCs or DN thymocytes causes cellular transformation, we first analyzed VP and LP lymphomas by flow cytometry using antibodies that recognize cell surface markers of specific lymphocyte lineages and developmental stages. For lymphomas found within the thymus, we assessed cell surface expression of TCR\(\delta\), TCR\(\beta\), CD4, CD8, and the CD3\(\varepsilon\) molecule through which \(\alpha\beta\) TCRs signal (Bell and Bhandoola 2008). For lymphomas found in lymph nodes, we assessed cell surface expression of the CD43, B220, IgM, Ig\(\kappa\), and Ig\(\lambda\) molecules. Due to the variation in cell
staining observed for some tumors, we simplified our classification by denoting a lymphoma as positive for an epitope if more than half of the cells fell within a positive gate established from flow cytometry of non-malignant lymphocytes. Of the 17 VP thymic lymphomas assayed, most were TCRβ⁺CD3⁺CD4⁺CD8⁺, but a substantial number were also TCRβ⁻CD3⁻ with or without CD4 and CD8 expression (Figure 1B; Table 1; Supplementary figure 3). Lymphoma cells in the spleens of the two VP mice (nos. 421 and 426) that lacked thymic lymphomas were B220⁺IgM⁺ (Figure 1B; Table 1; Supplementary figure 3). Lymphoma no. 421 contained Igκ⁺ and Igκ⁻ cells, while lymphoma no. 426 contained mostly Igκ⁻ cells (Figure 1B; Table 1; Supplementary figure 3). Of the 15 LP thymic lymphomas assayed, 10 were TCRβ⁺CD3⁺CD4⁺CD8⁺ only one was TCRβ⁻CD3⁻ (Figure 1B; Table 2; Supplementary figure 3). Many VP and LP thymic lymphomas displayed subpopulations with different expression patterns of TCRβ, CD3, CD4, and/or CD8, indicating that they represent either oligoclonal lymphomas arising from distinct initiating cells or clonal lymphomas with subpopulations that have differentially silenced and/or re-expressed genes. VP thymic lymphomas more often showed TCRβ, CD4, CD8, and CD3 expression characteristic of immature T cell developmental stages than LP thymic lymphomas. Collectively, our flow cytometry analysis of VP and LP lymphomas indicates that deletion of Tp53 in mouse HSCs or DN thymocytes causes predominantly immature T cell lymphomas, with HSC deletion leading to a higher percentage of tumors from an earlier T cell developmental stage and occasional mature B cell lymphomas.

To determine the lymphocyte lineage of the four TCRβ⁻CD3⁻CD4⁻CD8⁻ VP thymic lymphomas and to distinguish between oligoclonal and clonal immature T cell lymphomas in VP and LP mice, we next analyzed Tcrβ gene rearrangements in VP and
LP lymphomas. For DN thymocytes to survive and differentiate, Tcrβ rearrangements must occur on one allele. (Jiang et al. 1996) To characterize Tcrβ rearrangements, we conducted Southern blotting on HindIII-digested genomic DNA of VP and LP lymphomas with 3’Jβ1 and 3’Jβ2 probes (Figure 2A-C). We isolated DNA from the tumor-containing organ without further purification, under assumption that most cells were malignant. This was confirmed by flow cytometry. Further, this approach does not detect unique rearrangements in single cells, such as those occurring in normal lymphocytes. Thus, only germline configurations in pro-lymphocytes or in non-lymphoid cells will be detected as background. We detected Tcrβ rearrangements in the TCRβ-CD3-CD4-CD8- VP thymic lymphomas (nos. 228, 602, 618, and 773) (Figure 2B), demonstrating that these malignancies are immature T cell lymphomas. We also found that 16 of 18 VP and all 15 LP thymic lymphomas analyzed contained one or two rearranged Tcrβ alleles and therefore arose from the expansion of a single cancer-initiating cell (Figure 2B,C). These data indicate that the diverse expression of surface epitopes observed within some VP and LP lymphomas represents tumor subpopulations that have differentially silenced and/or re-expressed these genes. The remaining two VP thymic lymphomas (nos. 124 and 618) contained three Tcrβ rearrangements (Figure 2B), indicating that these cancers either arose from the expansion of two cancer-initiating cells or one cancer-initiating cell that continued to assemble Tcrβ genes after malignant transformation. We also performed Southern analysis of Tcrβ rearrangements on VP lymphomas found in the spleen or lymph nodes of mice. We found that three of these four VP lymphomas analyzed contained the same Tcrβ rearrangements as the thymic lymphomas from the same animals (Figure 2B), demonstrating that these mice succumbed to a disseminated clonal immature T cell lymphoma. VP lymphoma (no. 202) displayed an additional Tcrβ
rearrangement that was not present in the thymic lymphoma of this mouse (Figure 2B), suggesting that this splenic lymphoma developed from the thymic lymphoma in association with ongoing Tcrβ rearrangement. Our Southern analysis of Tcrβ rearrangements in VP and LP lymphomas indicates that inactivation of Tp53 in mouse HSCs or DN thymocytes causes mainly clonal immature T cell malignancies.

To determine whether the two B lineage lymphomas that arose in VP cohort mice were clonal and whether they arose from developing or mature B cells, we analyzed lgh and Igκ rearrangements in these tumors. To characterize lgh rearrangements, we conducted Southern blot analysis of EcoRI-digested genomic DNA of VP lymphomas nos. 421 and 426 with 3'JH and 3'Sμ probes (Figure 3A-C). We found that these tumors contained one (no. 426) or two (no. 421) JH rearrangements and therefore arose from the clonal expansion of a single cancer-initiating cell (Figure 3B). We also detected Sμ recombination in lymphoma no. 421 (Figure 3C), suggesting that this tumor may have developed from a B cell that had attempted CSR. Southern blot analysis of BamHI-digested genomic DNA from VP lymphomas nos. 421 and 426 with the 3'Jκ probe showed that each of these tumors contained Jκ rearrangements (Figure 3D,E). This suggests that IgM+Igκ- VP lymphoma no. 426 developed from a B lymphocyte that had developed at least to the pre-B cell stage. Our Southern analysis of these two VP B lineage lymphomas indicates that deletion of Tp53 in mouse HSCs can cause clonal B lineage lymphomas.

**Conditional inactivation of Tp53 in HSCs or thymocytes causes lymphomas with clonal translocations.**
To determine whether conditional deletion of *Tp53* initiating in HSCs or DN thymocytes causes lymphomas with chromosomal translocations, we conducted Spectral Karyotyping (SKY) on seven *VP* and eight *LP* lymphomas. SKY is a molecular cytogenetic approach to visualize all chromosomes in a single metaphase spread to identify translocations and fusions between chromosomes (Liyanage et al. 1996). We defined clonal translocations as those found in greater than half of the metaphases analyzed for a given tumor. SKY revealed that two of the five *VP* thymic lymphomas (nos. 118 and 820) analyzed had clonal translocations, with lymphoma no. 773 containing three different clonal chromosome fusions (Figure 4A; Table 1; Supplementary figure 4). One *VP* thymic lymphomas (no. 975) contained two non-clonal chromosome fusions (Table 2; Supplementary figure 4). The remaining *VP* thymic lymphoma (no. 207) lacked translocations but exhibited aneuploidy (Table 1; Supplementary figure 4). None of the clonal translocations in *VP* thymic lymphomas involved chromosomes on which *Tcr* or *Ig* loci reside. In contrast, SKY revealed that both *VP* B lymphomas contained clonal translocations involving chromosomes on which the *Igh* (chromosome 12 in no. 421) or *Igκ* (chromosome 6 in no. 426) locus resides (Figure 4B; Table 1; Supplementary figure 4). SKY also demonstrated that three of the eight *LP* thymic lymphomas (nos. 826, 902, and 976) analyzed harbored clonal translocations, with lymphoma no. 902 also containing a clonal chromosome fusion (Figure 4A; Table 2; Supplementary figure 4). Notably, two of these tumors (no. 902 and 976) contained clonal translocations involving chromosome 14 which carries the *Tcrα/δ* locus. The other five *LP* thymic lymphomas lacked translocations but exhibited aneuploidy (Table 2). Our SKY analysis shows that conditional deletion of *Tp53* deletion...
in HSCs or thymocytes causes lymphomas with clonal translocations, with thymocyte deletion also causing aneuploid lymphomas.

To determine whether conditional deletion of \(Tp53\) initiating in HSCs or DN thymocytes causes lymphomas with clonal \(Ig\) or \(Tcr\) translocations, respectively, we conducted FISH on the four tumors with potential clonal \(Igh\), \(Ig\kappa\), or \(Tcra/\delta\) translocations. For this purpose, we hybridized 5’ and 3’ \(Ig\) or \(Tcr\) locus probes and identified \(Ig\) or \(Tcr\) translocations by detection of probe signals on different chromosomes (Figure 4B,C). Since the \(c-myc\) oncogene on chromosome 15 is activated by \(Igh\) or \(Tcra/\delta\) translocations in mouse lymphomas (Rooney et al. 2004, Rowh et al. 2011), we also used a \(c-myc\) probe to identify potential \(Igh;c-myc\) and \(Tcra/\delta;c-myc\) translocations in \(VP\) lymphoma no. 421 and \(LP\) lymphoma no. 902. FISH revealed co-localization of 3’\(Igh\) and \(c-myc\) probe signals on one chromosome derivative in \(VP\) B lineage lymphoma no. 421 (Figure 4B), indicating that the clonal t(12;15) translocation in this tumor is an \(Igh;c-myc\) translocation. Unfortunately, we were unable to determine potential involvement of the \(Ig\kappa\) locus in the clonal t(6;4) translocation of \(VP\) B lineage lymphoma no. 426 due to insufficient numbers of metaphases from this tumor. FISH revealed splitting of 5’ and 3’ probes on the clonal t(16;14) translocation in metaphases from \(LP\) thymic lymphoma no. 976 (Supplementary figure 4 and not shown), indicating that this translocation tumor involves the \(Tcra/\delta\) locus. Finally, FISH showed co-localization of multiple copies each of 5’\(Tcra/\delta\) and \(c-myc\) probe signals on one chromosome derivative in metaphases from \(LP\) thymic lymphoma no. 902 (Figure 4C and not shown), indicating that the clonal t(14;15;4) translocation in this tumor is a \(Tcra/\delta;c-myc\) translocation with amplification of the \(c-myc\) oncogene and \(Tcra\) sequences. Our FISH analysis of metaphases from \(VP\) and \(LP\) lymphomas
demonstrates that deletion of *Tp53* initiating in HSCs or DN thymocytes can cause, respectively, B lineage lymphomas with oncogenic *Igh* translocations or thymic lymphomas with oncogenic *Tcrα/δ* translocations.
DISCUSSION

Our study demonstrates that the context of Tp53 inactivation influences lymphoma predisposition, and that inactivation of Tp53 in HSCs or in thymocytes predisposes mice to thymic lymphomas with clonal translocations including those involving the Tcrα/δ locus. We generated mice with conditional deletion of Tp53 initiating in HSCs or in DN thymocytes and compared their tumor predisposition to the well-characterized cancer phenotype of germline Tp53-deficient mice. We found that HSC initiation of Tp53 inactivation predisposed mice to predominantly thymic lymphomas with clonal translocations not involving antigen receptor loci and occasionally to peripheral B cell lymphomas with clonal Ig translocations. Inactivation of Tp53 in DN thymocytes predisposed mice to thymic lymphomas that exhibited aneuploidy or contained clonal translocations frequently involving Tcrα/δ loci. In contrast, germline inactivation of Tp53 predisposes mice to aneuploid thymic lymphomas lacking clonal translocations (Donehower et al. 1992, Jacks et al. 1994, Celeste et al. 2003). Other than the timing of Tp53 inactivation, the only difference between VP and LP mice and Tp53−/− mice is constitutive expression of Cre. Constitutive Cre expression causes genomic instability, at least in mouse embryonic cells cultured in vitro (Loonstra et al. 2001, Silver and Livingston 2001), suggesting that the translocations found in VP and LP lymphomas could be Cre-induced lesions. Since the lymphoma predisposition of Vav-cre:Tp53−/− mice has not been reported, we cannot conclude whether the clonal translocations and chromosome fusions found in VP thymic lymphomas arise independently of Vav-cre expression. Yet, considering that Vav-cre mice are not predisposed to cancer (Georgiades et al. 2002), our findings demonstrate that Tp53 serves important functions in HSCs and/or thymocytes to suppress malignant transformation in association with
genomic instability. Notably, constitutive Cre expression from Lck-cre initiating in DN thymocytes of Tp53−/− mice does not alter onset or karyotype of thymic lymphomas that arise in these mice (Cheung et al. 2002). Therefore, we conclude from the cancer predisposition of LP mice that Tp53 serves critical functions in suppressing generation and/or oncogenic potential of Tcra/δ translocations during αβ T cell development.

The objective of our study was to determine whether oncogenic lesions arising during embryogenesis and/or in HSCs precludes development of thymic lymphomas with clonal translocations including Tcr translocations. VP and LP mice succumb to tumors at similar ages as Tp53−/−, Lck-cre:Tp53−/− mice, and Mb1-cre:Tp53floxflox mice (Donehower et al. 1992, Jacks et al. 1994, Celeste et al. 2003, Rowh et al. 2011). In contrast to the aneuploid thymic lymphomas that arise in Tp53−/− mice, we found that VP and LP mice developed lymphomas with aneuploidy or clonal translocations, including Ig or Tcra/δ translocations. The distinct cancer phenotypes of these mice indicate that loss of Tp53 during embryogenesis, in cells before lymphocyte commitment, and/or in thymocytes masks development of lymphomas with oncogenic translocations in germline Tp53-deficient mice. In addition, the development of LP thymic lymphomas with aneuploidy or clonal translocations indicates that functions of Tp53 in response to both chromosome missegregation and DSBs are each critical for preventing malignant transformation of thymocytes.

T-cell acute lymphoblastic leukemia (T-ALL) remains a significant cause of cancer morbidity and mortality in both children and adults (Smith et al. 2010, Maloney et al. 2012). Advances have been made in treatment of patients with T-ALL, however drug-resistance and relapse are common causes of treatment failure and most patients with tumors that fail treatment do not survive (Smith et al. 2010). T-ALL is typically treated
using genotoxic drugs that can cause serious health issues through effects on normal cells, demonstrating a need to develop more specific and less toxic therapies (Bhatia 2012). T-ALLs have heterogeneous karyotypes, with about half being aneuploid and the remainder containing translocations including oncogenic Tcrα/δ translocations (Graux et al. 2006, Mrozek et al. 2009, Le Noir et al. 2012). Although inactivating TP53 mutations are not common in T-ALL, these genetic lesions are often associated with drug resistance, rapid disease progression, and poor survival (Cheung et al. 2009). Therefore, LP mice may provide a useful pre-clinical model to evaluate the potential efficacy of more specific and less toxic treatments for T-ALL with TP53 inactivation.
Figure 1. Mice with conditional inactivation of Tp53 initiating in HSCs or DN thymocytes reproducibly succumb to thymic lymphomas. (A) Kaplan–Meier curves comparing tumor-free survival of 22 VP and 20 LP mice. All tumors were thymic lymphomas other than two VP mice that succumbed to B lineage lymphomas (indicated by asterisks). Additionally, one LP mouse succumbed to a sarcoma, and one mouse of each cohort did not succumb to any tumors by one year of age. Curves were compared using the log-rank (Mantel–Cox) test, \( P = 0.134 \) (B) Flow cytometry analyses of VP lymphomas no. 118 and 975 and of LP lymphomas no. 740 and 976 showing their surface expression of CD3 and TCRβ or CD4 and CD8. Gates were drawn on normal T cells analyzed in parallel with the lymphomas. The percentages of cells in each gate are indicated. (C) Flow cytometry analyses of VP lymphomas no. 421 and 426 showing their surface expression of B220 and IgM or Igκ and Igλ. Gates were drawn on normal B cells analyzed in parallel with lymphomas. The percentages of tumor cells in each gate are indicated.
Figure 2. Mice with conditional inactivation of Tp53 initiating in HSCs or DN thymocytes develop clonal thymic lymphomas. (A) Schematic of the mouse TCRβ locus showing relative locations of representative upstream Vβs, the two Dβ-Jβ-Cβ clusters, and the Vβ14 segment. The positions of the HindIII restriction sites (H3) and 3'Jβ1 and 3'Jβ2 probes used for Southern analyses are also shown. (B and C). Southern blot analysis of HindIII-digested DNA isolated from the indicated (B) VP or (C) LP lymphomas or from kidneys of C57BL/6 (B6) or 129/SvEv (129) control mice using the 3'Jβ1 or 3'Jβ2 probe. Germline (GL) bands for each probe are indicated. Membranes were hybridized with 3'Jβ1 probes, stripped, and then hybridized with 3'Jβ2 probes to reveal which lymphomas contained Vβ rearrangements to the Dβ2-Jβ2 cluster on both alleles.
Figure 3. Mice with conditional inactivation of Tp53 initiating in HSCs also develop clonal B lineage lymphomas. (A) Schematic of the mouse IgH locus showing relative locations of representative D\(\beta\)H segments, the four J\(\beta\)H segments, the S\(\mu\) region, and the first C\(\beta\)H exon, C\(\mu\). The positions of the EcoRI restriction sites (RI) and 3′J\(\beta\)H and 3′S\(\mu\) probes used for Southern blots are also shown. (B and C) Southern blot analysis of EcoRI-digested DNA isolated from VP lymphomas no. 421 or 426 or from kidneys of C57BL/6 (B6) or 129/SvEv (129) control mice using the (B) 3′J\(\beta\)H and (C) 3′S\(\mu\) probes. Germline (GL) bands for each probe are indicated. (D) Schematic of the mouse Igκ locus showing relative locations of the five Jκ segments and the Cκ exon. The positions of the BamHI restriction sites (BHI) and 3′Jκ probe used for Southern blots are also shown. (E) Southern blot analysis of BamHI-digested DNA isolated from VP lymphomas no. 421 or 426 or from kidneys of C57BL/6 (B6) or 129/SvEv (129) control mice using the 3′Jκ probe. Germline (GL) band for the 3′Jκ probe is indicated. (F) Schematic of the mouse c-Myc locus showing relative locations of the three c-Myc exons, and of the EcoRI restriction sites (RI) and 3′Myc probe used for Southern blots. (G) Southern blot analysis of EcoRI-digested DNA isolated from VP lymphomas no. 421 or 426 or from kidneys of C57BL/6 (B6) or 129/SvEv (129) control mice using the 3′Myc probe. Germline (GL) band for the 3′Myc probe is indicated.
Figure 4. Mice with conditional inactivation of Tp53 initiating in HSCs or DN thymocytes develop lymphomas with oncogenic antigen receptor locus translocations. (A) Cytogenetic analysis of a metaphase from VP lymphoma no. 820 with the clonal t(2;17) translocation circled or isolated. (A, i) Spectral image. (A, ii) DAPI image. (A, iii) Karyotype table. (B) Cytogenetic analyses of a metaphase or chromosome from VP lymphoma no. 421 with the clonal t(12;15) translocation circled. (B, i) SKY image. (B, ii) DAPI image. (B, iii) Karyotype table. (B, iv) SKY (left) or FISH image (right) of the t(12;15) translocation hybridized with 3'IgH (green) and c-Myc (red) probes. (C) Cytogenetic analyses of a metaphase or chromosome from LP lymphoma no. 902 with the clonal t(4;15;14) translocation circled or isolated. (B, i) SKY image. (B, ii) DAPI image. (B, iii) Karyotype table. (B, iv) SKY (top) or FISH images of the t(4;15;14) translocation. (middle) hybridized with TCRVδ3/Vα6 (green) and TCRCα (red) probes. (bottom) hybridized with c-Myc (green) probe.
Table 1. Summary of VP tumor cohort.

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Lifespan, days</th>
<th>Gross phenotype</th>
<th>Karyotype¹</th>
<th>Surface expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>820</td>
<td>108</td>
<td>thy lymph</td>
<td>t(2;17) (28/33)</td>
<td>CD3+ TCRβ+ CD4/8 var</td>
</tr>
<tr>
<td>202</td>
<td>159</td>
<td>thy lymph + spleen</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>105</td>
<td>106</td>
<td>thy lymph</td>
<td>t(11;15) (16/26),</td>
<td>CD3+ TCRβ+ DP</td>
</tr>
<tr>
<td>118</td>
<td>119</td>
<td>thy lymph</td>
<td>t(12;19) (9/26)</td>
<td>CD3+ TCRβ+ DP</td>
</tr>
<tr>
<td>974</td>
<td>99</td>
<td>thy lymph</td>
<td>t(12;12) (12/36),</td>
<td>CD3+ TCRβ+ CD4/8 var</td>
</tr>
<tr>
<td>975</td>
<td>103</td>
<td>thy lymph</td>
<td>t(11;11) (15/36)</td>
<td>CD3- TCRβ- DP</td>
</tr>
<tr>
<td>112</td>
<td>134</td>
<td>thy lymph</td>
<td>--</td>
<td>CD3- TCRβ- DP</td>
</tr>
<tr>
<td>979</td>
<td>102</td>
<td>thy lymph</td>
<td>--</td>
<td>CD3+ TCRβ+ DP</td>
</tr>
<tr>
<td>977</td>
<td>119</td>
<td>thy lymph</td>
<td>--</td>
<td>CD3+ TCRβ+ DN</td>
</tr>
<tr>
<td>124</td>
<td>176</td>
<td>thy lymph</td>
<td>--</td>
<td>CD3- TCRβ- DP</td>
</tr>
<tr>
<td>930</td>
<td>93</td>
<td>thy lymph</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>207</td>
<td>231</td>
<td>thy lymph</td>
<td>aneuploid</td>
<td>CD3+ TCRβ+ DP</td>
</tr>
<tr>
<td>228</td>
<td>123</td>
<td>thy lymph + spleen</td>
<td>--</td>
<td>CD3- TCRβ- DN</td>
</tr>
<tr>
<td>306</td>
<td>129</td>
<td>thy lymph</td>
<td>--</td>
<td>CD3+ TCRβ+ DP</td>
</tr>
<tr>
<td>919</td>
<td>155</td>
<td>thy lymph</td>
<td>--</td>
<td>CD3+ TCRβ+ DN</td>
</tr>
<tr>
<td>421</td>
<td>176</td>
<td>LN tumor</td>
<td>t(12;15) (24/25)</td>
<td>B220+ IgM+ Igκ+/-</td>
</tr>
<tr>
<td>426</td>
<td>305</td>
<td>LN tumor</td>
<td>t(6;4) (23/24)</td>
<td>B220+ IgM+ Igκ-</td>
</tr>
<tr>
<td>618</td>
<td>213</td>
<td>thy lymph</td>
<td>--</td>
<td>CD3- TCRβ- DN</td>
</tr>
<tr>
<td>602</td>
<td>217</td>
<td>disseminated</td>
<td>--</td>
<td>CD3- TCRβ- DN</td>
</tr>
<tr>
<td>623</td>
<td>260</td>
<td>thy lymph + spleen</td>
<td>--</td>
<td>CD3+ TCRβ+ DN</td>
</tr>
<tr>
<td>773</td>
<td>216</td>
<td>thy lymph</td>
<td>t(4;4) (15/15), t(5;5)</td>
<td>CD3- TCRβ- DN</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(14/15), t(15;17) (12/15)</td>
<td></td>
</tr>
<tr>
<td>720</td>
<td>365</td>
<td>none apparent</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

¹ For each prevalent structural aberration, numbers in parentheses indicate proportion of metaphases analyzed carrying that aberration.

-- Dashes indicate parameters not assessed.

**Abbreviations:**
- thy lymph: thymic lymphoma
- CD4/8 var: variable levels of CD4 and/or CD8 expression
- DP: CD4+CD8+ double positive
- DN: CD4-CD8- double negative
- LN: lymph node
<table>
<thead>
<tr>
<th>Mouse</th>
<th>Lifespan, days</th>
<th>Gross phenotype</th>
<th>Karyotype†</th>
<th>Surface expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>722</td>
<td>72</td>
<td>thy lymph</td>
<td>--</td>
<td>CD3+ TCRβ+ CD4/8 var</td>
</tr>
<tr>
<td>734</td>
<td>76</td>
<td>thy lymph</td>
<td>aneuploid</td>
<td>CD3+ TCRβ+ DP</td>
</tr>
<tr>
<td>740</td>
<td>70</td>
<td>thy lymph</td>
<td>aneuploid</td>
<td>CD3+ TCRβ+ DP</td>
</tr>
<tr>
<td>824</td>
<td>105</td>
<td>thy lymph</td>
<td>aneuploid</td>
<td>CD3+ TCRβ+ CD4/8 var</td>
</tr>
<tr>
<td>826</td>
<td>111</td>
<td>thy lymph</td>
<td>t(11;19) (12/20)</td>
<td>CD3- TCRβ- DP</td>
</tr>
<tr>
<td>983</td>
<td>156</td>
<td>thy lymph</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>646</td>
<td>116</td>
<td>thy lymph</td>
<td>aneuploid</td>
<td>CD3+ TCRβ+ DP</td>
</tr>
<tr>
<td>902</td>
<td>158</td>
<td>thy lymph</td>
<td>t(4;15;14) (9/13), t(2;2) (10/13)</td>
<td>CD3+ TCRβ+DP</td>
</tr>
<tr>
<td>654</td>
<td>118</td>
<td>thy lymph</td>
<td>--</td>
<td>CD3- TCRβ- CD4/8 var</td>
</tr>
<tr>
<td>907</td>
<td>119</td>
<td>thy lymph</td>
<td>--</td>
<td>CD3+ TCRβ+DP</td>
</tr>
<tr>
<td>975</td>
<td>119</td>
<td>thy lymph</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>976</td>
<td>98</td>
<td>thy lymph</td>
<td>t(16;14) (8/9)</td>
<td>CD3+ TCRβ+ CD4+</td>
</tr>
<tr>
<td>913</td>
<td>129</td>
<td>thy lymph</td>
<td>--</td>
<td>CD3+ TCRγδ+ TCRβ+ DP</td>
</tr>
<tr>
<td>915</td>
<td>171</td>
<td>thy lymph</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>930</td>
<td>146</td>
<td>thy lymph</td>
<td>--</td>
<td>CD3+ TCRβ+DP</td>
</tr>
<tr>
<td>767</td>
<td>142</td>
<td>none apparent</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>111</td>
<td>153</td>
<td>thy lymph</td>
<td>--</td>
<td>CD3+ TCRβ+DP</td>
</tr>
<tr>
<td>126</td>
<td>168</td>
<td>thy lymph</td>
<td>--</td>
<td>CD3+ TCRβ+DP</td>
</tr>
<tr>
<td>119</td>
<td>192</td>
<td>thy lymph + spleen abdominal sarcoma</td>
<td>aneuploid</td>
<td>CD3+ TCRβ+DP</td>
</tr>
<tr>
<td>288</td>
<td>297</td>
<td>none apparent</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

Abbreviations explained in Table I.
Supplementary figure 1. Cre-mediated deletion of floxed *Tp53* exons is robust and limited to expected tissues. (A) Schematic of the *Tp53* locus showing relative locations of exons (red boxes) and loxP sites before or after Cre-mediated recombination (+/+Cre). Locations of *BgII* restriction sites and the plox probe are also shown. (B) Southern blot analysis of *BgII*-digested DNA isolated from indicated tissues of wild type C57BL/6 (B6), 129/SvEv (129), p53<sup>flox/flox</sup> (ctrl), VP, or LP mice using the plox probe. Lack of signal in 129 kidney and ctrl2 thymus sample lanes is due to inadequate DNA loading.
Supplementary figure 2.

A

Bone marrow

Spleen

CD43

IgM

IgLA

B

Thymus

C

Spleen

CD3

TCRβ

CD8

CD4

CD3

TCRβ

CD8

CD4

p53ff

pre-B

pre-B

pre-B

pre-B

VP

pre-B

pre-B

pre-B

pre-B

LP

pre-B

pre-B

pre-B

pre-B
Supplementary figure 2. Lymphocyte development is normal in young LP and VP mice. (A-C) Flow cytometry was performed on lymphocytes from (A) bone marrow and spleen, (B) thymus, or (C) spleen using the indicated antibodies. Numbers inside or next to gates indicate the percentage of cells in those gates. This experiment was performed twice with a total of two control, 3 VP, and 3 LP mice. (C) CD4 and CD8 expression is shown for CD3^+TCRβ^+ cells.
Supplementary figure 3.

A

WT spl

WT thy

B

VP 112

VP 207

VP 974

VP 802

VP 306

VP 979

C

LP 913

LP 646

D

LP 722

LP 824

LP 119

LP 913

LP 119
Supplementary figure 3. Flow cytometry analysis of VP and LP thymic lymphomas. (A-C) Flow cytometry analyses of (A) spleens (spl) and thymuses (thy) from normal mice, (B) VP lymphomas, or (C) LP lymphomas showing surface expression of CD3 and TCRβ or CD4 and CD8. Gates were drawn on normal cells and applied to lymphomas. The percentages of cells in each gate are indicated. Numbers were omitted when fewer than 10% of cells fell in a gate. (A) For splenocytes only, CD4 and CD8 expression is shown for CD3^+ TCRβ^+ cells. (D) Flow cytometry analysis of wild-type spleen and LP lymphoma no. 913 showing surface expression of CD3 and TCRδ. Percentages of CD3^+ TCRδ^+ cells are indicated. This was the only VP or LP lymphoma that expressed TCRδ.
Supplementary figure 4. Spectral karyotyping analysis of VP and LP lymphomas.
(A-F) Cytogenetic analysis of the indicated VP or LP lymphomas with their clonal or semi-
clonal chromosome translocations circled. Spectral images, DAPI images, and karyotype tables are shown for each tumor. (A) VP B lineage lymphoma no. 426 with a clonal t(6;4) translocation. (B) VP thymic lymphoma no. 207 with aneuploidy and no clonal translocations. (C) VP thymic lymphoma no. 975 with t(11;11) and t(12;12) centromeric fusions that were observed together in a third of metaphases. (D) VP thymic lymphoma no. 118 with a clonal t(11;15) and t(12;19) translocation found in a third of metaphases. (E) LP thymic lymphoma no. 976 with a clonal t(16;14) translocation. (F) LP thymic lymphoma no. 826 with a clonal t(11;19) translocation.
Chapter III: Lineage- and developmental stage-specific mechanisms regulate cyclin D3 expression in response to ionizing radiation

ABSTRACT

Proliferation begins in early G1 phase with upregulation of one or more D-type cyclins, which pair with cyclin-dependent kinases 4 or 6 (Cdk4/6). Active cyclin D-Cdk4/6 complexes drive cells into S phase, where DNA replication occurs. DNA double strand breaks (DSBs) occurring in G1 phase halt cell cycle pending repair in order to prevent genome instability. While the canonical p53-dependent G1/S checkpoint is well-described, other mechanisms that reinforce canonical pathways remain poorly understood. Mammalian cells express three D-type cyclins. In many tissues, D cyclins are functionally redundant. However, D3-deficient mice have severe blocks in B and T lymphocyte development and germinal center formation, despite increased expression of cyclin D2 in affected cells. Cell types requiring D3 are those in which proliferation closely follows programmed DSB induction. Thus, we hypothesized that the requirement for cyclin D3 in developing B and T cells reflects specific regulation of D3 in the context of DSB signaling. Following exposure of pre-B cells to ionizing radiation (IR), D3 is transcriptionally repressed. D3 loss correlates with arrest of cells in G1 phase, despite high expression of D2. In thymocytes, IR induces loss of D3 protein, likely through HuR-dependent modulation of D3 translation. Finally, overexpression of ectopic D3 that is not downregulated following IR in pre-B cells leads to a small but significant population of cells that fails to arrest in G1. These data suggest that developing B and T cells regulate D3 in response to IR through lineage-specific mechanisms, and suggest that D3 downregulation may facilitate the G1/S checkpoint in response to DSBs.
INTRODUCTION

The eukaryotic cell cycle comprises 4 main phases: G1, S, G2, and M. Progression through each phase of the cell cycle is controlled by cyclin dependent kinases (Cdks), whose activities depend on binding to appropriate cyclin partners (Malumbres and Barbacid 2009). In addition, Cdk activity is tightly regulated by both activating and inhibitory phosphorylation events (Malumbres and Barbacid 2009). In G1 phase, mitogenic signals upregulate D-type cyclins to stimulate the activity of Cdk4 or Cdk6. Active cyclin D-Cdk4/6 complexes drive early and mid-G1 progression and facilitate cyclin E/Cdk2 activity by both relieving transcriptional repression of cyclin E, and alleviating repression of cyclin E/Cdk2 by the CDK-inhibitors p21 and p27 (Malumbres and Barbacid 2001, Musgrove et al. 2011). Cyclin E/Cdk2 activation directly promotes movement from late G1 into S phase through phosphorylation of key pre-replication complex components and transcriptional activation of genes required for S phase (Malumbres and Barbacid 2009).

Movement through the cell cycle can be temporarily or permanently arrested by the activation of cell-cycle checkpoints (reviewed in (Bartek and Lukas 2007)). The G1/S checkpoint responds to DNA damage signaling to prevent initiation of DNA replication until DNA is repaired. Failure to delay S phase entry in the presence of damaged DNA contributes to genomic instability and can help drive tumor formation, as evidenced by the frequency of checkpoint or DNA damage response (DDR) factor inactivation in tumor cells (Malumbres and Barbacid 2001, Franco et al. 2006). In addition, malignant cells often fail to apoptose in the presence of high levels of DNA damage, allowing the propagation of genomically unstable cells, and exacerbating instability (Nussenzweig
and Nussenzweig 2010). The mechanisms responsible for activating the G1/S checkpoint by DSBs are well understood (Bartek and Lukas 2007). DSBs activate the kinase ATM, which phosphorylates target proteins at SQ/TQ motifs (Kim et al. 1999, Mazan-Mamczarz et al. 2011, Paull 2015). ATM activation and subsequent target phosphorylation causes activation of a number of downstream targets. For example, ATM phosphorylates Mdm2, the negative regulatory of p53 (Gannon et al. 2012). The subsequent stabilization of p53 results in transcriptional upregulation of targets including p21, which can bind and inhibit cyclinE/Cdk2 complexes, thereby delaying entry into S phase (el-Deiry et al. 1993, Macleod et al. 1995, Kannan et al. 2001). In addition, p53 is capable of inducing senescence or apoptosis, depending on the severity and persistence of the DNA damage (Kastan and Bartek 2004). While the canonical ATM-p53-p21 pathway is well-understood, much evidence suggests that other modes of regulation exist to delay G1 phase, or to re-inforce the canonical checkpoint to promote genome stability. For example, several studies have shown that downregulation of cyclin D3 can cause cell cycle arrest (Miyatake et al. 1995, Tiefenbrun et al. 1996). Similarly, loss of D1 protein can indirectly inhibit Cdk2 activity by releasing p21 from D-Cdk4/6 complexes (Agami and Bernards 2000). Overexpression of cyclin D1 is clinically correlated with an increase in genomic instability (Jares et al. 2007, Nosho et al. 2008), and evidence supports a role for D1 downregulation in G1 arrest following IR (Agami and Bernards 2000). These data suggest that downregulation of D-cyclins at the appropriate time within cell cycle is one means by which DNA damage signaling prevents inappropriate S phase entry of cells with unrepaired DSBs.

Lymphocytes provide a useful system to study the coordination of DDR and the G1/S transition because lymphocytes induce and repair DSBs during the processes of
V(D)J recombination and class switch recombination (CSR), during periods of development also characterized by rapid proliferation (Bednarski and Sleckman 2012). These recombination pathways, V(D)J recombination and class switch recombination (CSR), result in the diversification of antigen receptor genes that is required for functional adaptive immunity. Successful rearrangement in G1 cells, such as that resulting in expression of a functional pre-B cell receptor at the pro- to pre-B transition, induces rapid proliferative expansion and permits survival and further differentiation (Cooper et al. 2006, Clark et al. 2014). However, because V(D)J recombination and CSR both require DSB intermediates, cells must be maintained in G1 phase until repair has been successfully completed to avoid genomic instability and possible tumorigenesis (Nussenzweig and Nussenzweig 2010).

Three D-type cyclins are expressed in a tissue-specific manner in humans and mice. Mice deficient for all D-cyclins are not viable (Kozar et al. 2004). Models of single and double cyclin deficiency have shown that D-cyclins play largely redundant roles but have limited tissue-specific functions (Sicinski et al. 1995, Sicinski et al. 1996, Ciemerych et al. 2002, Sicinska et al. 2003, Carthon et al. 2005). In some cases, tissue-specificity has been attributed to differential expression patterns of the D-cyclins (Carthon et al. 2005). For example, defects in D1-/- mice can be partially rescued by knocking the D2 cDNA into the D1 genetic locus (Carthon et al. 2005). However, cyclin D3 has a unique role in lymphocyte development, as evidenced by developmental blocks observed in D3<sup>-/-</sup> mice (Sicinska et al. 2003, Cooper et al. 2006, Peled et al. 2010, Cato et al. 2011). Specifically, these mice display reduced numbers of pre-B and immature B cells in the bone marrow, resulting from an inability to stimulate proliferation after successful rearrangement of the immunoglobulin heavy chain (IgH) locus (Cooper...
et al. 2006). Interestingly, pre-B lymphocytes from D3<sup>−/−</sup> mice show a greatly increased expression of cyclin D2 protein, but this does not rescue pre-B cell proliferation (Cooper et al. 2006). Similarly, formation of germinal centers is impaired in mice lacking cyclin D3, due to a defect in proliferation that was not rescued despite abnormally high cyclin D2 expression in these cells (Peled et al. 2010, Cato et al. 2011). Cyclin D3 is again required during the rapid proliferation that occurs in activated B cells during the germinal center reaction, where CSR also occurs. Analogous to the pro- to pre-B cell transition, D3 drives proliferation of CD4<sup>-</sup>CD8<sup>-</sup> double negative (DN) thymocytes as they transition into CD4<sup>+</sup>CD8<sup>+</sup> double positive (DP) cells following productive V(D)J recombination at the TCRβ (Sicinska et al. 2003). Numbers of DP thymocytes are 12 fold lower in D3<sup>−/−</sup> mice than in WT littermates (Sicinska et al. 2003), consistent with a role for D3 in proliferation induced by expression of the pre-T cell receptor.

We hypothesized that the unique requirement for cyclin D3 for developing B and T cells and during the germinal center reaction reflects specific regulation of this D-type cyclin in the context of DSB signaling. We found that cyclin D3 expression is lost in pre-B cells and thymocytes following IR; however, the mechanism by which D3 is downregulated differs between the two cell types. D3 repression in irradiated pre-B cells occurs by an ATM-dependent loss of D3 transcription. In thymocytes from irradiated mice, D3 protein but not mRNA is lost, through a mechanism that likely depends on post-transcriptional regulation by the RNA-binding protein HuR. Finally, we demonstrate that overexpression of a misregulated D3 causes a small but significant population of pre-B cells to fail to arrest in G1 following IR, suggesting that D3 regulation may have important roles to play in DSB-dependent cell cycle control in developing lymphocytes.
RESULTS

IR induces an ATM- and p53- dependent loss of D3 protein expression in ex vivo mouse pre-B cells

Cyclin D3 is uniquely required to drive proliferation of pro-B cells, CD4+CD8+ thymocytes, and class-switching mature B cells (Sicinska et al. 2003, Cooper et al. 2006, Peled et al. 2010). We hypothesized that this requirement for cyclin D3 reflects distinct DSB-dependent regulation of cyclin D3 in the context of V(D)J recombination and CSR. We began to investigate this hypothesis by studying changes in cyclin D3 expression following IR-induced DSBs in ex vivo mouse pre-B cells. Culturing mouse bone marrow in medium containing IL-7 for 4-5 days results in a population of about 90-95% pre-B cells (Ray et al. 1998). We thus obtained pre-B cells from mice expressing an anti-apoptotic EμBcl2 transgene (Strasser et al. 1991) and treated these cells with 10Gy ionizing radiation. We quantified cyclin D3 and cyclin D2 protein expression in these cells at time points up to 6 hours following IR and compared them to unirradiated pre-B cells (Figure 1A). We did not assess cyclin D1 because it is not detectably expressed in non-malignant lymphocytes (Bartkova et al. 1998, Ciemerych et al. 2002). We found that IR induces 70% loss of cyclin D3 protein in 4 hours (Figure 1A). Cyclin D2 protein is not reduced, supporting the idea that D3 has important non-redundant functions in pre-B cells. We further verified that this effect was not an artefact of the anti-apoptotic EμBcl2 transgene by comparing D3 expression before and after IR in WT pre-B cells incubated with a pan-caspase inhibitor to pharmacologically limit apoptosis (not shown), and we saw the same reduction of D3 following IR. IR-dependent downregulation of cyclin D3 in pre-B cells is dependent on the DNA damage kinase ATM and the tumor suppressor
p53, as cells genetically deficient in either of these factors are unable to downregulate D3 following IR (Figure 1B).

**Reduced D3 protein expression in irradiated pre-B cells is due to an ATM-dependent loss of D3 transcription**

Because D-type cyclins, including D3, are sometimes regulated by induction of proteolytic degradation (Pontano et al. 2008, Chen et al. 2012, Thompson et al. 2015), we measured the half-life of D3 protein in irradiated and un-irradiated pre-B cells. We did this by treating pre-B cell cultures with IR or no IR, immediately adding cyclohexamid e to inhibit new protein synthesis, and then measuring D3 protein abundance over time. There was no significant difference in turnover of D3 protein in irradiated versus unirradiated pre-B cells (Figure 1C), suggesting that in primary pre-B cells, loss of D3 protein is not accomplished through protein destabilization.

The fact that protein turnover is not changed in irradiated pre-B cells compared with unirradiated controls led us to ask whether D3 might be post-transcriptionally regulated following IR. Since miRNAs regulate the stability and translational efficiency of many cellular targets (Johanson et al. 2014), we tested whether miRNAs are required to downregulate D3 express following IR. To address this question, we used a genetic model in which the gene encoding the miRNA processing enzyme Dicer is conditionally deleted in pro-B cells using Mb1-Cre and generated pre-B cell cultures from these mice and from Dicer-sufficient littermate animals. All animals in this experiment also expressed the EμBcl2 transgene to limit apoptosis following IR. We found that Dicer-deficient pre-B cells had a significantly impaired reduction in cyclin D3 protein at all
assessed timepoints after IR compared to Dicer-sufficient controls (Figure 1D),
consistent with the idea that Dicer-dependent generation of mature miRNAs is required
for normal downregulation of D3 expression following IR. These data are consistent with
either direct regulation of D3 mRNA stability of translation by miRNAs or an indirect
mechanism whereby miRNAs regulate factors required for the modulation of D3
expression after IR.

To address whether miRNAs directly or indirectly affected D3 expression, we
considered whether reduced cyclin D3 protein expression was the result of decreased
mRNA in irradiated cells compared to controls. Indeed, D3 mRNA levels are reduced by
50% at 1 hour after IR (Figure 2A). The fact that steady state mRNA levels decline faster
than protein levels (Figure 1A) in irradiated pre-B cells suggests that the IR-dependent
downregulation of D3 expression may be controlled at the level of mRNA abundance.

Steady-state mRNA abundance is the sum of new transcript generation and degradation
of existing transcripts. To determine whether IR inhibits D3 transcription and/or induces
D3 mRNA degradation, we used a “click” chemistry RNA-labeling approach. These
studies show that the rate of D3 mRNA turnover is not affected by IR in EμBcl2 pre-B
cells; however, synthesis of new D3 transcripts is dramatically lowered by IR (Figure 2B,
2C). As a control, we measured transcription and turnover of the cell cycle inhibitor p21,
which is both transcriptionally and post-transcriptionally induced by DSBs (Macleod et al.
1995). As expected, irradiation of EμBcl2 pre-B cells both increased p21 transcription
and promoted its mRNA stability compared to unirradiated controls (Figure 2B, 2C).

These data support a model in which inhibition of D3 transcription immediately following
IR together with a 1 hour half-life of D3 mRNA, account for the observed reduction in
steady-state D3 mRNA and protein levels. Consistent with the idea that ATM is required
for D3 downregulation, ATM-deficient pre-B cells failed to effectively inhibit D3 transcription following IR (Figure 2C). Because ATM activation induces activity of p53, which in turns activates p21 transcription, we were unsurprised to find that ATM-/- pre-B cells also have defects in IR-dependent upregulation of p21 transcription (Figure 2C).

**D3 downregulation in pre-B cells may be required for normal G1 arrest in response to IR**

We investigated whether cyclin D3 down-regulation has an effect on cell cycle progression in irradiated cells. We first assessed cell cycle progression in irradiated and unirradiated EμBcl2 pre-B cells by measuring BrdU incorporation in conjunction with DNA content staining. IR causes pre-B cells to arrest in G1 phase of the cell cycle, in correlation with reduced D3 expression (Figure 3A). These data are consistent with the idea that in pre-B cells, D3 is uniquely required to drive proliferation, since irradiated cells arrest in G1 despite increased D2 expression (Figure 1A). To test the hypothesis that forced D3 expression impairs the ability of cells to efficiently arrest in G1 following IR, we used retroviral transduction to ectopically express cyclin D3 from a constitutive promoter. We first tested that ectopically expressed D3 is not downregulated following IR. To this end we transduced D3-/- EμBcl2 pre-B cells with a MIGR1-based vector expressing GFP protein under control of an IRES (empty vector) or the same vector containing the D3 cDNA, including its full-length 3'UTR (D3-FL). Cells transduced with this construct, measured by co-expressed GFP, express about 3 times the normal level of D3 protein (data not shown). Consistent with the idea that ablation of D3 transcription following IR causes loss of D3 protein expression in pre-B cells, D3 expressed from a
constitutive promoter is not regulated by IR (Figure 3B). We next compared cell cycle progression of EμBcl2 pre-B cells transduced with empty vector, and D3-/- EμBcl2 pre-B cells transduced with the D3-FL construct, each with and without IR. We first noted that overexpression of the non-regulated D3 caused a slightly higher frequency of unirradiated cells to enter S phase during the time window studied, consistent with pro-proliferative functions of D3 in lymphocytes (Figure 3C,D). We then found that while the vast majority of irradiated of cells expressing non-regulated D3 arrested in G1 phase following IR treatment, a small but significantly higher number of cells escaped into S phase when D3 could not be downregulated (Figure 3C,D). Importantly, unirradiated cells transduced with D3 have 1.5 fold more BrdU+ cells than control transduced cells before IR, but irradiated cells expressing D3 have a 2.5 fold increase in BrdU+ cells, supporting the idea that this G1-escape does not merely reflect increased proliferation of D3-transduced cells, independent of irradiation. These data suggest that overexpression of D3, and/or the inability to downregulate D3 following IR, increases the frequency with which pre-B cells bearing DSBs enter S phase.

D3 is not lost in irradiated B cells undergoing CSR in vitro

Having shown that cyclin D3 expression is regulated in response to IR in developing B cells, we next asked whether D3 is regulated in mature B cells undergoing class switch recombination, as proliferation at this stage also depends on D3 expression (Peled et al. 2010, Cato et al. 2011). We induced proliferation and class switch recombination in vitro by incubating splenic B cells from EμBcl2 mice with LPS and IL-4 then measured D3 protein expression in these cells with and without 10Gy IR (Figure 4).
We find that unlike in pre-B cells, IR treatment of mature B cells activated with LPS and IL-4 does not cause loss of D3 protein expression.

**Irradiated thymocytes reduce D3 expression by an ATM-dependent mechanism involving loss of HuR binding to the D3 mRNA**

We next asked whether IR-dependent downregulation of cyclin D3 expression occurred in developing T cells. Because cyclin D3 drives proliferation of DP thymocytes following TCRβ recombination (Sicinska et al. 2003), we asked whether thymocytes downregulate D3 in response to IR treatment. To do this, we first removed thymocytes from EμBcl2 mice, treated with IR, or withheld from IR, and cultured in vitro for up to 6 hours. We found that D3 protein levels decrease in both irradiated and unirradiated thymocytes, suggesting that factors present in the thymic environment are required to maintain D3 expression in unirradiated cells (Figure 5A). To circumvent this difficulty, we instead compared expression of D3 in freshly isolated thymocytes from unirradiated EμBcl2 mice or from littermates irradiated 4 hours prior to harvest. This experiment revealed that D3 protein is reduced at 4h post-IR in thymocytes; however, D3 mRNA expression levels remained the same in both irradiated and unirradiated thymocytes (Figure 5B). These data suggest that thymocytes, like pre-B cells, downregulate D3 in response to IR, but by a distinct mechanism.

As observed in pre-B cells, we found that ATM is required for the downregulation of D3 protein in thymocytes after IR (Figure 5C). ATM-dependent loss of D3 protein but not mRNA in irradiated thymocytes could result from increased D3 protein turnover, potentially mediated by post-translational modification. Alternatively, reduced D3 protein
levels after IR might reflect reduced translation of existing D3 mRNA. The ubiquitously expressed RNA-binding protein HuR (Elavl1) is a key effector of ATM signaling (Mazan-Mamczarz et al. 2011). Further, HuR has been shown to directly regulate cyclin D3 expression under other conditions of stress (Rodriguez et al. 2010). Thus, we hypothesized that IR could induce loss of HuR binding to cyclin D3, thereby reducing D3 translation. Indeed, RNA-immunoprecipitation (RIP) followed by qPCR shows that HuR binds D3 mRNA in total thymocytes from unirradiated mice, but not in thymocytes from mice irradiated 30 minutes prior to harvest (Figure 5D). These data suggest that HuR may promote translation of D3 mRNA in unstressed cells, and that IR may induce loss of this binding.

**HuR-deficient thymocytes have reduced D3 protein expression and an impaired DN to DP developmental transition**

The model wherein HuR binds to and promotes translation of the cyclin D3 mRNA in thymocytes also predicts that HuR-deficient thymocytes may have reduced D3 protein expression. D3 is highly expressed in DN4 and immature single positive (ISP) thymocytes, where it drives proliferation following TCRβ recombination (Sicinska et al. 2003). D3-/- mice have very low numbers of DP thymocytes, consistent with the established role of D3 in expanding the DN and ISP populations as they differentiate into DP thymocytes (Sicinska et al. 2003). Phenotypically, we find that mice with thymus-specific HuR deletion initiating at the DN2 stage of development (LckCre HuRflox/flox or LH) have 4-fold lower numbers of DP and SP thymocytes compared to Cre negative (HuRflox/flox or WT) littermates (Figure 6A) (Lee et al. 2001, Ghosh et al. 2009). We
further show that this partial block in development in LH thymic development occurs at the DN3 to DN4 transition (Figure 6B). This partial developmental block is a likely cause of the reduced numbers of splenic T cells in LH mice (Figure 6C), although other thymic defects attributed to HuR deletion have been reported (Papadaki et al. 2009).

Although HuR likely regulates many targets important for thymocyte development, loss of DP thymocytes in LH mice is consistent with the notion that HuR promotes D3 expression in DN4 and ISP thymocytes to drive their proliferation. To ask whether reduced DP numbers are the result of intrinsically impaired TCRβ recombination, we introduced the pre-rearranged Vβ1NT allele, which obviates the need for V(D)J recombination at the TCRβ locus (Serwold et al. 2007, Brady et al. 2010). We find that although the Vβ1NT allele increases numbers of DP cells for both LckCre Vβ1NT/+ HuRf/f and Vβ1NT/+ HuRf/f control mice, mice with HuR-deficient thymocytes still have significantly lower DP numbers than their WT counterparts (Figure 6A). These data support the idea that HuR-deficient thymocytes are unable to fully upregulate D3 to promote expansion of DN4 and ISP cells downstream of signaling from a functional TCRβ chain.

To directly test whether HuR regulates D3 expression in thymocytes, we assessed D3 protein abundance in total thymocytes from WT and LH mice. We find that expression of D3 is 2.5 fold lower in LH thymuses compared to WT controls (Figure 6D). Consistent with HuR-dependent D3 translation, thymic D3 mRNA levels were not affected by loss of HuR (Figure 6E). Expression of D2 in total thymus was not affected by loss of HuR (Figure 6D). These data support a model wherein HuR promotes translation of the D3 mRNA in unstressed DN4 and ISP thymocytes, thereby facilitating
their expansion. In irradiated thymocytes, HuR binding to the D3 mRNA is lost, likely causing the reduction in D3 protein expression.

Collectively, these data show that while D3 expression drives \textit{in vivo} expansion of developing B cells, thymocytes, and activated B cells, D3 regulation in response to IR is cell type-dependent. Further, in pre-B cells, overexpression of D3 and/or the inability to downregulate D3 following IR prevents normal G1 arrest in a small fraction of cells.
DISCUSSION

We set out to understand whether and how cyclin D3 expression might be regulated in lymphocytes in response to IR-induced DSBs. We found that D3 protein is reduced in developing B and T cells after treatment with IR. In pre-B cells, IR-dependent D3 repression is accomplished by rapid inhibition of D3 transcription that occurs through direct or indirect regulation by ATM, p53, and the miRNA pathway (Figure 7A). During B cell development, IL-7R signaling induces transcription of D3 (Mandal et al. 2009), while pre-BCR signaling further increases D3 expression by stabilizing the D3 protein (Cooper et al. 2006). In order to initiate IgL recombination, large cycling pro-B cells must exit the cell cycle as they differentiate into pre-B cells (Clark et al. 2014). Much evidence suggests that inhibition of D3 transcription causes pre-B cells to exit the cell cycle, and failure of this process is associated with a pro- to pre-B cell developmental block (Mandal et al. 2009, Ma et al. 2010, Venigalla et al. 2013). It will be interesting to see whether any of these factors are responsible for inhibition of D3 transcription following IR in pre-B cells.

We showed that IR induces loss of D3 protein expression in thymocytes; however, the mechanism is different as compared to pre-B cells. We propose a model wherein HuR promotes translation of the D3 mRNA in unstressed thymocytes and that irradiation causes loss of HuR binding to the D3 mRNA, leading to inefficient D3 translation and reduced protein output (Figure 7A). Consistent with this idea, we find that HuR-deficient thymocytes express less D3 protein but not mRNA. Mice whose thymocytes lack HuR also have a DN to DP developmental defect that is analogous to but less severe than the phenotype of mice with complete deletion of cyclin D3 (Sicinska et al. 2003).
Downregulation of D3 in response to normal developmental cues may also occur at the post-translational level. DYRK1A protein phosphorylates the D3 protein in pre-T cells, leading to de-stabilization of the D3 protein (Thompson et al. 2015). Further, ATM activated by DSBs phosphorylates many proteins at SQ/TQ motifs (Kim et al. 1999, Paull 2015). The mouse D3 protein contains 2 SQ/TQ motifs, one or both of which could be phosphorylated by ATM. Although our data are incompatible with increased degradation of D3 in irradiated pre-B cells (see Figure 1C), we have not evaluated post-translational modification as a mode to reduce D3 protein expression in irradiated thymocytes. Therefore, it is formally possible that in thymocytes, loss of D3 protein is accomplished through reduction of D3 mRNA translation by loss of HuR binding, as well as through de-stabilization of the protein by DYRK1A, ATM, or another factor.

We find that cyclin D3 is downregulated in response to IR in pre-B cells and thymocytes, but not in mature B cells undergoing CSR in response to LPS and IL-4. It is possible that D3 downregulation is not important for mature B cells undergoing CSR, suggesting that other modes of DDR signaling and cell cycle control are most important. It is also possible that stimulation with LPS and IL-4 fails to adequately mimic the in vivo germinal center response in which D3 is particularly important (Peled et al. 2010, Cato et al. 2011). The lack of D3 downregulation after IR is alternatively consistent with regulation of D3 activity, such as a change in binding to CDK4/6, or modulation of subcellular localization. For example, D3 was recently shown to exist in distinct fractions within the nucleus of pro-B cells, each with slightly different biochemical functions (Powers et al. 2012), raising the possibility that similar subcellular compartmentalization of D3 may also occur in mature B cells.

It remains unclear why the D-type cyclins display tissue-specific functions and expression patterns. The three D cyclin proteins are biochemically very similar
Yet, phenotypic data from mice bearing individual or multiple knockouts of D-cyclins suggests that specific D cyclins have non-redundant functions in specific tissues (Ciemerych et al. 2002, Peled et al. 2010). In addition, D3 expression is high in certain quiescent tissues, belying its canonically pro-proliferative function (Bartkova et al. 1998). Several lines of evidence suggest that D cyclins may have roles outside of their ability to bind CDK4/6 proteins. D3 binds the retinoic acid receptor and influences transcription of retinoic acid-dependent genes (Despouy et al. 2003). D1 collaborates with the estrogen receptor to promote expression of estrogen-responsive target genes independent of CDKs (Zwijsen et al. 1997). Distinct pools of D3 exist within the nucleus, including a fraction bound to CDK4/6, a soluble fraction, and a fraction bound to the nuclear matrix (Powers et al. 2012). The latter fraction represses expression of V gene segments in WT pro-B cells, and may directly or indirectly influence expression of hundreds of other genes differentially expressed between WT and D3-/- pro-B cells (Powers et al. 2012). Collectively, these findings raise the possibility that DSB-dependent regulation of D3 may have important roles in lymphocyte development outside of its ability to bind CDKs.

We hypothesized that D3 downregulation in pro-B cells and DN4 thymocytes in response to unrepaired RAG or AID-induced DSBs reinforces canonical G1/S checkpoint pathways to allow repair of broken DNA and maintain genome stability. Our mechanistic analysis employed high doses of IR to induce DSBs. This treatment generates roughly 10-20 DSBs per cell per Gy of γ-radiation, although these numbers vary based on the cell being irradiated and the method used to quantify breaks (Iliakis et al. 1991, Rothkamm and Lobrich 2003, Lobrich et al. 2010, Roch-Lefevre et al. 2010). Low doses of IR significantly lower D3 protein and mRNA expression in pre-B cells (Figure 7B), albeit with slower kinetics and a smaller magnitude of change relative to
high dose treatment. These data are consistent with our hypothesis that physiologic levels of DSBs downregulate D3 expression to slow G1 progression. Indeed, both genotoxic and Rag-induced DSBs trigger largely overlapping gene expression changes that include canonical checkpoint and repair proteins as well as factors involved in lymphocyte differentiation (Bredemeyer et al. 2008). Further, unpublished data from the Sleckman lab show that D3 downregulation also correlates with cell cycle arrest in response to Rag-induced DSBs. They showed that in pre-B cell cultures in which IL-7 was withdrawn to induce IgL recombination, cells with unrepaired breaks were less able to upregulate D3 and enter cell cycle. These data are consistent with our findings that IR-induced breaks downregulate D3 in correlation with G1 phase arrest. We further show that overexpression of a D3 cDNA that is not downregulated after IR leads to a small but significant increase in the number of pre-B cells escaping G1/S arrest. Even though this population is small, a 2.4-fold increase in the frequency of cells aberrantly entering S phase with unrepaired breaks may be physiologically relevant, since 5 x10^7 immature B lymphocytes are generated in the mouse bone marrow per day (Opstelten and Osmond 1983), and malignant transformation of just one cell could be lethal. Future studies will focus on extension of these data from IR-breaks to Rag-dependent breaks and more rigorously examine the in vivo role of this phenomenon in lymphocyte development and genome stability in both the B and T lineages.
Figure 1: Irradiation of primary mouse pre-B cells induces loss of D3 protein in an ATM-, p53-, and Dicer-dependent manner, but does not alter D3 protein stability. 

(A-D) IL-7 pre-B cell cultures of BM from mice of indicated genotype, or EμBcl2 where not specified were treated with 10Gy of IR. D3 protein expression was assessed before IR and at indicated time points. D3 expression was normalized to β-actin expression and values for unirradiated cells were set to 1.0. *p<0.05. ***p<0.001. (A) 6 hour time point is also significant (**). The experiment was performed 4 times. (B) No significant differences were found. The experiments were performed 3 times each. (C) Cyclohexamide (CHX) was added to cells immediately after IR or mock IR, and cells were harvested at indicated time points. The experiment was performed 5 times. Linear regressions of no IR and 10Gy data sets calculated by Prism software were not significantly different. (D) The experiment was performed 5 times. p values are the result of 2-way ANOVA.
Figure 2: Irradiation of mouse pre-B cells induces an ATM-dependent loss of D3 transcription. (A) IL-7 pre-B cell cultures from EμBcl2 mice were treated with 10Gy of IR. D3 mRNA expression was assessed by qRT-PCR before IR (0h) and at indicated time points. Ct values for D3 were normalized to 18S and resulting values for unirradiated cells were set to 1.0. ***p<0.001. Each subsequent time point is similarly significant. The experiment was performed 4 times. (B) pre-B cell cultures of BM from EμBcl2 mice were grown overnight in medium containing 5-ethynyl uridine (EU) to maximally label cellular RNAs. EU was washed out of culture medium immediately before IR or mock IR treatment. Labeled mRNA from cells at indicated time points was isolated from total RNA using “click” biotin labeling and streptavidin pulldown and D3 (top) or p21 (bottom) abundance within pulled down RNA was measured by qRT-PCR. Ct values for D3 or p21 were normalized to 18S and resulting values for t=0 were set equal to 1.0. ****p<0.0001 by 2-way ANOVA. No significant changes were observed for D3. The experiment was performed 4 times. (C) IL-7 pre-B cell cultures of BM from EμBcl2 or ATM-/- EμBcl2 mice. EU was added to culture medium immediately following IR or mock IR (t=0). Labeled mRNA from cells at indicated time points was obtained as in (B) and D3 or p21 abundance was measured by qRT-PCR. Ct values for D3 or p21...
were normalized to HPRT, since detection of new 18S transcripts was low. Data are a ratio of normalized expression in irradiated cells compared to unirradiated cells. Each experiment was performed 3 times.

Figure 3.

A. (A) IL-7 pre-B cell cultures of BM from EμBcl2 mice were treated with 10Gy of IR or no IR before returning to culture in medium containing BrdU. Cells were harvested at indicated time points and processed for BrdU and 7AAD flow cytometry. Shown are representative flow plots after 6h BrdU incubation. Cells were previously gated on live transduced (GFP+) lymphocytes, and numbers are frequency of: G1 cells (2N DNA, BrdU⁻/⁻), S (BrdU⁺), and G2/M (4N DNA) for that plot. Quantification is from one experiment of 3 performed with similar results. (B) D3−/−-EμBcl2 pre-B cells were transduced with empty MIGR1 vector or MIGR1 containing full
length D3 cDNA (FL-D3). 48h after transduction, cells were treated with 10Gy IR. D3 protein expression was assessed before IR and at indicated time points. Shown is a representative experiment of 3 performed. (C) EμBcl2 pre-B cells were transduced with empty MIGR1 vector or MIGR1 FL-D3. 48h after transduction, cells were divided into two groups. BrdU was added to cell cultures immediately following 10Gy IR or mock IR. Cells were harvested and stained for BrdU and 7AAD after 6h BrdU labeling. Shown is a representative experiment of 3 performed with 7 biological replicates in total. (D) Quantification of S phase (%BrdU+) cells from (C), including all biological replicates. *p<0.05 by 2-tailed paired t-test.

Figure 4.

**Figure 4: Mature B cells undergoing CSR in vitro do not downregulate D3 upon IR treatment.** Total splenic B cells were isolated from EμBcl2 mice and cultured with LPS and IL-4 for 72h. After checking that cells were blasting and undergoing CSR, cells were subjected to 10Gy IR. Cells were harvested for immunoblotting before IR (t=0) and at indicated time points after IR. Experiment is representative of 3 performed.
Figure 5: Thymocytes from irradiated mice show reduced D3 protein but not mRNA. (A) Thymocytes were isolated from EμBcl2 mice, treated with 9Gy IR or mock IR, then cultured in complete medium for up to 6 hours. D3 protein expression was assessed by western blotting before IR (0h) and at indicated time points. D3 expression was normalized to β-actin expression. Shown is a representative western blot from 1 of 2 experiments performed with similar results. (B) 4-6 week old littermate EμBcl2 mice were treated with 9Gy of IR or mock IR. After 4 hours, mice were euthanized and thymocytes were analyzed for D3 protein (left and representative immunoblot) and mRNA expression (right). D3 protein expression was normalized to β-actin, and D3 mRNA was normalized to 18S RNA. Each point represents an individual mouse. **p<0.01. The experiment was repeated 3 times with a total of 7 mice in each condition. (C) 4-6 week
old littermate ATM−/− Vβ14NT/NT EμBcl2 mice were treated with 9Gy of IR or mock IR.
After 4 hours, mice were euthanized and thymocytes were analyzed for D3 protein
expression. D3 protein expression was normalized to β-actin. Each point represents an
individual mouse. Differences are not significant. The experiment was repeated 3 times
with a total of 8 mice in each condition. (D) 4-6 week old littermate EμBcl2 Vβ14NT/NT
mice were treated with 9Gy of IR or mock IR and thymocytes were isolated 30 minutes
later. HuR binding to D3 mRNA was assessed by RNA-immunoprecipitation followed by
qRT-PCR. D3 mRNA abundance for each sample was normalized to GAPDH, and fold
enrichment is expressed as a ratio of normalized D3 found in HuR IP compared to IgG
IP. Each point represents an individual mouse. The experiment was performed 3 times.
*p<0.05.

Figure 6.
Figure 6: Thymocyte-specific deletion of HuR impairs DN to DP development and reduces D3 expression. (A-C) Flow cytometry of thymuses (A-B) or spleens (C) from 4-6 week old mice of indicated genotypes. Representative flow plots and quantifications are shown. *p<0.05, **p<0.01, ***p<0.001. The experiment was performed 4 times with 4 or more mice of each genotype. (A) Previously gated on live lymphocytes. (B) Previously gated on live lymphocytes that are negative for a DUMP gate consisting of antibodies against TCRβ, CD4, CD8, and various non-T lineage markers. DN1: CD25+ CD117-, DN2: CD25+ CD117+, DN3: CD25- CD117+, DN4: CD25- CD117-. (C) Previously gated on live splenocytes. (D-E) Representative immunoblots and quantification (D) or qRT-PCR (E) of total thymocytes from 4-6 week old WT (HuRf/f) or LckCre HuRf/f mice. Each point represents one animal. (D) D3 was normalized to actin expression. Lane 1 is a WT thymus and lanes 2-3 are LckCre HuRf/f thymi. The experiment was performed 3 times. (E) D3 Ct values were normalized to 18S RNA. The experiment was performed twice.

Figure 7.
A.

pre-B

DN thymocyte

B.

* p<0.05

** p<0.01
Figure 7: Model of D3 regulation in response to IR-induced breaks in developing lymphocytes and extension to physiologic DSB levels. (A) Proposed mechanisms for downregulation in irradiated pre-B cells and thymocytes. Due to the need for near immediate ablation of D3 transcription in irradiated pre-B cells, we have proposed a model where a repressive transcription factor (TF) trades places with an activating TF; however, this aspect remains speculative. CDS= coding sequence. The AU-rich element (ARE) located within the D3 3'UTR is the putative HuR binding site in unirradiated thymocytes based on knowledge of HuR's consensus sequence; however, we have not verified the exact binding sequence in our context. (B) IL-7 pre-B cell cultures from EμBcl2 mice were treated with 1Gy of IR or no IR and D3 protein (left) and mRNA (right) expression were measured at indicated time points after IR. D3 protein abundance was normalized to B-actin, mRNA was normalized to 18S, and values at t=0 were set equal to 1.0. p values represent the result of 2-way ANOVA testing.
Chapter IV: B cell-intrinsic expression of the RNA-binding protein HuR is required for the T cell-dependent immune response in vivo

ABSTRACT

Lymphocyte development and function require careful regulation of DNA break induction and repair, proliferation, and survival and differentiation decisions. These processes are controlled in part by post-transcriptional gene regulation, and many genes in these pathways are targets of the RNA-binding protein HuR. HuR (Elavl1) binds thousands of transcripts in cell lines; however, its binding patterns and effects are tissue- and context-specific. To understand roles of HuR in B cell development and function, we studied mice with B lineage-specific HuR gene deletion (HuRΔ/Δ mice). HuRΔ/Δ mice display a partial block in pro- to pre-B cell development and reduced numbers of transitional B cells, culminating in a 50% reduction of naïve mature splenic B cells. Since HuR is not essential for B cell development, we investigated its role in B cell function. In vitro stimulation of purified B cells under conditions mimicking both T-independent and -dependent activation shows that HuR-deficient cells have a mild proliferative defect, but can survive, class switch and enact stereotypic gene expression changes. In vivo, HuRΔ/Δ mice have very low serum titers of all antibody isotypes. This may be because, in vitro, HuR-deficient B cells inefficiently upregulate the alternatively polyadenylated transcript that encodes secreted antibody. Low antibody titers are also consistent with greatly reduced numbers of peritoneal B1 cells and activated splenic B2 cells in HuRΔ/Δ mice. Upon immunization with a T-dependent antigen, HuRΔ/Δ mice show almost complete ablation of the GC response and affinity maturation. This failure of GC production in HuRΔ/Δ mice contrasts with the ability of HuR-deficient B cells to become “GC-like” in vitro. These data suggest a role for B cell-expressed HuR in promoting the in
vivo GC response in ways that are not well-modeled in vitro, perhaps by promoting B-T communication within the lymphoid follicle.

INTRODUCTION

The development and function of cells such as B lymphocytes require finely tuned and dynamic changes in gene expression. These changes are instigated by cell-intrinsic and cell-extrinsic factors and controlled by a combination of transcriptional, post-transcriptional, and post-translational mechanisms. Post-transcriptional mechanisms allow cells to rapidly alter protein expression by modulating the stability and/or translation of specific mRNAs, or by modifying mRNA processing events, such as alternative polyadenylation and splicing. RNA-binding proteins (RBPs) are major regulators of post-transcriptional control of gene expression. RBPs bind their target sequences in 3’ untranslated regions (3’UTRs) or internal elements of mRNAs to positively or negatively regulate mRNA processing, stability, and/or translation in cellular context-dependent manners (Kim et al. 2010, Srikantan et al. 2012). RBPs are regulated at the levels of gene expression and/or post-translational modification, and also can interact with other factors to quickly and specifically modulate gene expression in response to developmental cues or other stimuli (Kim et al. 2010). Although RBPs are increasingly appreciated to play a role in lymphocyte biology and function, their role in regulating B cell development and function remains poorly understood (Turner and Hodson 2012).

B lymphocytes are comprised of two main populations, B1 and B2 cells, that develop through distinct programs that each link cell-intrinsic and cell-extrinsic signals with cellular survival, proliferation, and continued differentiation. The larger B2 cell
population arises in bone marrow (BM) from common lymphoid progenitors (CLPs) starting shortly after birth and extending throughout life (Sindhava et al. 2013, Clark et al. 2014). However, the smaller B1 population develops mainly from fetal liver precursors during fetal and early neonatal development, although these cells may continue to develop at a low frequency in adult BM (Sindhava et al. 2013, Zhang 2013). Developmental-stage specific assembly and expression of IgH and Igκ or Igλ Ig light chain (IgL) genes results in expression of BCRs on immature B cells (Clark et al. 2014). Depending on their antigen specificity, these BCRs signal gene expression changes to induce apoptosis or mediate differentiation of immature transitional B cells (Srivastava et al. 2005, Rickert 2013). Transitional B2 cells migrate to the spleen and differentiate into mature naive quiescent marginal zone (MZ) or follicular (Fo) B cells that express BCRs and traffic throughout lymphatic tissues (Srivastava et al. 2005, Sindhava et al. 2013). In contrast, the majority of transitional B1 cells migrate to serous cavities where they become mature B1 cells that continually proliferate and secrete antibodies (Sindhava et al. 2013, Zhang 2013).

B lymphocytes mediate protective humoral immunity through their ability to express cell surface BCRs and secrete antibodies. B2 cells recognize and respond to antigens through T cell-dependent or -independent mechanisms (Garraud et al. 2012, Sindhava et al. 2013). During a T cell-dependent germinal center (GC) immune response, Fo B cells are activated by direct and indirect signals from antigen-presenting effector T cells; these B2 cells induce expression of surface molecules and cytokines that activate CD4\(^+\) T cells and cause their differentiation into T follicular helper cells, which in turn further activate B cells and direct them to become GC B cells (Pereira et al. 2010, Vinuesa and Cyster 2011, Victor and Nussenzweig 2012). GC B cells rapidly
proliferate, conduct IgH isotype switching and Ig somatic mutation, and alter gene expression to differentiate into short-lived high-affinity antibody-secreting plasma cells or long-lived memory cells (Victora and Nussenzweig 2012). The GC reaction is critical for adaptive responses and affinity maturation of antibodies against an enormous number and variety of antigens and rapid secondary responses against previously encountered antigens (Victora and Nussenzweig 2012). B1 cells predominantly recognize and respond to antigens through T cell-independent mechanisms that occur outside of GCs and largely do not involve antigen-driven IgH isotype switching or Ig mutation, although T-dependent responses by B1 B cells do occur (Sindhava et al. 2013, Zhang 2013). B1 cells express BCRs that bind common pathogen epitopes and spontaneously secrete antibodies to protect against commensal and other opportunistic bacteria (Sindhava et al. 2013, Zhang 2013). Throughout life, B1 and B2 cells function together to protect host organisms from universally encountered common foreign organisms and random unanticipated infections.

The development and function of B cells requires exquisite regulation of gene expression to coordinate cellular survival, proliferation, and differentiation. The ubiquitously expressed HuR (also called Elavl1) protein controls post-transcriptional expression of many genes that mediate these cellular processes (Lopez de Silanes et al. 2004, Kishore et al. 2011, Mazan-Mamczarz et al. 2011, Mukherjee et al. 2011, Uren et al. 2011). Mice with germline or postnatal global deletion of HuR are not viable (Ghosh et al. 2009, Katsanou et al. 2009). Global HuR deletion in adult mice causes increased apoptosis and loss of immature but not mature B cells (Ghosh et al. 2009). However, since global HuR deletion leads to increased apoptosis of hematopoietic stem cells (HSCs) and early lymphoid progenitors (ELPs) concomitant with elevated expression of
the p53 transcription factor and its effectors of apoptosis (Ghosh et al. 2009), the B lineage-intrinsic role of HuR in regulating B cell development and function remains unknown. To determine roles of HuR in B lymphocyte development and function, we made and analyzed mice with B lineage-specific deletion of HuR initiating in pro-B cells. We found that HuR is required for normal numbers of splenic B2 cells and peritoneal B1 cells; however, HuR is not necessary for B cell development per se, enabling us to study roles of HuR in B cell function. Upon in vitro stimulation of splenic B cells, HuR is dispensable for B cell survival, isotype switching, and induction of GC B cell markers, and HuRΔ/Δ B cells exhibit only mild defects in proliferation and Ig secretion. In marked contrast, HuRΔ/Δ mice have dramatically low serum titers of all antibody isotypes and also fail to generate GC B cells and high-affinity antibodies after immunization with a T-cell dependent antigen, suggesting that HuR may promote the ability of B cells to interact with other immune cell types in the follicle. Consistent with this idea, HuRΔ/Δ mice have reduced numbers of Tfh cells. These studies demonstrate the critical role of HuR in promoting T-dependent immune responses in mice, and highlight developmental stage- and context-specific functions of HuR.

RESULTS

B lineage-specific deletion of HuR leads to decreased numbers of immature and mature B cells.

To identify roles of the HuR RBP in B lymphocyte development and function, we generated and analyzed Mb1Cre:HuR<sup>flox/flox</sup> (HuRΔ/Δ) mice with B lineage-specific deletion of the HuR gene initiating in pro-B cells. We performed cell counting and flow
Cytometry analyses for B cell developmental stage-specific markers on BM and spleen cells of 4 to 6 week-old HuRΔ/Δ mice and littermate HuRflox/flox (HuRff or WT) mice. Compared to control mice, HuRΔ/Δ mice have a 2-fold and 1.7-fold reduction in the numbers of B lymphocytes in their bone marrow and spleen, respectively (Fig. 1A). Although HuRΔ/Δ mice have higher than normal numbers of BM pro-B cells, they have reduced numbers of BM pre-B cells and BM B cells that represent immature B cells and re-circulating mature B cells (Fig. 1B). We also evaluated cell surface expression of CD43, BP-1, and CD24 to divide BM B2 cells into subpopulations referred to as "Hardy fractions" (Hardy et al. 1991). While HuRΔ/Δ mice have normal numbers of cells in each of the pro-B cell subpopulations (Hardy fractions A, B, C, and C'), they have fewer cells in each of the pre-B cell and mature B cell subpopulations (D, E, and F) (Fig. 1C). The spleens of HuRΔ/Δ mice contain reduced numbers of T1 transitional, T2 transitional, MZ, and Fo B cells (Fig. 1D). HuRΔ/Δ mice have normal ratios of Igκ+ to Igλ+ B cells in their bone marrow and spleen (data not shown). We detected comparable substantial deletion of floxed HuR exons in pro-B, pre-B and mature B2 cells of HuRΔ/Δ mice, and confirmed substantial loss of HuR protein in HuRΔ/Δ mature B cells (Supplemental Fig. 1A, 1B). Although Cre expression can negatively affect development independent of gene deletion (Higashi et al. 2009, Shi and Petrie 2012), we found no differences in numbers of mature B cells or B cells at each developmental stage in Mb1-Cre mice as compared to WT controls (Supplemental Fig. 1C). These data indicate that while HuR is not necessary for BM B2 cell differentiation per se, HuR is required to generate normal numbers of B lineage cells at each developmental stage beyond the pro-B cell stage.

Deletion of HuR impairs survival of B cells developing in the bone marrow.
The decreased numbers of B2 lineage cells at all developmental stages beyond
the pro-B cell stage could reflect important roles of HuR in promoting IgH gene
assembly, proliferation, and/or survival during B cell development. To investigate
potential roles of HuR in these processes, we first generated and analyzed HuRΔ/Δ mice
expressing an IgH transgene (IgHTg) that blocks IgH gene assembly and enables
formation of pre-BCRs to signal proliferation and survival in the absence of IgH gene
recombination (Mandik-Nayak et al. 2006). The numbers of BM pre-B cells and B cells in
the BM and spleens were all lower in 4 to 6 week-old IgHTg:HuRΔ/Δ mice relative to
age-matched control IgHTg:HuRf/f mice (Fig. 2A). Since these differences were
comparable to those between HuRΔ/Δ and HuRf/f mice (Fig. 1A), our data indicate that
IgH gene assembly is grossly normal in HuRΔ/Δ mice. We next compared cell cycle
profiles of pro-B cells and the most proliferating immature B cell subpopulation (fraction
C) between HuRΔ/Δ and HuRf/f mice. By combining cell surface staining of B cell
developmental markers with DNA content analysis, we detected similar percentages of
pro-B cells and fraction C cells in S phase between HuRΔ/Δ and control HuRf/f mice
(Fig. 2B). While these data suggest normal proliferation of immature HuRΔ/Δ B2 cells,
they cannot identify potential changes where cells spend proportionally more time in
each cell cycle phase. Thus, we also quantified immature and mature splenic B2 cell
populations in HuRΔ/Δ and HuRf/f mice at 12 to 14 weeks of age when the mouse B cell
compartment is replete (Allman et al. 1993), reasoning that the number of splenic B2
cells in HuRΔ/Δ mice should reach the number of cells in WT mice at this age if HuRΔ/Δ
B cells merely transit the cell cycle more slowly. We found that B cell numbers in the BM
and spleens of these older HuRΔ/Δ mice are not restored to normal (Fig. 2C), indicating
that the reduced number of splenic HuRΔ/Δ B cells is not caused by decreased
proliferation of immature HuRΔ/Δ B2 cells. Next, we assayed apoptosis of pro-B and
pre-B cells in HuRΔ/Δ and HuRf/f mice by performing flow cytometry for B cell developmental stage-specific surface markers and the annexinV protein that is expressed on apoptotic and dead cells. We detected a near significant increase in the fraction of annexinV+ pre-B cells in HuRΔ/Δ mice as compared to HuRf/f mice (Fig. 2D), consistent with a role for HuR in promoting survival of immature B cells. Since HuR has been proposed to counter pro-apoptotic p53 signaling in HSCs and ELPs (Ghosh et al. 2009), we generated and analyzed Mb1Cre:p53floxtfloxtHuRflox/floxt (p53Δ/Δ:HuRΔ/Δ) mice and control Mb1Cre:p53floxtfloxt (p53Δ/Δ) mice. We detected no significant differences in the numbers of BM pro-B cells and pre-B cells between p53Δ/ΔHuRΔ/Δ and p53Δ/Δ mice, (Fig. 2E), indicating that HuR promotes survival of pre-B cells by countering p53-dependant pro-apoptotic signals. Notably, deletion of p53 in HuRΔ/Δ B lineage cells did not restore to normal the numbers of mature splenic B2 cells (Fig. 2E), despite inactivation of p53 rescuing immature B2 cell numbers. Collectively, these data indicate that HuR supports B2 lineage cells via developmental stage-specific mechanisms or cellular processes that antagonize p53-dependent elimination of immature but not mature B2 cells.

In vitro stimulated HuRΔ/Δ B cells exhibit a slight proliferation defect and impaired ability to produce mRNAs that encode IgH chains of secreted antibodies.

Despite the reduced numbers of immature and mature B cells in HuRΔ/Δ mice, we found that HuR is not necessary for the development of mature splenic B cells. Thus, we investigated roles of HuR in B cell function beginning with tractable in vitro approaches to monitor survival, proliferation, IgH isotype switching, and gene expression
changes during B cell activation. We isolated total splenic B cells from HuRΔ/Δ and HuRf/f mice and incubated equal numbers of cells with LPS and IL-4, which mimics T cell-independent activation of B cells. Cell counting showed that HuRΔ/Δ cells expanded less than HuRf/f cells after 72 hours of stimulation (Fig. 3A). Although Cre expression can antagonize cell growth (Loonstra et al. 2001, Silver and Livingston 2001), Mb1Cre+ B cells expand normally following addition of LPS and IL-4 (Supplemental Fig. 2A), suggesting that in vitro stimulated HuRΔ/Δ B cells exhibit increased apoptosis and/or reduced proliferation. To determine whether LPS- and IL-4-stimulated splenic HuRΔ/Δ B cells exhibit increased apoptosis, we labeled HuRΔ/Δ and HuRf/f cells with an amine-reactive viability dye to identify dead cells. We detected similar frequencies of dead cells in HuRΔ/Δ and HuRf/f B cell cultures after 72 hours stimulation and a lower frequency of dead HuRΔ/Δ cells after 96 hours stimulation (Fig. 3B). These data indicate that HuRΔ/Δ B cells do not exhibit increased apoptosis but instead survive better than HuRf/f B cells after in vitro stimulation with LPS and IL-4. To determine whether splenic HuRΔ/Δ B cells exhibit reduced proliferation, we labeled HuRΔ/Δ and HuRf/f B cells with the fluorescent dye CFSE, which is diluted 50% by each round of cell division, before 72 hours incubation with LPS and IL-4. We detected fewer cell divisions in HuRΔ/Δ cultures (Fig. 3C), indicating that LPS and IL-4 stimulated HuRΔ/Δ B cells exhibit reduced proliferation. As an independent means to monitor proliferation, we measured incorporation of BrdU into replicating DNA combined with DNA content staining to quantify cells in each cell cycle phase. We found that fewer HuRΔ/Δ cells entered S phase during the 45 minute BrdU pulse (Fig. 3D), providing further evidence that HuRΔ/Δ B cells stimulated by LPS and IL-4 exhibit reduced proliferation. To monitor IgH isotype switching, we measured surface expression of IgG1 and IgE, since LPS and IL-4 promote IgH class switch recombination (CSR) to these isotypes. After 72 hours stimulation, HuRΔ/Δ cultures
harbor IgG1+ B cells at the same frequency as HuRf/f cells, whereas HuRΔ/Δ cultures exhibit a 2-fold lower frequency of IgE+ cells (Fig. 3E). Reduced isotype switching to IgE could arise from impaired initiation of IgH CSR due to defective signaling downstream of IL-4. However, we detected normal levels of non-coding germline transcripts for IgG1 and IgE in stimulated HuRΔ/Δ cells (Supplemental Fig. 2B) (Lumsden et al. 2004), suggesting that signaling activation of CSR is normal in HuRΔ/Δ B cells. Since IgH isotype switching to IgE requires a minimum of 5 cell divisions (McCall and Hodgkin 1999), the lower frequency of IgE+ HuRΔ/Δ B cells could result indirectly from reduced proliferation. Consistent with this notion, we observed equivalent frequencies of IgE+ B cells in HuRΔ/Δ and HuRf/f cells after 96 hours of stimulation when similar fractions of cells in each culture had divided five or more times (Fig. 3E; Supplemental Fig. 2C). The data from our analyses of HuRΔ/Δ and HuRf/f B cells following in vitro stimulation with LPS and IL-4 indicates that HuR is necessary for optimal proliferation of B cells, yet dispensable for survival and isotype switching to IgG1 and IgE.

In addition to expressing cell surface BCRs, the ability to secrete antibodies is crucial for B cell function. Thus, we used ELISA assays to quantify IgM and IgG1 antibodies secreted into the supernatants of HuRΔ/Δ and HuRf/f cells stimulated in vitro with LPS and IL-4. Despite equivalent frequencies of IgM+ and IgG1+ B cells in HuRΔ/Δ and HuRf/f cultures, we detected modestly lower levels of IgM and IgG1 in supernatants from HuRΔ/Δ stimulations (Fig. 4A). Accounting for differences in cell numbers arising from impaired proliferation of HuRΔ/Δ B cells, these in vitro stimulated HuRΔ/Δ B cells secrete normal amounts of IgG1 but 50% less IgM than HuRf/f B cells (Fig. 4A). Membrane-bound and secreted IgH chains are generated from mRNAs that differ in 3’ translated sequences, with only membrane-bound mRNA forms encoding a
transmembrane domain (Fig. 4B) (Bruce et al. 2003, Peterson 2011). Alternative polyadenylation controls the relative abundance of membrane-bound versus secreted transcripts (Bruce et al. 2003, Peterson 2011). Since HuR can regulate pre-mRNA processing (Lebedeva et al. 2011), we investigated whether HuRΔ/Δ B cells normally generate mRNAs encoding secreted antibodies. We used distinct primer sets to amplify mRNAs encoding the membrane-bound or secreted isoforms of IgM after 72 hours of stimulation. We found that the levels of secreted IgM mRNAs were reduced while levels of membrane-bound IgM mRNAs were unchanged in HuRΔ/Δ cells as compared to HuRf/f cells (Fig. 4C), reflecting the relative amounts of secreted and membrane-bound IgM in the cultures. These data show that HuR is required for generation of alternatively-polyadenylated IgH mRNAs and secretion of antibodies at normal levels following in vitro stimulation by LPS and IL-4.

We next assayed in vitro activation of HuRΔ/Δ B cells by conditions that mimic a T cell-dependent B cell response. For this purpose, we isolated total splenic B cells from HuRΔ/Δ and HuRf/f mice and incubated equal numbers of cells with anti-IgM and anti-CD40 with or without IL-21. To assess whether splenic HuRΔ/Δ B cells also exhibit reduced proliferation when stimulated by these conditions, we labeled cells with CFSE prior to incubation for 60 hours with anti-IgM, anti-CD40, and/or IL-21. We observed an equivalent number of cell divisions in HuRΔ/Δ and HuRf/f cultures (Fig. 4D), indicating that HuRΔ/Δ B cells exhibit normal proliferation following in vitro stimulation under conditions that mimic T cell-dependent activation. We also noted that cell viability after stimulation was not decreased by HuR deletion (Fig. 4D). To determine whether HuR affects the induction of GC markers on HuRΔ/Δ B cells in vitro, we measured expression of the BCL6, CD95, and TACI proteins in un-stimulated and stimulated cells. After
stimulation for 60 hours, we found similarly changed expression of each of these GC B cell markers in HuRΔ/Δ and HuRf/f cultures (Fig. 4E), revealing that HuRΔ/Δ B cells show normal expression of GC markers following in vitro stimulation by conditions that mimic T cell-dependent activation. These data demonstrate that HuR expression in B-lineage cells is dispensable for the ability of naive B cells to become activated and "GC-like" in vitro.

HuRΔ/Δ mice have impaired humoral immunity and dysfunctional B-T cell interactions during an in vivo T cell-dependent immune response.

To determine whether HuR has a role in humoral immunity in vivo, we first analyzed standing serum antibody levels in non-immunized 8 to 10 week-old HuRΔ/Δ and WT mice. We observed that serum levels of all Ig isotypes were reduced in HuRΔ/Δ mice (Fig. 4F), indicating that HuR expression in B lineage cells is necessary for optimal antibody production in vivo. Since B1 B cells produce the majority of standing IgM and IgA serum titers, we assessed the effects of HuR deletion on these B lineage cells. We detected 75% fewer peritoneal B1 B cells in HuRΔ/Δ mice (Fig. 4G), revealing that HuR expression in B lineage cells is required to support normal numbers of B1 cells. We also quantified GC cells, plasma cells (PC), and switched memory (Sw-mem) B cells, which arise from T cell-dependent B cell responses and together generate the majority of standing IgG and IgE serum titers. We detected reduced numbers of GC (50-fold lower) and PC (2-fold lower) cells, and a trending reduction in Sw-mem (2.5-fold lower) B cells in HuRΔ/Δ mice (Fig. 5A), indicating that expression of HuR in B lineage cells is required to support normal numbers of antibody secreting B2 cells. Collectively, these data
demonstrate that B-lineage intrinsic functions of HuR are necessary for normal numbers of antibody-secreting B1 and B2 cells and normal titers of standing antibodies of all isotypes.

We next evaluated the role of HuR in T cell-dependent humoral immunity *in vivo* by testing the ability of HuRΔ/Δ mice to mount a B cell response to immunization with a T cell-dependent antigen. For this purpose, we injected 8 week-old HuRΔ/Δ or WT mice with NP-OVA precipitated in alum. After 9 or 14 days, we quantified numbers of naive B cells, PCs, total GC B cells, and NP-specific GC B cells in immunized mice and non-immunized littermate controls. Since some antibodies generated from NP-OVA injection will recognize epitopes within OVA rather than NP, we measured total GCs as well as NP-specific GCs. The numbers of naive B cells in HuRΔ/Δ mice remained 50% lower than normal after immunization (Fig. 5B). WT mice mounted a robust humoral response, generating half a million or more total and NP-specific GC B cells by 9 and 14 days post-immunization (Fig. 5B). In contrast, HuRΔ/Δ mice were impaired substantially in ability to mount a humoral immune response, generating only thousands of total and NP-specific GC B cells over the same period following immunization (Fig. 5C). Reflecting fewer GC B cells, the production of plasma cells was reduced 4.5-fold in HuRΔ/Δ mice relative to WT mice (Fig. 5C). We also performed ELISAs to quantify low- and high-affinity NP-specific antibodies in sera of HuRΔ/Δ and WT mice at 9 or 14 days post-immunization. We detected 6-fold reduced levels of low-affinity IgG NP-specific antibodies in HuRΔ/Δ mice at each time point assayed (Fig. 5D). Consistent with the requirement of GCs for robust affinity-maturation, we found a 20-fold increase in high-affinity IgG anti-NP antibodies in immunized WT mice between days 9 and 14, but only a 4-fold increase in HuRΔ/Δ mice over the same time (Fig. 5D). Further, HuRΔ/Δ mice had reduced levels of
IgM anti-NP antibodies (Fig. 5D), consistent with the idea that low IgG antibody titers are not the result of a CSR defect in HuRΔ/Δ B cells. Additionally, we found that deletion of p53 in HuR-deficient B cells did not rescue the ability to form germinal centers relative to p53-deficient HuR-sufficient B cells (Fig. 5E). This is consistent with our finding that HuRΔ/Δ B cells survive well in vitro, although we cannot rule out that apoptosis may still occur independently of p53 activation in HuRΔ/Δ B cells in vivo.

The compromised ability of HuRΔ/Δ mice to produce GC B cells and high-affinity antibodies in vivo, which contrasts with ability of HuRΔ/Δ B cells to become "GC-like" in vitro, we investigated aspects of GC formation that may not be measured accurately by the in vitro assays used thus far. Since the ability of B cells to form GCs depends upon their ability to appropriately localize within lymphoid organs, we examined the structure of WT and HuRΔ/Δ spleens by histological means. The gross architecture as measured by H&E staining was normal in both WT and HuRΔ/Δ spleens (Fig 6A). After immunization, GCs were visible in follicles of WT but not HuRΔ/Δ mice, as evidenced by H&E and immunohistochemical (IHC) staining for PNA, which marks GC B cells (Fig 6A). Since the light zone of the germinal center consists of rapidly dividing B cells (Cato et al. 2011), we also performed IHC staining for Ki67, which marks proliferating cells. Indeed, we found clusters of Ki67+ cells only in immunized WT mice, but not in immunized HuRΔ/Δ mice or un-immunized mice of either genotype (Fig 6A and data not shown), consistent with the lack of GC formation in HuRΔ/Δ mice.

The severe lack of GCs in HuRΔ/Δ mice suggests that GCs fail to initiate in mice with HuR-deficient B cells. GC initiation requires B cells to interact with activated CD4+ T cells at the periphery of the lymphoid follicle. Appropriate B-T interactions allow migration of both cells into the follicle and promote their differentiation into GC B cells.
and GC T follicular helper (Tfh) cells, respectively (Linterman and Vinuesa 2010, Goenka et al. 2011). Failure of B cells to interact with T cells prevents GC Tfh formation and also ablates the in vivo GC response (Linterman and Vinuesa 2010, Baumjohann et al. 2013). We quantified total and mature (GC) Tfh cells in the spleens of immunized HuRΔ/Δ and WT mice. Immunized HuRΔ/Δ mice had 1.6 and 17-fold lower numbers of total and mature Tfh cells, respectively, in their spleens relative to WT controls (Fig. 6B), suggesting that HuR may promote the ability of B cells to effectively interact with T cells to promote Tfh differentiation.

Because lack of Tfh cells could also result from failure of B cell activation, we investigated B cell-intrinsic factors that promote Tfh differentiation prior to GC initiation. Activated B and T cells interact in part through contact between co-stimulatory receptors and ligands expressed on each cell’s surface (Borst et al. 2005, Baumjohann et al. 2013, Cubas et al. 2013). We first asked whether HuR-deficient B cells appropriately expressed known co-stimulatory molecules. We assessed expression of a panel of such markers by flow cytometry on WT or HuRΔ/Δ B cells stimulated in vitro for 72h with α-IgM and α-CD40 (Fig 6C). HuRΔ/Δ B cells showed reduced expression of the CD81, CD70, and CD86 proteins (Fig 6C) (Borriello et al. 1997, Maecker and Levy 1997, Miyazaki et al. 1997, Tsitsikov et al. 1997, Akiba et al. 1999, Borst et al. 2005, Denoeud and Moser 2011, Levy 2014). Since CD86 binding to CD4+ T cells also activates B cell intracellular signals to promote antibody secretion (Rau et al. 2009), this reduced CD86 expression might impair humoral immunity through distinct mechanisms. We also found that HuR binds to CD81, CD70, and CD86 transcripts (Fig 6D), consistent with the notion that HuR directly regulates expression of these molecules in activated B cells. These small but significant reductions in co-stimulator expression may be biologically
meaningful in light of clinical data from human patients with immunodeficiency (Denz et al. 2000, Groth et al. 2002). However, there are likely many other contributions of HuR to the in vivo GC response.

We failed to identify any major defects in HuR-deficient B cell function in vitro; therefore we hypothesize that HuR facilitates functional interactions between B cells and T cells in the follicular milieu. However, it is plausible that the physiologic demands placed upon B cells during a T-dependent immune response are not accurately mimicked by standard in vitro assays, emphasizing the need to better understand GC formation in its in vivo context. Collectively, our data demonstrate that B cell-intrinsic functions of the HuR RBP are necessary for T cell-dependent humoral immunity.
DISCUSSION

Post-transcriptional regulation of gene expression allows dynamic and precise regulation of specific functional programs in many cell types including lymphocytes. Studies in other cell types show that the RNA-binding protein HuR can regulate genes that control proliferation, cell survival, differentiation, and DNA damage responses (Abdelmohsen et al. 2007, Rodriguez et al. 2010, Mazan-Mamczarz et al. 2011). Each of these processes is essential for normal lymphocyte development and function.

Therefore, we set out to understand roles of HuR in B cells by analyzing mice with B lineage-specific HuR gene deletion. We discovered important roles of HuR in several subsets of B cells throughout their development and function. Further, these data point to interesting developmental-stage and context-specific roles of HuR, which were previously unexplored.

Our data show that B cell-specific HuR-deficiency reduces numbers of immature and mature B2 B cells. HuRΔ/Δ mice have a partial block in pro- to pre-B cell development, in correlation with the increased apoptosis of HuR-deficient pre-B cells. Consistent with the notion that HuR suppresses apoptosis of pre-B cells, co-deletion of the pro-apoptotic p53 with HuR restores pre-B cell numbers to the level of p53Δ/Δ control animals. However, numbers of mature B cells are not restored in HuRΔ/Δ p53Δ/Δ mice suggesting additional p53-independent roles of HuR in promoting the development or maintenance of normal mature B cell numbers. A role for HuR in limiting p53-dependent apoptosis of immature B cells was shown in the context of global HuR deletion in adult mice using Tamoxifen-inducible Cre (Ghosh et al. 2009). Ghosh and colleagues also reported death of BM CLPs and HuR-deficient thymic progenitors. These results contrast the effects of HuR in a mouse model of thymus-specific HuR
knockout using LckCre, where HuR-deficient thymocytes had reduced p53 expression, increased proliferation, and a concomitant increase in numbers of thymocytes (Papadaki et al. 2009). Comparison of these mouse models highlights important lineage- and developmental stage-specific differences in roles of HuR in lymphocyte biology.

Steady state numbers of mature B cells are determined by the balance between generation of new cells, proliferation, and death of existing cells (Sindhava et al. 2013). These mechanisms are dynamic, and allow a restoration of normal lymphocyte numbers following infection, disease, or genetic mutation. The reduced numbers of naïve splenic HuRΔ/Δ B2 cells in older mice and in the situation where p53 inactivation restores immature B2 cell numbers suggest that HuR has B lineage-intrinsic functions in controlling homeostasis of naïve B2 cells. The BLyS family cytokine BAFF secreted from fibroblastic reticular cells and radiation-resistant stromal cells promotes B cell homeostasis by signaling through BAFF-R, TACI, and BCMA receptors expressed on B cells (Gorelik et al. 2003, Rauch et al. 2009, Sindhava et al. 2013, Cremasco et al. 2014). We detected normal expression of TACI on stimulated and un-stimulated WT and HuRΔ/Δ cells; however HuR might regulate expression of BAFF-R or BCMA, or their downstream signaling factors, such as the mTORC2 complex (Lee et al. 2013). Further, the decreased numbers of peritoneal HuRΔ/Δ B1 cells may reflect a role for HuR in promoting homeostasis of naïve B1 cells. Evidence suggests that the BLyS family cytokine APRIL secreted from peritoneal macrophages promotes B1 cell homeostasis (Sindhava et al. 2014); however, homeostatic regulation of B1 cells is incompletely understood. Identifying HuR mRNA targets in splenic B2 and peritoneal B1 cells may yield novel molecular insight into the molecular factors and mechanisms that govern B cell homeostasis.
While HuR is not strictly required for B cell development in mice, it is crucial for humoral immunity in vivo. Serum titers for all antibody isotypes were greatly reduced in HuRΔ/Δ animals. The low levels of serum antibodies are consistent with our finding that HuRΔ/Δ mice have greatly reduced numbers of peritoneal B1 cells, which secrete the majority of IgM and IgA, as well as reduced numbers of plasma cells, which secrete the majority of IgG isotypes. However, using in vitro stimulation of WT and HuR-deficient splenic B cells, we discovered an additional role for HuR in promoting antibody secretion by regulating alternative polyadenylation of IgM mRNA that may extend to other IgH mRNAs as well. One way that HuR might regulate IgM polyadenylation is by binding the IgM pre-mRNA in the nucleus and directly regulate its processing at the level of splicing or polyadenylation, since these processes are in competition at the IgH locus (Peterson 2011). This would be consistent with a well-established function of nuclear HuR in regulating splicing of many transcripts (Lebedeva et al. 2011, Mukherjee et al. 2011, Diaz-Munoz et al. 2015). RNA-IPs performed on lysates from stimulated WT cells did not detect immature IgM mRNA bound to HuR (not shown); however, these assays were done on whole cell lysate, rather than nuclear fractions, and the abundance of immature IgM mRNA is expected to be very small since processing occurs co-transcriptionally (Lee and Tarn 2013). Alternatively, HuR may regulate expression of an mRNA processing factor acting on the IgH mRNA. The trans-acting factors that regulate the switch from receptor- to antibody-coding IgH mRNAs are not completely understood, however, factors including ELL2, XBP-1, PTB, and hnRNPF have been implicated (Calfon et al. 2002, Bruce et al. 2003, Shell et al. 2007, Peterson 2011, Benson et al. 2012). Our investigation of protein expression of these factors showed no obvious differences in expression between stimulated WT and HuR-deficient cells (not shown). These data may suggest that a previously undescribed factor that controls IgM mRNA
polyadenylation might be mis-expressed in the absence of HuR. Taken together, these
data show that HuR is required for the production of adequate quantities of serum
antibodies of all isotypes, and highlights the critical *in vivo* role of HuR in humoral
immunity.

As we were submitting our work, another group published that mice with B
lineage-specific HuR deletion are defective in the GC reaction and antibody generation
in response to T cell-dependent and -independent antigens (Diaz-Munoz et al. 2015).
Based on their analysis of B cells stimulated *in vitro* with LPS, this group suggests that
HuR regulates cellular metabolism of activated B cells such that the impaired GC
reaction *in vivo* might result from death of HuR-deficient B cells (Diaz-Munoz et al.
2015). This group observed a much greater defect in proliferation of HuR-deficient B
cells stimulated *in vitro* than we report here. We wondered whether our inclusion of
sodium pyruvate, which also acts as a scavenger of reactive oxygen species, as a
medium additive might be masking a viability defect in HuRΔ/Δ cells, as sodium pyruvate
was reported by Diaz-Munoz and colleagues to have this effect. However, we compared
class switching, viability, and proliferation of cells stimulated without sodium pyruvate,
and found no substantial differences between WT and HuRΔ/Δ (Supplemental Fig.
3D,E). These divergent results may be due to strain-specific differences or other subtle
alterations in culture conditions. However, our data that HuRΔ/Δ B cells proliferate,
survive, and induce expression of GC B cell markers normally *in vitro* following
stimulation under conditions that mimic a T cell-dependent response suggests that
altered B cell metabolism is not a major cause of the impaired GC reaction and T cell-
dependent immune response of HuRΔ/Δ mice.
B cell function \textit{in vivo} is dependent upon direct interactions of B cells with other cell types, for example through contacts between cell surface receptors, as well as indirect interactions via cytokines and chemokines secreted by surrounding immune cells (Vinuesa and Cyster 2011, Victora and Nussenzweig 2012). That HuR-deficient B cells can survive, proliferate, class-switch, and differentiate normally \textit{in vitro} is in contrast with the lack of GC response in HuRΔ/Δ mice immunized with NP-OVA. This major disconnect between the \textit{in vitro} normalcy of HuR-deficient B cells, and their inability to function in the T-dependent immune response \textit{in vivo} could point to a number of mechanistic defects. B cells traffic in and out of the follicle with defined kinetics in order to encounter antigen and subsequently engage antigen-primed CD4⁺ T cells (Pereira et al. 2010, Vinuesa and Cyster 2011). Activated B cells must physically interact with antigen-primed CD4⁺ T cells to induce pre-Tfh cells to migrate into the follicle and differentiate into mature Tfh cells that provide survival and differentiation signals essential for generating GC B cells and high-affinity antibodies (Linterman and Vinuesa 2010, Goenka et al. 2011, Baumjohann et al. 2013, Cubas et al. 2013). B cells drive contact with CD4⁺ T cells through B cell-surface MHC II molecules loaded with processed antigen fragments and through co-stimulatory proteins expressed on B cells (Vinuesa and Cyster 2011, Baumjohann et al. 2013, Yuseff et al. 2013). In addition to physical interactions with CD4⁺ T cells, B cells enhance Tfh differentiation by secreting IL6 and other cytokines (Eto et al. 2011, Karnowski et al. 2012). We found that HuRΔ/Δ B cells exhibit normal expression of chemokine receptors CXCR5 and CCR7, which are important for B cell trafficking, as well as MHCII proteins and IL6 mRNA (data not shown); however, it is possible that antigen processing and/or B cell trafficking are impaired in HuRΔ/Δ cells through dysregulation of unexplored factors.
Co-stimulatory receptors and ligands that are expressed on both B and T cells provide important survival and differentiation signals that in turn are required for both the B cell and the T cell (Xu et al. 1994, Denz et al. 2000, Linterman and Vinuesa 2010). We found that HuRΔ/Δ cells stimulated in vitro have subtle but significant reductions in expression of CD81, CD70, and CD86. Impaired expression of CD81, CD70, or CD86 has been observed on B cells of humans with Common Variable Immune Deficiency (CVID) (Denz et al. 2000, Groth et al. 2002, van Zelm et al. 2010). Notably, the subtle differences in CD86 and CD70 expression on HuRΔ/Δ B cells mimic the modest reduction of these co-stimulatory molecules on B cells from CVID patients (Denz et al. 2000, Groth et al. 2002). Although to our knowledge, mutations or polymorphisms of the HuR gene have not been associated with CVID, identifying HuR target mRNAs in naïve B2 cells and antigen-activated GC B cells could lead to greater understanding and perhaps improved therapies for humans with impaired T cell-dependent immune responses.
FIGURE 1. B lineage-specific deletion of HuR leads to decreased numbers of immature and mature B cells. (A - D) Representative flow cytometry analysis and quantification of B lineage cell populations in the BM and spleens of 4-6 week old HuRΔ/Δ and HuRF/f mice. The population of live cells analyzed is depicted above each flow plot and the percentages of analyzed cells in each of the indicated gates are shown. Data are from three or more independent experiments conducted on a total of at least five HuRΔ/Δ mice and five control littermate HuRf/f mice. *p<0.05, **p<0.01. (A) B220+ cells in BM and spleen. (B) BM B220+CD43+ pro-B, B220+CD43- pre-B, and B220+IgM+ cells. (C) BM Hardy fractions A (BP1-HSA-), B (BP1-HSA+), C (BP1+), C’ (BP1+HSA-), D (B220+IgM), E (B220+IgM+), and F (B220-high IgM). (D) Splenic IgM-high T1 (CD23CD21+), T2 (CD23CD21-), MZ (CD23CD21-), and Fo (IgM-low CD21) B cells.
Figure 2

A. 

B. 

C. 

D. 

E.
FIGURE 2. HuR protects immature B cells from p53-dependent elimination. (A - E) Representative flow cytometry analysis and quantification of B lineage cell populations, cell cycle distribution, or apoptosis in BM and spleens of the indicated mice. The population of live cells analyzed is depicted above each flow plot and the percentages of analyzed cells in each of the indicated gates are shown. Data are from three or more independent experiments conducted on a total of five or more mice of each genotype. *p<0.05, **p<0.01, ***p<0.001 (A) B220+CD43+ pro-B, B220+CD43− pre-B, and B220+IgM+ B cells in BM or B220+IgM+ B cells in spleens of 4-6 week old IgHTg HuRΔ/Δ or IgHTg HuRf/f mice. (B) DNA content cell cycle profile of pro-B cells or Hardy fraction C cells (see Fig 1C) from 4-6 week old HuRΔ/Δ or HuRf/f mice. (C) Flow cytometry analysis of pro-B, pre-B, and B220+IgM+ B cells in BM or B220+IgM+ cells in spleens of 12-14 week old HuRΔ/Δ or HuRf/f mice. (D) AnnexinV staining of BM pro-B or pre-B cells of 4-6 week old HuRΔ/Δ or HuRf/f mice. (E) pro-B, pre-B, and B220+IgM+ B cells in BM or B220+IgM+ B cells in spleens of 4-6 week old p53Δ/Δ or p53Δ/Δ HuRf/f mice.
FIGURE 3. *In vitro* stimulated HuRΔ/Δ B cells exhibit a mild proliferation defect, enhanced survival, and normal IgH isotype switching. (A) Quantification of the fold expansion of splenic B cells from HuRΔ/Δ or HuRff/f mice cultured for 72 hours in LPS and IL-4. Data are from three independent experiments performed on a total of ten
animals of each genotype. (B - E) Representative flow cytometry analysis and quantification of live cells (B), cellular divisions (C), cell cycle distribution (D), or switched Ig expression (E) following culture of HuRΔ/Δ or HuRf/f splenic B cells for 72 hours or where indicated 96 hours in LPS and IL-4. Data are from three or more independent experiments conducted on at least five mice of each genotype. *p<0.05, ** p<0.01, ***p<0.001.
FIGURE 4. HuR is required for antibody production and numbers of peritoneal B1 cells but dispensable for in vitro functions. (A) ELISA quantification of IgM or IgG1 secreted by HuRΔ/Δ or HuRf/f splenic B cells during a 72 hour culture in LPS and IL-4. Data are
presented as raw values (left graph) or values normalized to the numbers of cells in each culture (right graph). Shown is a representative of three independent experiments. (B) Schematic of the final three exons of the μ constant region showing the genomic configuration and the mRNA forms generated by alternative polyadenylation. Arrows above the exons indicate primers used to detect secreted (sec) or membrane-bound (mem) IgM transcripts by qRT-PCR. (C) Quantification of IgM transcript variants in LPS and IL-4 stimulated HuRΔ/Δ or HuRf/f splenic B cells presented as their relative abundance to 18S mRNA. Data are from three independent experiments. *p<0.05, **p<0.01, ***p<0.001. (D - E) Representative flow cytometry analysis and quantification of live cells and cell divisions (D) or expression of GC and plasmablast markers (E) following culture of HuRΔ/Δ or HuRf/f splenic B cells for 60 hours without stimulation or with stimulation by anti-IgM, anti-CD40, with or without IL-21. Data are representative of two experiments performed on a total of four mice of each genotype. (F) ELISA quantification of serum Ig isotypes in non-immunized HuRΔ/Δ and HuRf/f mice. Data are from three or more independent experiments conducted on at least five 6-8 week old mice of each genotype. (G) Representative flow cytometry analysis and quantification of peritoneal B1 B cell subsets (CD11b⁺CD5⁺ B1a cells and CD11b⁺CD5⁻ B1b cells) following gating on live CD19⁺ lymphocytes. Bar graph shows numbers of total B1 cells from three experiments performed with 6-8 week old mice, 6 HuRf/f and 9 HuRΔ/Δ.
FIGURE 5. HuR is required in vivo for generation of GC B cells and high-affinity antibodies. (A) Representative flow analysis and quantification of splenic GC, PC, and Sw-mem B cells from non-immunized mice. Flow plots are shown following gating on live
IgD-dump lymphocytes. Data are from two independent experiments conducted on at least six 6-8 week-old mice of each genotype. *p<0.05, **p<0.01, ***p<0.001. (B - C) Quantification of B cell populations and NP-specific antibodies in HuRΔ/Δ and HuRf/f mice immunized with NP-OVA in alum. Data are from two independent experiments involving three and five mice of each genotype at 9 and 14 days after immunization, respectively. (B) Representative flow cytometry analysis and quantification of splenic naive, total GC cells, NP+ GC cells, and PCs. (C) ELISA quantification of low- and high-affinity anti-NP specific antibodies, indicated by NP25 and NP4, respectively. IgM anti-NP ELISAs were run without a standard, thus are displayed as a dilution series for each group. For IgM ELISAs, p-values are the result of two-way ANOVA. (D) Quantification of GC B cells from p53Δ/Δ and HuRΔ/Δ p53Δ/Δ mice. Representative plots show cells previously gated on live IgD- DUMP-, CD138- CD19+ lymphocytes. The experiment was performed three times with 5 mice per genotype in total.

Figure 6. HuR is required for in vivo formation of germinal centers in context. (A) Left two panels show representative H&E staining on spleens of non-immunized and immunized HuRf/f or HuRΔ/Δ mice 14d after NP-OVA administration. Right two panels show representative immunohistochemical (IHC) staining for PNA or Ki67 in brown on spleens from mice 14d after NPOVA immunization. Staining was performed on spleens...
from two separate immunizations, with a total of two un-immunized mice, and 6 immunized mice of each genotype. Scale bars are 400μm for H&E and 500μm for IHC. Arrows point to GCs. (B) Representative flow analysis of TCRβ⁺CD4⁺CD62L⁻ lymphocytes from spleen of un-immunized mice or mice 14d after NP-OVA administration. Percentages of PD-1⁺ CXCR5⁺ total Tfh cells and PD-1ʰ élevé CXCR5ʰ élevé GC Tfh cells are shown on plots and quantified on right. Quantification shown is from a single experiment of two performed with similar results. (C) Flow cytometry to measure expression of co-stimulatory surface molecules on B cells after 48h stimulation with α-IgM and α-CD40. Representative histograms following gating on live cells is shown for significantly changed surface markers. Quantification of median fluorescence intensity (MFI) of HuRΔ/Δ cells is expressed as fold change over HuRf/f cells from the same experiment. Significant differences are the result of a one-sample t-test. **p<0.01 and ***p<0.001. The experiment was repeated at least twice for each marker shown, with a minimum of 5 animals per genotype. (D) RNA-IP qRT-PCR analysis performed on WT splenic B cells stimulated for 72h with α-IgM and α-CD40. Ct values for indicated transcripts were normalized to GAPDH for each IP, and HuR IP values are expressed as fold enrichment compared to IgG IP. Results are average +/-SEM for four biological replicates over two individual experiments. No statistical tests were performed on these results, but typically RNA-IP enrichment over 2-fold (dotted line) is considered meaningful.
Supplemental Figure 1. Characterization of HuRΔ/Δ and Mb1Cre+ mice. (A) qPCR assay to measure HuR gene deletion from the indicated subsets in HuRΔ/Δ mice and control HuRf/f and HuRf/- mice. Pro-B and pre-B cells were obtained by sorting, with purities ranging from 90-98%. Splenic B cells were obtained by bead-based negative selection, yielding 93-98% purity. Data are the average of at least three mice per genotype from three experiments. n.d. - not detected. (B) Representative western blotting for HuR protein in splenic B cells purified from HuRf/f or HuRΔ/Δ mice. HuR levels were normalized to actin and WT HuR levels were set to 1.0. (C) Representative flow cytometry analysis and quantification of B lineage cell populations in BM and spleens of 4-6 week old Mb1-Cre+ or WT mice. The population of live cells analyzed is depicted above each flow plot and the percentages of analyzed cells in the indicated gates are shown. Data are from two experiments conducted on a total of five mice of each genotype.
Supplemental Figure 2. Characterization of HuRΔ/Δ and Mb1Cre+ B cell function. (A) Expansion of WT or Mb1Cre+ B cells following 72h stimulation with LPS and IL-4. This experiment was performed twice with a total of five mice of each genotype. (B) qRT-PCR quantification of germline transcripts emanating from the IgG1 or IgE constant regions conducted on splenic B cells of the indicated genotypes following 18 hours of stimulation with LPS and IL-4. Each point represents a mouse analyzed in one of the two experiments performed. (C) CFSE dilutions in HuRff and HuRΔ/Δ B cells stimulated in vitro for 96 hours with LPS and IL-4. This experiment was performed twice with three mice of each genotype in total. Error bars indicated the SEM. *p<0.05. (D-E) Flow cytometry analysis of IgG1 switching and proliferation (D) or viability (E) from WT or HuRΔ/Δ cells stimulated in vitro for 72h. The experiment was performed twice with 4 biological replicates in total. Composition of complete medium is described in methods. In (E) each point represents cells obtained from an individual animal.
D-cyclin misregulation and transformation of developing lymphocytes

The integration of cell cycle regulation with DNA damage signaling is essential for maintaining genome integrity. The G1/S checkpoint is particularly important for preventing unrepaired breaks during V(D)J recombination from entering S phase, where they are more likely to be misrepaired. The canonical G1/S checkpoint pathway involving ATM-dependent p53 activation is well-described and undoubtedly contributes to suppression of RAG-induced genome instability in developing lymphocytes (Dicker et al. 2009, Rowh et al. 2011, DeMicco et al. 2013). Indeed, chapter II of this thesis describes roles of p53 in preventing translocations at antigen receptor (AgR) loci. However, this p53-dependent checkpoint relies on upregulation of cell cycle inhibitors at the transcriptional level, thus is relatively slow (von Boehmer and Melchers 2010). Further, evidence suggests that checkpoint mechanisms do not necessarily operate identically in all cell types. For instance, chapter II demonstrates that the karyotype of tumors arising following deletion of p53 in hematopoietic stem cells as compared to DN thymocytes is subtly different, with the latter more likely to give rise to AgR translocations. In contrast, germline deletion of p53 in mice leads to thymic tumors with aneuploidy, for reasons that remain incompletely understood.

We propose here that downregulation of cyclin D3 following induction of DSBs may be another mechanism whereby developing lymphocytes with unrepaired breaks delay S phase entry. Mice with deletion of p53 beginning in pro-B cells develop immature and mature B cell tumors with a high frequency of AgR translocations (Rowh et al. 2011). Although p53 certainly suppresses AgR translocations in developing B cells through D3-independent mechanisms, our data demonstrate that p53-/- pre-B cells fail to
downregulate D3, and that D3 misregulation causes some pre-B cells to aberrantly enter S phase following IR. This raises the interesting, yet untested, possibility that p53 suppresses AgR translocations during B cell development in part through downregulation of D3 in response to unrepaired Rag breaks.

To rigorously test the hypothesis that DSB-dependent loss of D3 expression indeed promotes genome stability during B lymphocyte development, we need to better understand the transcriptional regulation of the D3 promoter in the context of DSB signaling. One possibility is that p53 directly inhibits D3 promoter activity, since p53 can repress transcription of some genes (Ho and Benchimol 2003), although this view is somewhat controversial (Fischer et al. 2014). Hypotheses about the identity of other transcription factor(s) (TFs) regulating D3 inhibition after IR might be based on known transcription factors that regulate D3 in response to physiologic stimuli. For example, Aiolos suppresses D3 expression downstream of pre-BCR signaling (Ma et al. 2010, Venigalla et al. 2013) and E2F1 is a potent activator of D3 transcription (Ma et al. 2003). Further, the candidate TF must be linked to DSB signaling through ATM and p53, although such a link may be as yet unreported in the literature. Finally, the conjectured mechanism of downregulation at the D3 locus should be compatible with the seemingly immediate ablation of transcription following IR. Specifically, it seems likely that IR induces either an immediate removal of an activating TF or deposition of a repressive TF that is already present in the vicinity of the D3 promoter. Such an event could conceivably occur due to post-translational modification of a TF, perhaps mediated by ATM or Chk2, although this would not fully explain the role of p53. The model proposed in chapter III posits such a mechanism (Figure 7A). Ultimately, mutation of the promoter element(s) found to be required for DSB-dependent D3 downregulation, perhaps using CRISPR technology in mice (Ma et al. 2014) would enable us to more rigorously test
whether DSB-dependent downregulation of D3 promotes genome stability during B lymphocyte development.

A teleological explanation for the existence of three D-type cyclins in mammalian cells is lacking; however, single and combinatorial D-cyclin knockout mouse models demonstrate the importance of tissue-specific expression patterns. Moreover, while enforced expression of some D-cyclins can replace the absence of another D-cyclin in some contexts, it is clear that each cyclin has specific biological roles to play (Bartkova et al. 1998, Ciemerych et al. 2002, Kozar et al. 2004, Musgrove et al. 2011). For example, thymocytes, pro- and pre-B cells, and GC B cells from D3-/- mice have increased expression of D2 protein; however, this increased D2 protein does not rescue the proliferative defects of these cells (Sicinska et al. 2003, Cooper et al. 2006, Peled et al. 2010). Consistent with these published findings, our data show that D2 expression is not affected by IR in ex vivo pre-B cells but that this continued D2 expression likewise does not drive proliferation in the absence of D3 protein. However, the mechanisms by which D3, but not D2, can drive proliferation of pro-B cells and DN thymocytes remain elusive. Even though each of the D-cyclins has the ability to bind to CDK4 and CDK6, it is possible that the ability of D2 and D3 to form a complex with their CDK partners might be regulated in a unique way in lymphocytes. For example, a tertiary factor may bind D2 to prevent its association with CDKs, or D2 might be post-translationally modified to similarly prevent CDK binding. These alternatives could be addressed by immunoprecipitating D2 and D3 in lymphocytes and non-lymphoid cell types and directly investigating the ability of D2 and D3 to exist in a complex with CDK4/6. In fact, this was partially addressed in a recent study, showing that D3 but not D2 associates strongly with CDK4 in pro-B cells (Powers et al. 2012). Further investigation into the mechanisms
by which D-CDK complex formation is regulated will enhance our understanding of tissue-specific roles of D-cyclins in driving proliferation.

Another intriguing possible reason for the inability of D2 to compensate for D3 loss in lymphocytes is that D3 promotes proliferation of these cells independent of its binding to CDK4/6. The literature suggests many examples of non-catalytic functions of D cyclins, mostly cyclin D1, which has been more extensively studied. Specifically, a high-throughput study showed that the D1 protein physically interacts with many factors that regulate transcription, including chromatin modifiers, and transcriptional co-activators (Bienvenu et al. 2010). This group and others further showed that D1 is bound to many developmentally-regulated promoters and that D1 regulates transcription of these genes (Reutens et al. 2001, Fu et al. 2005, Fu et al. 2005, Bienvenu et al. 2010). Evidence that D3 can also regulates genes at the level of transcription comes from data that in pro-B cells a specific sub-cellular fraction of D3 protein regulates abundance of many mRNA transcripts (Powers et al. 2012). These data suggest that cyclin D3 may be specifically required to drive lymphocyte proliferation in part due to non-catalytic functions of D3. Further works remains to elucidate such mechanisms and how they are integrated with canonical roles of D3-CDK4/6 complexes during lymphocyte development.

It is clear that D3 protein promotes B and T cell development in ways that are not redundant with D2; however, why did these mechanisms evolve? Our model proposes that the unique requirement for D3 in driving the proliferation of developing B and T lymphocytes at times closely following V(D)J recombination may reflect a need to downregulate D3 in response to unrepaired DSBs, potentially providing a reason why lymphocytes evolved to require D3 at these stages. Although D-type cyclins including D3 are often overexpressed in leukemias and lymphomas (Musgrove et al. 2011), our model
suggests that D3 might be relatively less oncogenic than D1 or D2. Although the picture is complicated, some clinical data supports this hypothesis. D cyclin dysregulation has not been as well studied in immature B cell tumors as in mature B cell tumors; however, D1 overexpression in B cell acute lymphoblastic leukemia (B-ALL) is correlated with relapse and poor prognosis (Sauerbrey et al. 1999). Further, mutation or deletion of negative regulators including Aiolos have been reported in a subset of B-ALL cases (Mullighan et al. 2007). Given that, in immature B cells, D3 downregulation in response to IR occurs at the level of transcription, these aberrations might be expected to render D3 insensitive to DSB signaling.

Consistent with tissue-specific regulation of D3, we showed that D3 repression in irradiated thymocytes occurs by a post-transcriptional mechanism involving loss of binding of the HuR RBP to the D3 mRNA. This mechanism depends on the expression of the tumor-suppressive kinase ATM. Because ATM-deficiency in the thymus is correlated with T cell ALLs (T-ALLs) (Liberzon et al. 2004), especially aggressive subtypes with unstable genomes (Meier et al. 2005), we suggest that ATM might suppress genome instability in part by repressing D3 expression in response to unrepaired breaks during T cell development. However, this interpretation is complicated by the finding that D3 is sometimes required for malignant transformation of immature T cells. Work from our lab has shown that D3 deletion spares mice from T-ALL driven by ATM inactivation (Ehrlich et al. 2015), and D3-/- mice are likewise resistant to Notch-driven T-ALL (Sicinska et al. 2003). However, D3-/- mice are lymphopenic; therefore, we propose that normally regulated D3 is able to promote appropriate proliferation of thymocytes while still remaining responsive to DSB-dependent restraint. Further studies are needed to validate our model wherein activated ATM causes HuR to release D3 mRNA, leading to reduced D3 translation, and subsequent G1 delay. Following these
mechanistic studies, we would then investigate whether the inability of D3 to be downregulated in response to DSBs in the thymus indeed causes genome instability.

We wondered whether HuR had any role in controlling expression of D3 in pre-B cells. To investigate this, we generated ex vivo IL-7 pre-B cell cultures from WT and HuRΔ/Δ mice. We first noticed that we could only achieve about 10% deletion of HuR alleles in a population of IL-7 cultured pre-B cells from HuRΔ/Δ mice (Figure A-2-A); despite the fact that pre-B cells isolated from HuRΔ/Δ mice showed robust deletion (see Chapter IV, Supplemental figure 1A). We found that transgenic overexpression of Bcl2 increased the extent of deletion in pre-B cell cultures to 50% (Figure A-2-A), suggesting that HuR is important for survival of ex vivo cultured pre-B cells. However, despite the incomplete deletion of HuR in EμBcl2 HuRΔ/Δ IL-7 cultured pre-B cells, we found that D3 protein expression was increased in these cells, relative to HuR-sufficient controls (Figure A-2-B). Because our finding that D3 downregulation may be important for preventing unrepaired RAG-induced DSBs from progressing into S phase, overexpression of D3 at the pre-B cell stage in HuRΔ/Δ cells may lead to increased genome instability and potentially increased cell death. Therefore, we crossed HuRΔ/Δ mice onto a D3-/- background, generating HuRf/f D3-/- and HuRΔ/Δ D3-/- mice. Contrary to our expectations that overexpression of D3 in HuRΔ/Δ pre-B cells might be inhibiting the pro- to pre-B cell transition, HuRΔ/Δ D3-/- mice had an exacerbated loss of pre-B cells, even compared to HuRf/f D3-/- controls (Figure A-2-C). These data demonstrate that while HuR likely directly or indirectly regulates D3 expression in developing B cells, this mode of regulation is dissimilar to the proposed role of HuR in regulating D3 expression in thymocytes.

Candidate functions of HuR in genome stability and cell cycle in activated B cells
Although the work in chapter IV focuses on the immunologic aspects of the HuR-deficient GC defect, additional data suggest that HuR may also play roles in cell cycle and genome stability control in activated B cells. These findings are consistent with literature firmly linking DNA damage signaling with HuR regulation (Kim et al. 2010, Mazan-Mamczarz et al. 2011). As discussed, our in vitro studies of HuR-deficient B cell function did not reveal any serious defects in proliferation, survival, or class-switch recombination. Yet these data are in contrast with the severe in vivo GC defect of mice with HuR-deficient B cells. In an attempt to understand this GC defect mechanistically, and bearing in mind that the lack of GC B cells in HuRΔ/Δ mice precludes analysis of these cells in vivo, we performed a proteomic analysis of WT and HuR-deficient B cells stimulated in vitro under conditions mimicking a T-dependent immune response. These data (Figure A-1, Table A-1), showed many proteins that are likely misexpressed in HuRΔ/Δ cells relative to WT controls. We were not surprised to find more proteins decreased in HuRΔ/Δ cells, since HuR’s molecular role is canonically to promote expression of target genes (Mazan-Mamczarz et al. 2011, Mukherjee et al. 2011). We noted that the genes found in Table A-1 participate in diverse cellular processes, and that there are no genes with an immediately obvious link to germinal center function. These data might suggest that HuR promotes germinal center formation by controlling a factor or factors not previously known to be involved in B cell function. The diversity of the genes found to be differentially expressed in HuRΔ/Δ cells also raises the hypothesis that HuR likely has many molecular targets, and the combined direct and indirect effects of these changes subtly alters pleiotropic cellular processes whose dysregulation together results in a striking biologic phenotype. However, this hypothesis may be difficult to directly test, since it predicts many small changes in gene expression.

Although we did observe misregulation of a few proteins involved in metabolism, these
were not the majority of the proteins found, in disagreement with a mechanism proposed by another group to explain lack of GCs in HuRΔ/Δ mice (Diaz-Munoz et al. 2015).

We found that HuR-deficient cells had reduced abundance of the Ncapg2, Odf2, and Cdc27 proteins, which have established or proposed functions in chromosome segregation during mitosis (Krauss et al. 2008, Kim et al. 2014, Sivakumar and Gorbsky 2015). This is consistent with a pathway analysis performed using the online tool DAVID (Huang et al. 2007) where we submitted all proteins decreased in HuRΔ/Δ cells regardless of p value (data not shown). To our surprise, the list was enriched for genes involved in mitosis and mitotic cell cycle regulation, including microtubule organization. Cdc27 is part of the anaphase promoting complex, which is important for allowing cells to traverse through mitosis (Sivakumar and Gorbsky 2015). We also observed enrichment of a set of proteins known to be involved in DNA damage and DNA replication, including Mum1, Claspin, INO80c, and Tipin (Huen et al. 2010, Smith-Roe et al. 2013, Gerhold et al. 2015). COP7SB, a component of the Cop9 signalosome, is involved in regulation of signaling through pathways important for B cell function, including NFκB, as well as being involved in regulation of p53 activity (Schweitzer and Naumann 2010, Meir et al. 2015). While we observed only mild defects in proliferation in vitro, we speculate that cell cycle defects, including chromosome mis-segregation and DNA damage defects could plausibly manifest a phenotype exclusively during an in vivo GC response. GC B cells proliferate very rapidly (Victora and Nussenzweig 2012), and this accelerated timeline of cell division, even relative to in vitro stimulated cells, might heighten the importance of checkpoints to maintain genome stability and cell viability.

Although we posit that the primary role of HuR in the GC response is to allow B cells to effectively communicate with T cells in the follicle, it will be interesting to see whether HuR additionally promotes survival and/or genome stability in GC B cells in vivo.
Concluding remarks

The data described here describe diverse mechanisms that collectively promote lymphocyte development and function while suppressing malignant transformation. These mechanisms highlight the fact that lymphocytes, like other cell types, use a wide variety of gene expression modulators to precisely tune biologic responses. Further, our data emphasize that many of these processes occur in ways that are both lineage- and developmental stage-specific, emphasizing the need to study molecular mechanisms in vivo, in the cell types in which they are biologically relevant.
Methods

Mouse strains

All mice were on a background of mixed 129SvEv and C57BL/6, with the 129SvEv strain predominant. Studies were performed in accordance with national guidelines, and approved by the Institutional Animal Care and Use Committee of the Children’s Hospital of Philadelphia. Three tissue-specific Cre-recombinase strains were used: Vav-cre (Georgiades et al. 2002), Lck-cre (Lee et al. 2001), and Mb1-Cre (Hobeika et al. 2006). HuR<sup>flox/flox</sup> mice were provided by Dr. Timothy Hla (Ghosh et al. 2009), and Dr. Piotr Sicinski provided D3/- mice (Sicinska et al. 2003). The following other mouse strains were used: p53<sup>flox/flox</sup> (Jonkers et al. 2001), EμBcl2 (Strasser et al. 1991), VH147 IgH transgenic (Mandik-Nayak et al. 2006), and Vβ1NT (Serwold et al. 2007). For most breedings, Cre+ males were bred to Cre-negative females to minimize germline activation of Cre (Rowh et al. 2011). However, Mb1-Cre*HuR<sup>flox/flox</sup> females were bred with HuR<sup>flox/flox</sup> males to generate HuR<sup>+/−</sup> mice with one germline HuR-deleted allele.

In vitro stimulation of B cells

For IL-7 pre-B cell cultures, bone marrow was harvested from mice of indicated genotypes and cultured at a density of 5 x 10<sup>6</sup> per ml in medium containing 5ng/ml IL-7 for 4-5 days. Cells were re-plated in fresh IL-7 containing medium each day except for day 2 (48h after harvest). For mature B cell assays, splenic B cells were isolated using EasySep negative selection B cell isolation kits (Stem Cell Technologies) or follicular B cells were isolated by positive selection using biotinylated anti-mouse CD23 (B3B4, BD) in conjunction with streptavidin microbeads (Miltenyi Biotec) on an LS column (Miltenyi
Biotec). Isolated cells were labeled with CFSE (Life Technologies) as described (Lyons and Parish 1994, McCall and Hodgkin 1999). Equal numbers of cells were stimulated at a density of $1 \times 10^6$ per ml for indicated time periods with 25μg/ml LPS (0111:B4, Sigma) and 80ng/ul recombinant mouse IL-4 (R&D Systems), or 10μg/ml anti-mouse CD40 (HM40-3, Biolegend) and 10μg/ml F(ab')2 fragment goat anti-mouse IgM (Jackson Immunoresearch) with or without 50ng/ml IL-21 (Shenandoah Biotechnolgoy). Where not specified, cells were stimulated in RPMI-1640 supplemented with 10% heat-inactivated FBS, antibiotics, 50μM β-mercaptoethanol, 2mM L-glutamine, 10mM HEPES, 1mM sodium pyruvate, and non-essential amino acids.

**γ-Irradiation**

Cell suspensions were collected and irradiated in conical tubes for appropriate times to yield desired IR dose. Cells were then returned to the tissue culture incubator. For whole mouse irradiation, mice were placed in a pie restrainer (Braintree) and dosed with 9 Gy IR. Mice were returned to their holding cages for 30min to 4h before being euthanized.

**qPCR and qRT-PCR**

Genomic DNA was isolated as described (Steinel et al. 2010). Total RNA was isolated using Trizol reagent (Life technologies) and DNase treated according to manufacturer directions (Promega), primed with random nonamer (New England Biolabs), and reverse transcribed with M-MuLV (NEB). qPCR and qRT-PCR reactions were performed with
SYBR green mastermix (Applied Biosystems) and run on an Applied Biosystems 7500 Fast machine. Primers used for qPCR and qRT-PCR reactions are found in Table 3.

**RNA-Immunoprecipitation**

RNA-IPs for HuR were performed as previously described (Yoon et al. 2012). Per IP, 100 μl protein G dynabeads (Life Technologies) were incubated with 15μg anti-HuR (3A2, Santa Cruz) or 15μg normal mouse IgG (Santa Cruz). B cells stimulated for 72h with LPS + IL-4 (6-9 x 10^7 cells per IP pair) were lysed in polysome lysis buffer containing 0.5% NP-40 supplemented with protease inhibitor (Roche) and RNase inhibitor (NEB). Half of each sample lysate was added to IgG or HuR-coated dynabeads and incubated with shaking for 2h at 4°C. After washing of beads, RNA was extracted using Trizol as for total RNA and reverse transcribed.

**Click-it nascent RNA labeling**

Click-it nascent RNA labeling kit was obtained from Life Technologies. For mRNA turnover assays, ethynyl uridine (EU) was added to medium of IL-7 cultures at a final concentration of 0.2mM for the last 16h of culture time. Immediately before irradiation, EU was washed out of the medium. Cells were collected for RNA isolation immediately following EU removal, and at indicated times after IR or no IR. For transcriptional studies, cells were grown in medium without EU. Immediately following IR or no IR, EU was added to medium to a final concentration of 0.5mM. Cells were collected for RNA at indicated times. RNA was isolated using Trizol reagent as above, and click chemistry
and streptavidin pulldown were performed as per kit instructions. RNA obtained in this way was reverse transcribed as described for total RNA.

**Western blotting**

Cells were resuspended in a Tween-20 containing lysis buffer, and sonicated at intervals of 30 sec on 30 sec off for 5 min at 4°C. Cells were incubated for 5 min on ice then spun to remove insoluble material. 100μg lysate prepared under reducing conditions were loaded into each well of a NuPage 10% Bis-Tris gel (Life Technologies). Electrophoresed proteins were transferred to PVDF, and membranes were blocked with Odyssey blocking buffer (Li-Cor) and incubated with antibodies indicated in Table 4. After washing, blots were incubated with appropriate IRDye800 secondary antibodies (LiCor). Following washing, blots were scanned on an Odyssey infrared scanner (Li-Cor).

**Cyclohexamide chase assays**

Cyclohexamide chase assays were performed on IL-7 pre-B cell cultures by treating cells with 10Gy IR or mock IR, and immediately adding cyclohexamide (Sigma) to a final concentration of 100ng/ml. Cells were harvested for immunoblotting immediately after addition of cyclohexamide (t=0) and at indicated times thereafter.

**Southern blotting.**
Genomic DNA (~20 μg) from lymphomas, non-transformed lymphocytes, or kidneys was digested with 100 units of indicated restriction enzymes (New England Biolabs), separated on 0.8% TAE agarose gels, transferred onto Zeta-probe membrane (BioRad), and hybridized with $^{32}$P-labeled Tcrβ, Igh, Igκ, or c-myc DNA probes as described. (Bassing et al. 2003, Savic et al. 2009, Yin et al. 2011).

**Cytogenetics.**

Metaphase spreads were prepared as previously described (Bassing et al. 2003). Spectral karyotyping and fluorescence in situ hybridization (FISH) were performed according to manufacturers’ instructions (Applied Spectral Imaging). FISH probes were labeled with digoxigenin or biotin according to manufacturer’s instructions (Roche). The TCRCα-232F19, TCRVδ3/Vα6-46G9, Igh C H BAC199, and c-myc-454G15 FISH probes have been described previously (Rowh et al. 2011, Yin et al. 2011). Slides were examined at room temperature under a BX61 microscope (magnification: 600x) from Olympus, controlled by a LAMBDA 10-B Smart Shutter from Sutter Instrument (Novato). Images were captured using a LAMBDA LS light source from Sutter Instrument, and a COOL-1300QS camera ASI, then analyzed through Case Data Manager Version 5.5 configured by Applied Spectral Imaging.

**Flow cytometry**

Peritoneal lymphocytes were obtained by peritoneal lavage with PBS, or cells were isolated from thymus, spleen, bone marrow, or tumors as previously indicated (Miyazaki
et al. 1997, Rowh et al. 2011). For some experiments, single cell suspensions were first incubated with live/dead viability dye (Life Technologies). For all experiments, equal numbers of cells were stained with antibodies against surface antigens in PBS with 3% FBS. Following washing, cells were either analyzed directly or treated with cytofix/cytoperm buffer (BD biosciences) and then stained with antibodies against intracellular antigens. The antibodies used are listed in Table 4. Samples were run on a FACSCalibur or LSR Fortessa cytometer (BD Biosciences) and analyzed with Flowjo software (Treestar). Sorting was performed on a MoFlo Astrios (Beckman-Coulter).

AnnexinV assays were performed according to manufacturer instructions (BD Biosciences) except that annexinV antibody was used at a 1:100 dilution. BrdU incorporation assays were performed by incubating cells in medium containing 10uM BrdU for indicated amounts of time before fixing and staining as instructed (BD Biosciences).

**ELISA assays**

96-well polystyrene assay plate (Corning) medium binding were coated with goat-mouse Ig(H+L), NP4-BSA, or NP33-BSA (Biosearch Technologies). After blocking with 2% BSA, serum and unlabeled isotype standard dilutions were applied. NP standard kindly provided by Garnett H. Kelsoe. Detection of antibody isotype was achieved with appropriate goat anti-mouse conjugated antibody (see supplemental table 1). TMB substrate (OptiEIA, BD) was used to develop according to manufacturer’s instructions and 2M sulphuric acid to stop the reaction. Signal was read at 450nm on a Molecular Devices Emax.
Histology

For H&E, mouse spleens were fixed in 10% neutral buffered formalin overnight, then sectioned and stained for H&E. For immunohistochemistry, mouse spleens were fresh frozen in optimal cutting temperature compound (Sakura Finetech), sectioned, stained with biotinylated antibodies (Table 4), and developed with appropriate secondary antibodies.

Cloning and retroviral transduction

Full-length cyclin D3 cDNA including its 3'UTR (NM_007632.2) was cloned into the MIGR1 retroviral vector (Pear et al. 1998) by PCR amplification using BAC # RP-160K24 as template. EcoRI restriction sites were included on the end of each PCR primer such that PCR products and MIGR1 were digested with EcoRI and ligated. Individual clones were screened for correct orientation and sequenced to ensure sequence identity. Viral supernatants were generated by co-transfection of 293Ts with either MIGR1 or MIGR1-D3 along with helper plasmids pCGP and pHIT123. IL-7 pre-B cultures were transduced by spinfection twice, 48h and 72h, after BM harvest. Spinfections were performed as described (Pear et al. 1998), including final concentrations of 10ug/ml polybrene and 5ng/ml IL-7. MIGR1, pCGP, and pHIT123 plasmids were kindly provided by the Pear lab.

Immunization

NP-OVA is the hapten 4-hydroxy-3-nitrophenylacetyl (NP) conjugated to the ovalbumin carrier protein. NP-OVA (Biosearch Technologies) resuspended in PBS was added to a
solution of 10% aluminum potassium sulfate and precipitated by dropwise addition of potassium hydroxide. Precipitate was washed thoroughly with sterile PBS before injection of 50μg NP-OVA in alum into the peritoneal cavity of 8-week old WT or HuRΔ/Δ. Injected mice were euthanized and analyzed at 9 or 14 days post-immunization.

Statistics and general data analysis

Kaplan-Meier curves were generated in Graphpad Prism 5 (Graphpad Software Inc.) and compared using the log-rank (Mantel-Cox) test. Except where otherwise indicated, p-values were generated by two-tailed unpaired Student's t test using Prism (GraphPad Software). Error bars represent the standard error.

LC-MS/MS

WT or HuRΔ/Δ B cells were stimulated for 48h with α-CD40 and α-IgM. Greater than 75% viability was confirmed before lysing cells. LC-MS/MS workflow is modified from the procedure described in (Mertins et al. 2013). Cells were lysed in urea buffer, protein concentration was measured via micro BCA assay (Thermo), and peptides were prepared 15x10^6 stimulated B cells at the start of stimulation yielded ~0.5mg of total protein. Tryptic digests were analyzed by LC-MS/MS on a hybrid LTQ Orbitrap Elite mass spectrometer (Thermofisher Scientific) coupled with a nanoLC Ultra (Eksigent). Peptides were separated by reverse phase (RP)-HPLC at high pH on a nanocapillary column, 75 um ID x 15 cm Reprosil-pur 3um (Dr. Maisch, Germany) in a Nanoflex chip
system (Eksigent). Mobile phase A consisted of 0.1% formic acid (Thermofisher Scientific) and mobile phase B of 0.1% formic acid/80% acetonitrile. Peptides were eluted into the mass spectrometer at 300 nl/min with each RP-LC run comprising a 90 minute gradient from 10 to 25 % B in 65 min, 25-40% B in 25 min, followed by column re-equilibration. The mass spectrometer was set to repetitively scan m/z from 300 to 1800 (R = 240,000 for LTQ-Orbitrap Elite) followed by data-dependent MS/MS scans on the twenty most abundant ions, with a minimum signal of 1500, dynamic exclusion with a repeat count of 1, repeat duration of 30s, exclusion size of 500 and duration of 60s, isolation width of 2.0, normalized collision energy of 33, and waveform injection and dynamic exclusion enabled. FTMS full scan AGC target value was 1e6, while MSn AGC was 1e4, respectively. FTMS full scan maximum fill time was 500 ms, while ion trap MSn fill time was 50 ms; microscans were set at one. FT preview mode; charge state screening, and monoisotopic precursor selection were all enabled with rejection of unassigned and 1+ charge states.

Proteomics data analysis

Whole proteomes were analyzed together in MaxQuant version 1.5.1.2, using the Uniprot complete mouse reference proteome including isoforms (updated Apr 9, 2015) and common lab contaminants with a minimum peptide length of 7 amino acids and 1% false discovery rate; re-quantify and match between runs were turned off. Label-free quantification was used to identify MS counts for relative abundance. Proteins analyzed had a minimum of 9 spectra across all four runs, and were identified in all four experiments. Heatmaps were generated by one-matrix CIM with columns and rows
clustered by average linkage and Euclidean distance with quantile bins
(http://discover.nci.nih.gov/cimminer/).
<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD81 F</td>
<td>CCTGCCTTGTGATCCTGTTT</td>
</tr>
<tr>
<td>CD81 RV</td>
<td>GATTTGGGCATCATCATC</td>
</tr>
<tr>
<td>HuR del qPCR F</td>
<td>CCCAATGTCTGTGATGACCAA</td>
</tr>
<tr>
<td>HuR del qPCR RV</td>
<td>TCCAGGGGAGGATAACAGA</td>
</tr>
<tr>
<td>IgM-mem F</td>
<td>TACCCACAGCATCCTGACTG</td>
</tr>
<tr>
<td>IgM-mem RV</td>
<td>AGGCTCAGGAGGAGGAGGAG</td>
</tr>
<tr>
<td>IgM-sec F</td>
<td>GGCAGCTGCTCTACATGATG</td>
</tr>
<tr>
<td>IgM-sec RV</td>
<td>CCGTCTGCTGTCAGCATGAT</td>
</tr>
<tr>
<td>GAPDH F</td>
<td>TGTTCCCATCCCCAATGAT</td>
</tr>
<tr>
<td>GAPDH RV</td>
<td>GGTTCTCAGTGTAGCAGCAA</td>
</tr>
<tr>
<td>18S F</td>
<td>CCGGTTTCTATTTGTGTTG</td>
</tr>
<tr>
<td>18S RV</td>
<td>AGTCGGCATTGGTTATGATG</td>
</tr>
<tr>
<td>GL IgG1 F</td>
<td>TCGAAGCCTGAGGAAATG</td>
</tr>
<tr>
<td>GL IgG1 RV</td>
<td>ATAGACAGATGGGGGATG</td>
</tr>
<tr>
<td>GL IgE F</td>
<td>CTGGCCAGCCACTCATTAT</td>
</tr>
<tr>
<td>GL IgE RV</td>
<td>CAGTGGCTTTTACAGGCTT</td>
</tr>
<tr>
<td>HPRT F</td>
<td>CTGGTGAAAGGACCTCAGG</td>
</tr>
<tr>
<td>HPRT RV</td>
<td>TAAATGACTCATTATAGCAGGAAGGCA</td>
</tr>
<tr>
<td>CD70 F</td>
<td>AGGGTGGATATCCAGGGT</td>
</tr>
<tr>
<td>CD70 RV</td>
<td>CAGGTATGTCAGGCCGCTGTA</td>
</tr>
<tr>
<td>CD86 F</td>
<td>CAGTTACTGTCAGGTCCTC</td>
</tr>
<tr>
<td>CD86 RV</td>
<td>TGGGTTTCAAGTTTCTCAG</td>
</tr>
<tr>
<td>D3 F</td>
<td>AGGAGATCAGGGCAGCATG</td>
</tr>
<tr>
<td>D3 RV</td>
<td>GGTAGTTCATAGCCAGGGGAAGA</td>
</tr>
<tr>
<td>p21 F</td>
<td>GACATTCCAGGCCACAGGCAC</td>
</tr>
<tr>
<td>p21 RV</td>
<td>GTCAAGGTTTCCAGGTTTCTC</td>
</tr>
<tr>
<td>Antigen</td>
<td>Clone</td>
</tr>
<tr>
<td>---------</td>
<td>-------</td>
</tr>
<tr>
<td>CD43</td>
<td>S7</td>
</tr>
<tr>
<td>B220</td>
<td>RA3-6B2</td>
</tr>
<tr>
<td>IgM</td>
<td>II/41</td>
</tr>
<tr>
<td>Igκ</td>
<td>187.1</td>
</tr>
<tr>
<td>Igλ</td>
<td>R26-46</td>
</tr>
<tr>
<td>CD21/35</td>
<td>7G6</td>
</tr>
<tr>
<td>CD23</td>
<td>B3B4</td>
</tr>
<tr>
<td>CD81</td>
<td>EAT-2</td>
</tr>
<tr>
<td>CD24/HSA</td>
<td>M1/69</td>
</tr>
<tr>
<td>Ly-51/BP-1</td>
<td>6C3</td>
</tr>
<tr>
<td>CD19</td>
<td>1D3</td>
</tr>
<tr>
<td>IgG1</td>
<td>A85-1</td>
</tr>
<tr>
<td>IgE</td>
<td>23G3</td>
</tr>
<tr>
<td>streptavidin</td>
<td>n/a</td>
</tr>
<tr>
<td>CD70</td>
<td>FR70</td>
</tr>
<tr>
<td>CD80</td>
<td>16-10A1</td>
</tr>
<tr>
<td>CD86</td>
<td>GL-1</td>
</tr>
<tr>
<td>IcosL</td>
<td>HK5.3</td>
</tr>
<tr>
<td>OX40L</td>
<td>RM134-L</td>
</tr>
<tr>
<td>CCR7</td>
<td>4B12</td>
</tr>
<tr>
<td>CD5</td>
<td>S3-7.3</td>
</tr>
<tr>
<td>CD11b</td>
<td>M1/70</td>
</tr>
<tr>
<td>GL7</td>
<td>GL-7</td>
</tr>
<tr>
<td>CD95/Fas</td>
<td>Jo2</td>
</tr>
<tr>
<td>PD-1</td>
<td>RMP1-30</td>
</tr>
<tr>
<td>CXCR5</td>
<td>L138D7</td>
</tr>
<tr>
<td>IgD</td>
<td>11-26c.2a</td>
</tr>
<tr>
<td>Bcl6</td>
<td>GI191E</td>
</tr>
<tr>
<td>TACI</td>
<td>eBio8F10-3</td>
</tr>
<tr>
<td>TCRβ</td>
<td>H57-597</td>
</tr>
<tr>
<td>CD8</td>
<td>S3-6.7</td>
</tr>
<tr>
<td>CD4</td>
<td>H129.19</td>
</tr>
<tr>
<td>CD4</td>
<td>RM4-5</td>
</tr>
<tr>
<td>CD62L</td>
<td>MEL-14</td>
</tr>
<tr>
<td>CD138</td>
<td>281-2</td>
</tr>
<tr>
<td>CD38</td>
<td>90</td>
</tr>
</tbody>
</table>
### For ELISA assays:

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Clone</th>
<th>Vendor</th>
<th>Conjugate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ig(H+L)</td>
<td>polyclonal</td>
<td>Southern Biotech</td>
<td>unlabeled</td>
</tr>
<tr>
<td>IgM</td>
<td>11E10</td>
<td>Southern Biotech</td>
<td>unlabeled</td>
</tr>
<tr>
<td>IgG1</td>
<td>15H6</td>
<td>Southern Biotech</td>
<td>unlabeled</td>
</tr>
<tr>
<td>IgG2a</td>
<td>HOPC-1</td>
<td>Southern Biotech</td>
<td>unlabeled</td>
</tr>
<tr>
<td>IgG2b</td>
<td>A-1</td>
<td>Southern Biotech</td>
<td>unlabeled</td>
</tr>
<tr>
<td>IgG2c</td>
<td>6.3</td>
<td>Southern Biotech</td>
<td>unlabeled</td>
</tr>
<tr>
<td>IgE</td>
<td>15.3</td>
<td>Southern Biotech</td>
<td>unlabeled</td>
</tr>
<tr>
<td>IgA</td>
<td>S107</td>
<td>Southern Biotech</td>
<td>unlabeled</td>
</tr>
<tr>
<td>anti-mouse lambda</td>
<td>polyclonal</td>
<td>Southern Biotech</td>
<td>HRP</td>
</tr>
<tr>
<td>anti-mouse kappa</td>
<td>polyclonal</td>
<td>Southern Biotech</td>
<td>HRP</td>
</tr>
<tr>
<td>IgM</td>
<td>polyclonal</td>
<td>Southern Biotech</td>
<td>HRP</td>
</tr>
<tr>
<td>IgG1</td>
<td>polyclonal</td>
<td>Southern Biotech</td>
<td>HRP</td>
</tr>
<tr>
<td>IgG2a</td>
<td>polyclonal</td>
<td>Southern Biotech</td>
<td>HRP</td>
</tr>
<tr>
<td>IgG2b</td>
<td>polyclonal</td>
<td>Southern Biotech</td>
<td>HRP</td>
</tr>
<tr>
<td>IgG2c</td>
<td>polyclonal</td>
<td>Southern Biotech</td>
<td>HRP</td>
</tr>
<tr>
<td>IgE</td>
<td>polyclonal</td>
<td>Southern Biotech</td>
<td>HRP</td>
</tr>
<tr>
<td>IgA</td>
<td>polyclonal</td>
<td>Southern Biotech</td>
<td>HRP</td>
</tr>
</tbody>
</table>

### For immunoblotting

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Clone</th>
<th>Vendor</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>cyclin D3 (C-16)</td>
<td>polyclonal</td>
<td>Santa Cruz Biotechnology</td>
<td>rabbit IgG</td>
</tr>
<tr>
<td>cyclin D2 (M-20)</td>
<td>polyclonal</td>
<td>Santa Cruz Biotechnology</td>
<td>rabbit IgG</td>
</tr>
<tr>
<td>HuR</td>
<td>3A2</td>
<td>Santa Cruz Biotechnology</td>
<td>mouse IgG</td>
</tr>
<tr>
<td>β-actin</td>
<td>polyclonal</td>
<td>Sigma</td>
<td>rabbit IgG</td>
</tr>
<tr>
<td>β-actin</td>
<td>D6A8</td>
<td>Cell Signaling Technologies</td>
<td>rabbit IgG</td>
</tr>
</tbody>
</table>

### For immunohistochemistry

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Clone</th>
<th>Vendor</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peanut agglutinin (PNA)</td>
<td>n/a</td>
<td>Vector Laboratories</td>
<td>n/a</td>
</tr>
<tr>
<td>Ki67</td>
<td>SP6</td>
<td>Abcam</td>
<td>rabbit IgG</td>
</tr>
</tbody>
</table>
Figure A-1. Proteins identified by proteomic analysis of WT and HuRΔ/Δ B cells stimulated \textit{in vitro} for 48h with α-IgM and α-CD40. Total proteins identified by LC-MS/MS analysis (6132) were limited to those found in 3 of the 4 total biological replicates (2 WT and 2 HuRΔ/Δ), and to those having at least 8 total spectra observed. The resulting list of 4678 proteins was plotted in the following way: the x-axis shows the log$_2$ ratio of the average MS counts for HuRΔ/Δ versus the average MS counts for WT cells, and the y-axis shows log$_{10}$ p value from a two-tailed student’s t-test. As expected Elavl1 (HuR) has the lowest p value as well as the greatest magnitude of change in MS counts. Points within boxes represent proteins listed in Table A-1 as potentially of interest based on p value and magnitude of change.
Table A-1: Proteins putatively misexpressed in HuR-deficient B cells

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Gene Symbol</th>
<th>log2 (HuR/WT)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELAV-like protein 1</td>
<td>Elavl1</td>
<td>-5.716968616</td>
<td>0.000431904</td>
</tr>
<tr>
<td>A-kinase anchor protein 12</td>
<td>Akap12</td>
<td>-2.495914591</td>
<td>0.01229924</td>
</tr>
<tr>
<td>Ig heavy chain V regions</td>
<td>Igvh1-69....</td>
<td>-2.183526204</td>
<td>0.001307999</td>
</tr>
<tr>
<td>Condensin-2 complex subunit G2</td>
<td>Ncapg2</td>
<td>-1.97259261</td>
<td>0.043093799</td>
</tr>
<tr>
<td>Nitric oxide-associated protein 1</td>
<td>Noa1</td>
<td>-1.964088242</td>
<td>0.029696139</td>
</tr>
<tr>
<td>NADH Dehydrogenase (Ubiquinone) Flavoprotein 3</td>
<td>Ndufv3</td>
<td>-1.861598109</td>
<td>0.001613143</td>
</tr>
<tr>
<td>Retinoic acid receptor RXR-beta</td>
<td>Rxb</td>
<td>-1.726651987</td>
<td>0.047049862</td>
</tr>
<tr>
<td>Leucine-rich repeat-containing protein 20</td>
<td>Lrrc20</td>
<td>-1.683526204</td>
<td>0.021012965</td>
</tr>
<tr>
<td>E3 ubiquitin-protein ligase synoviolin</td>
<td>Syvn1</td>
<td>-1.663475569</td>
<td>0.028304025</td>
</tr>
<tr>
<td>Small Integral Membrane Protein 20</td>
<td>Smim20</td>
<td>-1.663475569</td>
<td>0.028304025</td>
</tr>
<tr>
<td>COP9 signalosome complex subunit 7b</td>
<td>Cops7b</td>
<td>-1.531523111</td>
<td>0.028257322</td>
</tr>
<tr>
<td>Outer dense fiber protein 2</td>
<td>Odf2</td>
<td>-1.509737028</td>
<td>0.043399824</td>
</tr>
<tr>
<td>Ubiquitin carboxyl-terminal hydrolase</td>
<td>Usp34</td>
<td>-1.509737028</td>
<td>0.043399824</td>
</tr>
<tr>
<td>PWPP domain-containing protein MUM1</td>
<td>Mum1</td>
<td>-1.44656061</td>
<td>0.002393989</td>
</tr>
<tr>
<td>Pyruvate Dehydrogenase Phosphatase Catalytic Subunit 2</td>
<td>Pdp2</td>
<td>-1.44656061</td>
<td>0.002393989</td>
</tr>
<tr>
<td>Stromal membrane-associated protein 1</td>
<td>Smap1</td>
<td>-1.44656061</td>
<td>0.002393989</td>
</tr>
<tr>
<td>Cell division cycle protein 27 homolog</td>
<td>Cdc27</td>
<td>-1.44656061</td>
<td>0.002393989</td>
</tr>
<tr>
<td>INO80 complex subunit C</td>
<td>Ino80c</td>
<td>-1.361598109</td>
<td>0.040165349</td>
</tr>
<tr>
<td>WD repeat-containing protein 46</td>
<td>Wdr46</td>
<td>-1.361598109</td>
<td>0.040165349</td>
</tr>
<tr>
<td>Dual specificity mitogen-activated protein kinase kinase 7</td>
<td>Map2k7</td>
<td>-1.317546477</td>
<td>0.012265199</td>
</tr>
<tr>
<td>Bromodomain PHD Finger Transcription Factor</td>
<td>Bptf</td>
<td>-1.313010365</td>
<td>0.032754676</td>
</tr>
<tr>
<td>ATP-dependent RNA helicase DDX50</td>
<td>Ddx50</td>
<td>-1.282403904</td>
<td>0.020894207</td>
</tr>
<tr>
<td>HBS1-like protein</td>
<td>Hbs1l</td>
<td>-1.276635609</td>
<td>0.002979061</td>
</tr>
<tr>
<td>Cytoplasmic protein NCK1</td>
<td>Nck1</td>
<td>-1.271843967</td>
<td>0.028198903</td>
</tr>
<tr>
<td>Probable tRNA(His) guanylyltransferase</td>
<td>Thg1l</td>
<td>-1.266609759</td>
<td>0.03827026</td>
</tr>
<tr>
<td>A-kinase anchor protein 9</td>
<td>Akap9</td>
<td>-1.266609759</td>
<td>0.03827026</td>
</tr>
<tr>
<td>Phosphofurin acidic cluster sorting protein 1</td>
<td>Pacs1</td>
<td>-1.219195683</td>
<td>0.044861567</td>
</tr>
<tr>
<td>Inositol hexakisphosphate and diphosphoinositol-</td>
<td>Ppip5k2</td>
<td>-1.180972643</td>
<td>0.006319567</td>
</tr>
<tr>
<td>Phosphate kinase 2</td>
<td>Parp2</td>
<td>-1.15407936</td>
<td>0.019127435</td>
</tr>
<tr>
<td>Serine/threonine-protein kinase 38</td>
<td>Stk38</td>
<td>-1.091013962</td>
<td>0.048577367</td>
</tr>
<tr>
<td>Kinesin-like protein KIF20B</td>
<td>Kif20b</td>
<td>-1.069116859</td>
<td>0.031600764</td>
</tr>
</tbody>
</table>
Table A-1. List of proteins potentially misexpressed in HuR-deficient B cells after in vitro stimulation. Listed proteins fell within boxes in Figure A-1, representing proteins with both a log\(_{10}\) p value below 0.05 and whose absolute value of the log\(_2\) ratio of average HuR MS counts to average WT MS counts is greater than or equal to 1.0. Black bar separates proteins that are reduced in HuR\(\Delta/\Delta\) cells (above bar), and those that are increased in HuR\(\Delta/\Delta\) cells (below bar).

A.

<table>
<thead>
<tr>
<th>Protein Description</th>
<th>Symbol</th>
<th>Log(_{10}) p value</th>
<th>Log(_2) Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Branched-chain-amino-acid aminotransferase</td>
<td>Bcat1</td>
<td>0.031600764</td>
<td>1.069116859</td>
</tr>
<tr>
<td>Nuclear envelope pore membrane protein POM 121</td>
<td>Pom121</td>
<td>0.041906583</td>
<td>1.064325218</td>
</tr>
<tr>
<td>Malcavernin</td>
<td>Ccm2</td>
<td>0.038527961</td>
<td>1.025065227</td>
</tr>
<tr>
<td>Transcription factor E2-alpha</td>
<td>Tcf3</td>
<td>0.038527961</td>
<td>1.025065227</td>
</tr>
<tr>
<td>Catechol O-methyltransferase</td>
<td>Comt</td>
<td>0.024697716</td>
<td>1.018730118</td>
</tr>
<tr>
<td>Hydroxyacylglutathione hydrolase, mitochondrial</td>
<td>Hagh</td>
<td>0.041319731</td>
<td>1.054766667</td>
</tr>
<tr>
<td>Gasdermin-D</td>
<td>Gsdmc1</td>
<td>0.000883642</td>
<td>1.062400344</td>
</tr>
<tr>
<td>Protein NipSnap homolog 1</td>
<td>Nipsnap1</td>
<td>0.019037847</td>
<td>1.134155973</td>
</tr>
<tr>
<td>Inactive hydroxysteroid dehydrogenase-like protein 1</td>
<td>Hsd1</td>
<td>0.00683526</td>
<td>1.138401891</td>
</tr>
<tr>
<td>Ubiquitin-like protein ISG15</td>
<td>Isg15</td>
<td>0.020751496</td>
<td>1.277593377</td>
</tr>
<tr>
<td>Platelet glycoprotein 4</td>
<td>Cd36</td>
<td>0.027970818</td>
<td>1.566252636</td>
</tr>
<tr>
<td>Pyridoxine-5-phosphate oxidase</td>
<td>Pnpo</td>
<td>0.015702286</td>
<td>1.616195041</td>
</tr>
<tr>
<td>Liprin-beta-2</td>
<td>Ppifibp2</td>
<td>0.032751817</td>
<td>1.70170299</td>
</tr>
<tr>
<td>Golgi to ER traffic protein 4 homolog</td>
<td>Get4</td>
<td>0.004044886</td>
<td>1.723364391</td>
</tr>
<tr>
<td>N-6 Adenine-Specific DNA Methyltransferase 1</td>
<td>N6amt1</td>
<td>0.004044886</td>
<td>1.723364391</td>
</tr>
<tr>
<td>Stromal interaction molecule 1</td>
<td>Stim1</td>
<td>0.017806498</td>
<td>1.930883141</td>
</tr>
<tr>
<td>Leukotriene-B(4) omega-hydroxylase 2</td>
<td>Cyp4f3</td>
<td>0.0101085</td>
<td>2.45754135</td>
</tr>
<tr>
<td>Interferon-activatable protein 205-B</td>
<td>Mnda</td>
<td>0.046701879</td>
<td>3.05463192</td>
</tr>
</tbody>
</table>

Figure A-2

A.

B.

Legend:

- **Hif D3/-:**
- **HuR\(\Delta/\Delta\) D3/-:**

Graphs showing cell counts in pro-B, pre-B, bone marrow (BM), and spleen.
Figure A-2. HuR-insufficient cells have increased expression of cyclin D3 but HuR-deficient D3-deficient B cells have a pro- to pre-B developmental block. (A) D3 expression, normalized to actin expression, in ex vivo IL-7 cultured pre-B cells from WT or HuRΔ/Δ mice. Representative western blot shows cells from two WT mice (lanes 1 and 2), and one HuRΔ/Δ mouse (lane 3). Note that deletion of HuR alleles in the population of IL-7 cultured pre-B cells from HuRΔ/Δ mice was only about 50% (not shown). (B) B cell development in BM and spleen of HuRf/f D3-/- control mice and Mb1Cre HuRf/f D3-/- mice. Shown are representative flow plots with percentages. The experiment was repeated three times with a total of 4 control mice and 7 Mb1Cre HuRf/f D3-/- mice.
REFERENCES


alters cell cycle progression, and perturbs mitotic spindles and anaphase." Mol Cell Biol 28(7): 2283-2294.


