***Ddx3x* regulates B-cell development and light chain recombination in mice**

Running title: *Ddx3x* regulates B-cell development

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Abstract:

Ddx3x encodes a DEAD box RNA helicase implicated in antiviral immunity and tumorigenesis. We find that hematopoietic *Ddx3x* deficiency in *Vav1-Cre* mice ($\Delta Ddx3x$) results in altered leukocyte composition of secondary lymphoid tissues, including a marked reduction in mature B cells. This paucity of peripheral B cells is associated with deficits in B-cell development in the bone marrow, including reduced frequencies of small pre-B cells. Bone marrow chimera experiments reveal a B-cell intrinsic effect of *Ddx3x* deletion. Mechanistically, $\Delta Ddx3x$ small pre-B cells exhibit lower expression of *Brwd1*, a histone reader that restricts recombination at the immunoglobulin kappa (*Igk*) locus. In fact, the B-cell deficits in $\Delta Ddx3x$ mice resemble those of *Brwd1* mutant mice, and both strains of mice exhibit defective *Igk* rearrangement in small pre-B cells. The contribution of *Ddx3x* to *Brwd1* expression and light chain rearrangement constitutes the first evidence of a role for an RNA helicase in promoting B-cell development.

Introduction

RNA helicases are key regulators of gene expression that control almost all aspects of RNA metabolism from synthesis to degradation (1). The largest group of RNA helicases, the DEAD (Asp-Glu-Ala-Asp) box family, contain a characteristic DEAD-box and a helicase-C domain (2). Recent evidence reveals crucial unexpected roles for DEAD-box helicases in antiviral immunity (3-9).

DDX3X (DEAD-Box Helicase 3, X-linked) is a ubiquitously expressed helicase implicated in innate immunity and control of virus replication (9-12). *DDX3X* has a homolog on the Y chromosome, *DDX3Y* (91% identity), which is putatively only expressed in testis (13-15). The amino acid sequence homology between human and mouse *DDX3X* is >98%, implying evolutionary conservation (16). The ATP-dependent RNA helicase functions of *DDX3X* are involved in several steps of RNA processing that ultimately promote translation (17-19). These functions likely relate to the role of *DDX3X* in cell cycle control, apoptosis, and tumorigenesis (18-21). In addition, *DDX3X* participates in innate immune nucleic acid sensing that promotes interferon-beta (IFN- β) (12, 22). Given this myriad of functional roles, it is not surprising that *DDX3X* is targeted by several viruses to evade host immune responses and promote virus replication (10, 19, 23).

DDX3X is broadly expressed in hematopoietic cells (24) and mutations in *DDX3X* are linked to various cancers (18), including leukemia and lymphoma. Yet, the function of *DDX3X* in leukocytes remains largely unexplored. Since global deletion of *Ddx3x* is embryonically lethal (25), we generated a novel mouse model characterized by *Vav1-Cre*-driven deletion of floxed *Ddx3x* alleles in hematopoietic cells. We discovered a crucial function for *Ddx3x* in hematopoiesis,

69 particularly during B-cell development. *Ddx3x*-deficiency promotes abnormalities in B-cell
70 receptor (BCR) light chain recombination that result in marked defects in B-cell development and
71 a paucity of mature peripheral B cells.

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Materials and Methods

Mice. *Ddx3x* floxed mice (*Ddx3x^{fl/fl}*) were created by Dr. Josef Penninger (Vienna, Austria). The mice were backcrossed to the C57BL/6 background for eight generations and then bred with *Vav1-Cre* mice (MGI:3765313). Experiments made use of male hemizygous deficient mice (*Ddx3x^{fl/y}Vav1-Cre⁺* or $\Delta Ddx3x$) and wild-type (WT) littermate controls (*Ddx3x^{fl/y}Vav1-Cre^{neg}* or *Ddx3x^{wt/y}Vav1-Cre[±]*). We used C57BL/6 CD45.1⁺ (BoyJ) mice from Jackson Laboratory (#002014) in bone marrow transplantation experiments. Mice were housed in a specific pathogen-free barrier facility, and experiments conducted with approval from the Cincinnati Children's Institutional Animal Care and Use Committee. Mice were used when 6 to 12 weeks old unless otherwise specified.

Tissue harvest, cell isolation, and flow cytometry. To examine bone marrow cells, mouse femura and tibiae were flushed with 1× PBS (Corning) using a 10-mL syringe and a 26-gauge 3/8-inch needle (BD Worldwide). We filtered the resulting cell suspension through a 70µm filter mesh (Corning). Mouse spleens and lymph nodes were harvested and mashed on top of the 70µm filter mesh. Red blood cells were lysed using an ACK lysing buffer (Thermo Fisher).

Cells were stained with fluorochrome-labeled antibodies in 100µL flow buffer (2% fetal bovine sera in PBS) for 30 minutes at 4°C, then fixed with 100µL fixation buffer for 30 minutes at 4°C. Data were acquired on LSR II or Fortessa cytometers and analyzed by FACSDiva (BD Biosciences) and FlowJo (TreeStar). Bone marrow small pre-B cells (CD3⁻Ly6C⁻Ter119⁻CD11b⁻Gr-1⁻B220⁺CD43⁻IgM⁻IgD⁻) and splenic IgM⁺ B cells (CD3⁻CD19⁺IgM^{hi}) were sorted using a FACS Aria II (BD Biosciences).

Bone marrow chimeras. We harvested bone marrow cells from CD45.1⁺ WT mice and CD45.2⁺

$\Delta Ddx3x$ mice and mixed these cells at 1:1, 1:2 or 1:4 ratios. We intravenously injected 2.5×10^6 cells into lethally irradiated (700 rad + 475 rad) CD45.1⁺ WT recipient mice via the lateral tail vein. Mice were examined 14 to 17 weeks after transplantation.

ELISA. Blood was collected by cardiac puncture using a 1-mL syringe and 26-gauge 3/8-inch needle (BD Worldwide) and centrifuged at 3,000 rpm for 10 minutes. Supernatant was aliquoted and stored at -80°C. Mouse IgA, IgG, IgM, IgE, IgG1, IgG2c, IgG2b or IgG3 measured using ELISA Ready-SET-Go kits according to manufacturer's protocols (eBioscience).

Microarray analysis. We isolated total cellular RNA from sorted small pre-B cells using mirVanaTM miRNA Isolation Kit (Thermo Fisher Scientific). RNA concentration and integrity were measured on an Agilent Bioanalyzer. Ovation Pico WTA kit (NuGen) was used to amplify and label RNA library. The RNA libraries were evaluated using GeneChip Mouse Gene 2.0 arrays (Affymetrix). RNA library preparation and GeneChip assays were performed by Cincinnati Children's Gene Expression Core. Raw data were used for analysis with GeneSpring GX software (v13.0, Agilent Technologies). We first applied standard RMA normalization and baseline transformation to all samples (n=4/group), then removed noise by applying a filter requiring a raw expression value >50. Unpaired t-tests were performed, with p<0.05 and fold change >1.5 were set as benchmarks for differential gene expression between experimental groups. A hierarchical clustered heatmap of the top 20 up-regulated and top 15 down-regulated genes were made using R software (64bit, v3.2.2). Genome data have been deposited in GEO (<https://www.ncbi.nlm.nih.gov/geo/>) under accession number GSE112549.

Quantitative PCR analysis. We isolated total cellular RNA from sorted small pre-B cells and splenic IgM⁺ B cells using mirVana Kits. We performed reverse transcription using PrimeScriptTM

RT reagent Kit (Clontech). For qPCR, 10μL SYBR Green master mix (SYBR Premix Ex Taq™ II, Clontech), 0.4μL Rox dye, 1.6μL of 10μM primer mix, 6μL water, and 2μL cDNA template were used in a total 20μL system per sample. Samples were run in triplicates on ABI 7500 Real-Time PCR System (Applied Biosystems). Target genes were normalized to actin or β2-Microglobulin (B2M) mRNA expression levels. Forward and reverse primers used for quantitative PCR (qPCR) are as follows: *Ddx3x*-F 5'-ACCCCTATCCCAAAGTGCAT-3', *Ddx3x*-R 5'-TCATGACTGGAATGGCTTGT-3', *Actin*-F 5'-ATGCTCCCCGGGCTGTAT-3', *Actin*-R 5'-CATAGGAGTCCTTCTGACCCATTC-3', *Brwd1*-F 5'-TTGCTTCTGGCAGTGGGATTT-3', *Brwd1*-R 5'-GCTTTCAAGCTCGGCGATTT-3', *B2m*-F 5'-AGACTGATACATACGCCTGCA-3', *B2m*-R 5'-GCAGGTTCAAATGAATCTTCA-3', *Igκ*-Germline-F 5'-GAGGGGGTTAAGCTTTTCGCCTACCCAC-3', *Igκ*-Germline-R 5'-GTTATGTCGTTTCATACTCGTCCTTGGTCAA-3', *degVκ* 5'-GGCTGCAGSTTCAGTGGCAGTGGRTCWGGRAC-3', and *κ-J1-R* 5'-AGCATGGTCTGAGCACCGAGTAAAGG-3'.

PCR analysis of *Igκ* rearrangement. We performed semi-quantitative PCR assay with reverse transcribed cDNA using an updated protocol (26). Instead of genomic DNA, we performed PCR assays from cDNA using *Vκ-FW* (AGCTTCAGTGGCAGTGGRTCWGGRAC) and *Cκ* (CTTCCACTTGACATTGATGTC) primers, which target rearranged *Vκ-Jκ* transcripts. We sorted small pre-B cells from WT and $\Delta Ddx3x$ mice. cDNA from WT splenic IgM⁺ B cells was used as a positive control. *B2m* was amplified as an internal control. PCR products were evaluated on 1% agarose gel and quantified using ImageLab software (BIO-RAD). The intensity of the band for each rearrangement product (*Vκ-Jκ1/2/4/5*) was divided by that of the corresponding *B2m* band, and the resulting value was then normalized to the values obtained from IgM⁺ B cells.

140 ***Statistical analyses.*** Experiments were analyzed using GraphPad Prism (Version 6). Data are
 141 presented as mean $\pm$ standard deviation (SD). Unless otherwise specified, significant differences
 142 were calculated using unpaired, two-tailed Student's t-test for two groups and one-way ANOVA
 143 for three groups, where p value is indicated by * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, or **** $p \leq$
 144 0.0001.

145

Results

Ablation of Ddx3x restricts peripheral B-cell compartment

To evaluate the role of *Ddx3x* in leukocyte development and function, we utilized mice in which exon 2 of *Ddx3x* was flanked by *LoxP* sites (*Ddx3x^{fl/fl}*). Expression of *Cre* recombinase facilitates deletion of exon 2 and introduction of an early stop codon in exon 3 (**Figure 1a**). We bred female *Ddx3x^{fl/fl}* mice to male *Vav1-Cre* mice (27) to facilitate deletion of *Ddx3x* in hematopoietic cells. We generated hemizygous male mice (*Ddx3x^{fl/y} Vav1-Cre⁺*, henceforth denoted $\Delta Ddx3x$), but never recovered female mice with a homozygous *Ddx3x^{fl/fl} Vav1-Cre⁺* genotype. This may reflect a role for hematopoietic cells in the embryonic lethality associated with germline *Ddx3x*-deficiency (25), and a potentially compensatory role for *Ddx3y* in embryogenesis. *Ddx3x* mRNA expression was reduced 80% in bone marrow and 60% in spleen CD45⁺ cells from hemizygous male $\Delta Ddx3x$ mice relative to Cre-negative littermate controls (**Figure 1b**).

In $\Delta Ddx3x$ mice, there was a 4-fold reduction in the proportion of circulating B cells as well as marked reductions in the numbers of CD3⁺CD19⁺ B cells in the inguinal lymph nodes (iLN) and spleen relative to WT mice (**Figure 1c**). Similar frequencies of B cells were observed in the spleen of all strains of control mice examined, including mice expressing *Cre* but no floxed alleles of *Ddx3x* (*Ddx3x^{WT/Y} Vav1-Cre⁺*) and *Cre*-negative mice with (*Ddx3x^{WT/Y} Vav1-Cre^{neg}*) or without (*Ddx3x^{fl/y} Vav1-Cre^{neg}*) a floxed allele of *Ddx3x* (**Supplemental Figure 1**). In the periphery, when immature B cells egress from bone marrow, they go through transitional stages (T1, T2, T3) before acquiring maturity. The number of CD19⁺CD93⁺ B cells in stage T1 (IgM⁺CD23⁻), T2 (IgM⁺CD23⁺), and T3 (IgM^{low}CD23⁺), were reduced in the spleen of $\Delta Ddx3x$ mice (**Figure 1d**). Mature B cells differentiate into two main subpopulations in spleen: follicular B cells (FOB,

CD19⁺CD23⁺CD21^{int}) and marginal zone B cells (MZB, CD19⁺CD21^{hi}CD23⁻). The spleens of $\Delta Ddx3x$ mice harbor 6.5-fold fewer FOB, but exhibit a concomitant 1.8-fold increase in the number of B cells with a MZ phenotype (**Figure 1e, Supplemental Figure 1**). In addition, there were 5.4-fold fewer germinal center (GC) phenotype B cells (GC-B, CD19⁺CD95⁺GL7⁺) and 4.2-fold fewer plasmablasts (CD19^{int}CD138⁺) in the spleen of $\Delta Ddx3x$ mice relative to controls animals (**Figure 1f**). Thus, peripheral B cells are markedly reduced in $\Delta Ddx3x$ mice.

Ddx3x deficiency is associated with enhanced immunoglobulin production

To assess whether reduced expression of *Ddx3x* affects B-cell activity, we measured expression of activation markers on the surface of splenic B cells and the baseline levels of different immunoglobulin classes in the sera of the $\Delta Ddx3x$ and WT mice. Splenic B cells in $\Delta Ddx3x$ mice exhibit high expression levels of the costimulatory receptors, CD80 and CD86, relative to control B cells (**Figure 2a**). Likewise, $\Delta Ddx3x$ B cells exhibit higher expression of MHC class II than their counterparts in WT mice (**Figure 2a**). These activation markers were expressed at higher levels on both FOB (CD19⁺CD1d^{low}) and MZB (CD19⁺CD1d^{hi}) cells (data not shown). Despite a reduced number of B cells and plasmablasts (**Figure 1**), sera titers of IgA, IgG, and IgM were elevated 1.8- to 4.1-fold in $\Delta Ddx3x$ mice relative to littermate controls (**Figure 2b**). In contrast, sera IgE levels were decreased almost 9-fold in mice with reduced expression of *Ddx3x* (**Figure 2b**). Among IgG subclasses, IgG1, IgG2b, and IgG3 titers were increased 1.7 to 4.2-fold while IgG2c levels remained unaltered (**Figure 2c**). These data reveal hypergammaglobulinemia in $\Delta Ddx3x$ mice despite clear deficiencies in development and composition of the B-cell compartment.

In addition to B cells, reduced numbers of T cells and natural killer (NK) cells were recovered

from the spleen of $\Delta Ddx3x$ mice (**Supplemental Figure 2a,b**). As the frequencies of thymic precursors at the double negative (DN), double positive (DP), and single positive (CD4 or CD8) stages appeared normal in the absence of $Ddx3x$ (**Supplemental Figure 2c**), the reduction of splenic T cells likely occurs in the periphery at a post-developmental stage. The profound lymphopenia observed in $\Delta Ddx3x$ mice likely contributes to homeostatic proliferation of the remaining cells. In fact, the heightened expression of CD21 and reduced levels of CD23 on B cells (**Figure 1**), as well as the increased immunoglobulin titers (**Figure 2**), are consistent with reported responses of B cell to lymphopenia (28, 29). Moreover, CD4 and CD8 T cells in the spleens of $\Delta Ddx3x$ mice also exhibit phenotypes consistent with lymphopenia-induced homeostatic proliferation (30), including elevated expression levels of activation markers CD44, CD11a, and CD69 (**Supplemental Figure 2d-f**). The effector CD8 T cells in $\Delta Ddx3x$ mice also expressed high levels of CD62L, consistent with the central memory like phenotype of CD8 T cells in lymphopenic hosts(31).

Ddx3x supports B-cell lymphopoiesis

The peripheral B cell lymphopenia in $\Delta Ddx3x$ mice portends a defect in B-cell development and maturation in the bone marrow. Notably, the frequency of B220⁺ B cells in the bone marrow was reduced 1.8-fold in $\Delta Ddx3x$ mice relative to WT counterparts (**Figure 3a**). Frequencies of common lymphoid progenitor (CLP, Flk2⁺IL7R α ⁺) cells were similar in the bone marrow of $\Delta Ddx3x$ and control mice. B-cell development in bone marrow is a sequential process of proliferation and BCR rearrangement that occurs in discrete stages that can be delineated by flow cytometry according to the Hardy classification system (32). The frequency of pre-pro-B (BP-1⁻CD24⁻), late pro-B (BP-1⁺CD24^{int}), and large pro-B (BP-1⁺CD24^{high}) cells among lineage^{neg}CD43⁺B220⁺ precursors

appeared normal in mice with targeted deletion of *Ddx3x* (**Figure 3b,c**). However, $\Delta Ddx3x$ bone marrow contained 3.4-fold fewer cells at the early pro-B cell ($\text{lin}^- \text{CD43}^+ \text{B220}^+ \text{BP-1}^{\text{low}} \text{CD24}^{\text{high}}$) stage (**Figure 3c**). Of note, the frequencies of late and large pro-B cells were normal, with similar expression levels of intracellular IgM ($\Delta Ddx3x = 47.2 \pm 1.6\%$, $\text{WT} = 47.5 \pm 1.3\%$, $p=0.90$, $n=4$, Student's t-test), suggesting B cells progressing through early pro-B cell stage properly rearranged heavy chain. Among lineage^{neg} $\text{CD43}^- \text{B220}^+$ precursors, $\Delta Ddx3x$ mice harbored 1.8-fold fewer small pre-B cells ($\text{IgM}^- \text{IgD}^-$), 2.1-fold fewer immature B cells ($\text{IgM}^+ \text{IgD}^-$), and 7.2-fold fewer circulating mature B cells ($\text{IgM}^+ \text{IgD}^+$) (**Figure 3c**). Thus, there is a progressive and lasting defect in B-cell lymphopoiesis beginning at the small pre-B cell stage that may contribute to the peripheral B-cell lymphopenia in $\Delta Ddx3x$ mice.

B cell-intrinsic role for Ddx3x

Since *Ddx3x* expression is broadly reduced in the hematopoietic lineage of $\Delta Ddx3x$ mice, the changes observed in the B-cell compartment could be due to intrinsic effects of *Ddx3x* in developing B cells. To test intrinsic role of *Ddx3x* in B cells, we mixed bone marrow cells from $\text{CD45.2}^+ \Delta Ddx3x$ mice and CD45.1^+ B6 WT mice (BoyJ), and then transplanted these cells into lethally irradiated CD45.1^+ B6 WT recipient mice. When donor bone marrow cells were mixed at a 1:1 or 1:2 ratio, very few $\text{CD45.2}^+ \Delta Ddx3x$ cells were apparent in recipient mice 14 to 16 weeks after bone marrow reconstitution (data not shown). Therefore, we generated mixed chimera mice with 4-fold more $\Delta Ddx3x$ than WT donor cells (**Figure 4a**). Although $\text{CD45.2}^+ \Delta Ddx3x$ donor cells became detectable by 16 weeks in this context, these cells were still outnumbered 2:1 and 12:1 by CD45.1^+ WT-derived cells in recipient mouse bone marrow and spleen, respectively (**Figure 4b, c**). Thus, $\Delta Ddx3x$ cells have a competitive disadvantage compared to WT cells.

Despite developing in a predominately WT environment, CD45.2⁺ $\Delta Ddx3x$ donor cells in the mixed bone marrow chimeric mice still demonstrated defects similar to cells in non-chimeric $\Delta Ddx3x$ mice (**Figure 4d, e**). Specifically, the percentage of late/small pre-B, immature B and mature B cells among CD45.2⁺ $\Delta Ddx3x$ cells in the bone marrow did not reach the normal proportions apparent among WT B cells in the CD45.1⁺ donor compartment (**Figure 4d, e**) or in non-chimeric mice (**Figure 2**). Therefore, reduced *Ddx3x* expression confers both a competitive disadvantage and an intrinsic defect in B-cell differentiation in the bone marrow. In the spleen of the chimeric mice, the percentage and absolute number of FOB derived from CD45.2⁺ $\Delta Ddx3x$ donor were 18-fold lower than that derived from the CD45.1⁺ WT donor precursors, suggesting that FOB deficit is B-cell intrinsic phenomenon (**Figure 4d, e**). However, the percentage of MZB derived from the two donors in these chimeric mice is similar (**Figure 4d, e**), suggesting that the increased frequency of MZB cells in non-chimeric $\Delta Ddx3x$ mice (**Figure 1**) is a result of B cell-extrinsic factors.

Ddx3x regulates Brwd1 and Igκ rearrangement in small pre-B cells

The loss of developing B cells apparent in $\Delta Ddx3x$ mice is likely a result of *Ddx3x* activity in one or more stages of B-cell development. The appearance of a prominent and lasting phenotype at the pre-B stage (fraction D) of B-cell development (**Figure 3**), suggests that *Ddx3x* likely plays an important role in pre-B cells. Thus, we extracted RNA from small pre-B cells (CD3⁻Ly6C⁻Ter119⁻CD11b⁻Gr-1⁻B220⁺CD43⁺IgM⁺IgD⁻) sorted out of the bone marrow of $\Delta Ddx3x$ and WT mice. We then analyzed gene expression using Affymetrix mouse gene 2.0 arrays. We found 477 differentially expressed entities/transcripts that have more than 1.5-fold expression differences between $\Delta Ddx3x$ and WT late/pre-B cells with *p*-value smaller than 0.05 using unpaired Student's

t-test. Among the 477 transcripts, 374 transcripts were expressed at higher levels in the $\Delta Ddx3x$ late/pre-B cells compared to their WT counterparts, while the remaining 103 transcripts were expressed at lower levels when *Ddx3x* expression was reduced. Within each experimental group, each individual mouse ($n = 4$) had a relatively similar gene expression pattern to other mice in the same group (**Figure 5a**).

Of note, $\Delta Ddx3x$ pre-B cells exhibit 2.77-fold lower expression of *Brwd1* relative to controls (**Figure 5a**). *Brwd1* (bromodomain and WD repeat domain containing 1) encodes a protein that targets and restricts recombination at the immunoglobulin *Kappa* (*Igκ*) locus in B cells (26). Notably, *Brwd1* mutant mice exhibited a similar B-cell phenotype to that observed in $\Delta Ddx3x$ mice, with both mouse strains exhibiting a deficiency at the late/small pre-B cell stage of B-cell development in bone marrow (**Figure 3**) linked to peripheral B-cell lymphopenia (**Figure 2**) (26). *Brwd1* mRNA expression is highest in bone marrow B-cell fractions D, E, and F relative to fractions A, B, and C (Immgen (24)). We therefore hypothesized that *Ddx3x* promotes upregulation of *Brwd1* expression at the pre-B cell stage, such that reduced expression of *Ddx3x* causes a decrease in the expression of *brwd1* and defects in B-cell light chain recombination.

To test our hypothesis, we first confirmed the expression of *Brwd1* by quantitative PCR (qPCR). We found that *Brwd1* expression was decreased by ~9-fold in late/small pre-B cells of $\Delta Ddx3x$ mice (**Figure 5b**). Before *Kappa* DNA rearrangement happens, a germline transcript is expressed as a consequence of chromatin accessibility of the *Kappa* locus (33). Of note, we found decreased germline *Igκ* expression in the $\Delta Ddx3x$ mice (**Figure 5c**). We used semi-quantitative PCR to demonstrate that rearrangement of Vκ-Jκ was also diminished 20-30% in the $\Delta Ddx3x$ late/small pre-B cells compared to WT cells (**Figure 5d,e**). In this assay, spleen-derived WT IgM⁺ B cells

278 served as positive control for *Kappa* rearrangement. In addition, qPCR specifically for V κ -J κ 1
 279 showed a similar 2.3-fold reduction in V κ -J κ 1 rearrangement in cells with reduced *Ddx3x*
 280 expression compared to controls (**Figure 5f**). These data demonstrate that B-cell light chain kappa
 281 rearrangement is deficient in the $\Delta Ddx3x$ mice. In order to facilitate light chain rearrangement,
 282 developing B cells must efficiently exit the cell cycle at the small pre-B cell stage. In conjunction
 283 with reduced light chain rearrangement, more small pre-B cells in $\Delta Ddx3x$ mice stain positive for
 284 Ki67, a marker of proliferation (**Figure 5g**). In summary, these data show a defect in *Brwdl*
 285 expression, cessation of cell proliferation, and κ chain rearrangement in late/small pre-B cells of
 286 $\Delta Ddx3x$ mice.

Discussion

We report that reduced hematopoietic expression of *Ddx3x* results in a marked peripheral lymphopenia and paucity of mature B-cells that is associated with defective B-cell development in the bone marrow. T and B cells in $\Delta Ddx3x$ mice exhibit activated phenotypes and contribute to elevated sera immunoglobulin titers that are consistent with lymphopenia driven homeostatic proliferation. Mixed bone marrow chimera experiments demonstrated that the loss of B-cells in $\Delta Ddx3x$ mice is largely attributable to cell autonomous defects in B-cell development. Gene expression analyses revealed that *Ddx3x* deficiency correlates with low expression of the histone reader *Brwd1* and restricted κ light chain recombination. This represents a crucial new cellular function for *Ddx3x* and the first demonstration of a role for an RNA helicase in B-cell development.

Among the various hematopoietic abnormalities in $\Delta Ddx3x$ mice, the peripheral B-cell lymphopenia and marked defects at various stages of B-cell development are most notable. These effects are apparent as early as development of B-cell poised progenitors in the bone marrow, and significantly impact several B cell subpopulations in peripheral lymphoid tissues. The substantial reduction in the number of mature B cells should contribute to hypogammaglobulinemia in $\Delta Ddx3x$ mice. However, $\Delta Ddx3x$ mice exhibited elevated sera titers of several immunoglobulin classes, including IgA, IgM, and IgG. Exaggerated production of these isotypes, reduced expression of CD23, and increased levels of CD21 as well as activation markers on B cells are phenotypes consistent with lymphopenia associated proliferation (28, 29). In addition to B-cell defects, $\Delta Ddx3x$ mice exhibit a marked reduction in splenic T-cell numbers despite relatively unaltered thymic composition. The remaining peripheral T cells in $\Delta Ddx3x$ mice were characterized by increased effector and central memory phenotypes that are similarly consistent

with lymphopenia-driven homeostatic proliferation (30, 31). NK cell numbers were also reduced in the spleen of $\Delta Ddx3x$ mice, while macrophage and dendritic cell numbers appeared normal. Overall, these phenotypes argue that *Ddx3x* plays an important role in development of multiple lymphocyte lineages, such that *Ddx3x*-deficiency contributes to marked lymphopenia and associated modulation of peripheral lymphocyte phenotype.

The mixed bone marrow chimera experiments revealed that defects in B-cell development and FOB frequency in $\Delta Ddx3x$ mice were attributed to B-cell intrinsic mechanisms. In contrast, the increased frequency of CD21-expressing B cells with MZB characteristics cells was dependent on extrinsic factors (e.g. lymphopenia). The B-cell intrinsic defect in the absence of *Ddx3x* was apparent as early as the early pro-B cell stage, which is associated with heavy chain recombination. Although the nature of this defect remains to be explored, two subsequent stages of pro-B-cell development appeared relatively normal in terms of cell number and intracellular expression of IgM. Thus, efficiency of heavy chain recombination may be regulated by DDX3X, but the process can still proceed successfully at low expression levels of *Ddx3x*.

A more substantial defect becomes apparent in B cell development beginning at the small pre-B cell stage and increasing in magnitude in the subsequent immature and mature B cell stages. Notably, light chain recombination occurs at the small pre-B cell stage of development. Our microarray analysis identified *Brwdl* as a potential target of *Ddx3x*-mediated dysregulation in small pre-B cells. The reduced level of *Brwdl* expression in $\Delta Ddx3x$ small pre-B cells was associated with diminished transcription of germline Igk and impaired Igk rearrangement. Defects in light chain recombination are likely to result in loss of B cells at the second checkpoint in B cell development, which may explain the reduced numbers of B cell progressing past this stage to

populate the periphery of $\Delta Ddx3x$ mice. These results largely mirror defects seen in mice lacking *Brwd1* (26, 34). We suggest that DDX3X may enhance transcription of *Brwd1* or stabilize *Brwd1* transcripts to increase levels of this crucial histone reader protein at key points in B-cell development.

Hematopoietic lineage differentiation and specification is regulated by expression of lineage specific genes, cell cycle progression, and environmental growth factors (35). Cyclin and cyclin-dependent kinase (CDK) control of cell cycle progression is pivotal to lineage differentiation (36). *Ddx3x* can influence the expression levels of cyclins, including cyclin E1 (37). In our microarray data analysis of small pre-B cells, *Ccnd2* (encodes cyclin D2) is upregulated in the absence of *Ddx3x*. Consistent with heightened expression of *Ccnd2*, pre-B cells from $\Delta Ddx3x$ mice exhibit increased staining with Ki67 that indicates defective exit from the cell cycle. Failure to properly exit the cell cycle likely contributes to reduced *Brwd1* upregulation and light chain recombination at the pre-B cell stage. Incidentally, the ephemeral defect in B-cell development at the early pro-B cell stage in $\Delta Ddx3x$ mice coincides with coordinated changes in cell cycle and expression of various cyclin and CDK genes (38). Thus, *Ddx3x* may control cell cycle in developing lymphocytes by affecting the expression of the cyclin or CDK family proteins.

We demonstrate for the first time that *Ddx3x* plays a role in B-cell development and function. The link between *Ddx3x* and *Brwd1* represents an excited new area of exploration that will further illuminate mechanisms governing B-cell development that may be impacted in immunodeficiency or autoimmune disease states.

Author contributions

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Figure legends.

Figure 1. Ablation of *Ddx3x* expression affects B cell compartment. (a) Strategy for conditional deletion of *Ddx3x* (*Ddx3x^{fl/fl}*) using targeting construct with *Loxp* sites (triangles) inserted flanking exon 2 of *Ddx3x* gene. Following removal of neomycin cassette, conditional expression of Cre causes deletion of exon 2 ($\Delta Ddx3x$) and disruption of transcription by an early stop codon in exon 3. Localization of *Ddx3x* genotyping primers are denoted as 64, 27, and 65. *Ddx3x* mRNA expression quantified by qRT-PCR (mean $\pm$ SD) in CD45⁺ leukocytes from (b) bone marrow and (c) spleen in WT (*Vav1^{Cre}-Ddx3x^{fl/y}*) and hemizygous *Vav1^{Cre}-Ddx3x^{fl/y}* ($\Delta Ddx3x$) male mice (n=4/group). (c) Flow cytometry was used to determine the proportion and number of B cells (CD19⁺CD3⁻) in the blood, inguinal lymph nodes (iLN), and spleens of $\Delta Ddx3x$ and WT mice (n=4-6/group). (d) Number of transitional B cells in the spleen determined by flow cytometric gating of CD3⁻CD19⁺CD93⁺ B cells into T1 (IgM⁺CD23⁻), T2 (IgM⁺CD23⁺), and T3 (IgM⁻CD23⁺) subsets (n=6/group). (e) Proportion and number of follicular (FOB, CD21^{int}CD23⁺) and marginal zone (MZB, CD21^{high}CD32⁻) subsets of CD3⁻CD19⁺ B cells in the spleen (n=7/group). (f) The number of germinal center B cells (GC-B, CD3⁻CD19⁺CD95⁺GL7⁺) and plasmablasts (CD3⁻CD19^{mid}CD138⁺) in the spleen is shown (n=5/group). Data were reproduced in at least two independent experiments. Significant differences were calculated using Student's t-test. * $p \leq 0.05$, *** $p \leq 0.001$, or **** $p \leq 0.0001$.

Figure 2. Hypergammaglobulinemia in $\Delta Ddx3x$ mice. (a) Splenic B cells (CD19⁺Lineage^{neg}) were analyzed (n=4/group) by flow for expression of CD80, CD86, and MHC class II. Representative histogram and mean MFI of staining ($\pm$ SEM) on gated B cells is displayed. (b,c) Baseline serum levels of IgA (n=7/group), IgG (n=7/group), IgM (n=7/group) and IgE (n=6/group), IgG1 (n=8/group), IgG2b (n=8/group), IgG2c (n=5/group), and IgG3 (n=8/group) in

WT and $\Delta Ddx3x$ mice were determined by ELISA. Immunoglobulin levels were measured in 2 independent experiments. Significant differences were calculated using Student's t-test. $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$, or $****p \leq 0.0001$.

Figure 3. *Ddx3x* supports B-cell lymphopoiesis. In bone marrow, flow cytometry was used to determine total number of (a) B cells ($B220^+$) and lymphoid progenitors (LP, $Ter119^+Mac1^+Gr1^+CD5^-CD3^-CD4^-CD8^-B220^-$), including common (CLP, $Flk2^+IL7R\alpha^+$) and B-cell poised (BLP, $Flk2^+IL-7R\alpha^+Ly6d^+$) progenitors in WT and $\Delta Ddx3x$ mice ($n=4/\text{group}$). (b) Representative flow cytometry analysis of B-cell development in bone marrow of $\Delta Ddx3x$ and WT mice using the Hardy classification system. The top panel is gated on $Lin^-CD43^+B220^+$ to delineate fractions A, B, C, and C', which correspond to pre-pro- early pro-, late pro-, and early/large pre-B cell stages of development. The lower panel is gated on $Lin^-CD43^-B220^+$ to delineate fractions D (late/small pre-B), E (immature B), and F (mature B) of B-cell development ($n=7/\text{group}$). (c) The number of cells in each subset is plotted. Results were repeated in 2 independent experiments. The histogram insets represent ($n=4/\text{group}$) intracellular IgM staining on $CD19^+sIgM^-CD43^+BP-1^+$ pro-B cells (fraction C and C'). Significant differences were calculated using Student's t-test. $**p \leq 0.01$, $***p \leq 0.001$, or $****p \leq 0.0001$.

Figure 4. Cell intrinsic contribution of *Ddx3x* to B cell biology. (a) Schematic of bone marrow transplantation experiments. Lethally irradiated $CD45.1^+$ WT mice received total of 2.5 million bone marrow cells from $CD45.1^+$ WT mice and $CD45.2^+$ $\Delta Ddx3x$ mice mixed at 1:4 ratio. (b,c) Percentage of $CD45.1^+$ and $CD45.2^+$ donor cells among bone marrow and splenic leukocytes in the chimeric mice ($n=6/\text{group}$). (d) Representative contour plots from flow cytometry analysis of $lineage^{neg}CD43^-B220^+$ bone marrow cells (left panel) and $CD19^+CD3^-$ spleen cells revealing proportions of late/small pre-B cell (fraction D, $IgM^{neg}IgD^{neg}$), immature B cells (fraction E,

IgM⁺IgD^{neg}), mature B cells (fraction F, IgM⁺IgD⁺), FOB (CD21^{int}CD23⁺), and MZB (CD21^{hi}CD23⁻) among the CD45.1⁺ or CD45.2⁺ donor cell populations in the chimeric recipient mice (n=6/group). (e) Percentage and absolute number of CD45.1-derived or CD45.2-derived late/small pre-B cells, immature B cells, and mature B cells in bone marrow, as well as splenic FOB and MZB cells spleens of chimeric mice. Data are representative of two independent experiments. Significant differences were calculated using Student's t-test. *p ≤ 0.05, ***p ≤ 0.001, or ****p ≤ 0.0001.

Figure 5. *Ddx3x* deficiency associated with reduced *Brwd1* expression and *Kappa* chain rearrangement in small pre-B cells. (a) Clustered heat map of top 15 up-regulated and down-regulated genes in late/small pre-B cell from $\Delta Ddx3x$ mice compared to WT mice (n=4/group). (b) *Brwd1* and (c) germline *Igk* mRNA expression in late/small pre-B cell from $\Delta Ddx3x$ mice compared to WT mice as determined by qPCR (n=4/group). (d,e) Semi-quantitative PCR analysis of rearranged *Igk* in bone marrow late/small pre-B cell from $\Delta Ddx3x$ and WT mice. WT splenic CD19⁺IgM⁺ cells were used as positive control (n=4/group). Quantification of V κ -J κ 1,2,4,5 band intensity is plotted. (f) Quantitative real-time PCR analysis for expression of V κ -J κ 1 in late/small pre-B cell from $\Delta Ddx3x$ and WT mice (n=4/group). (g) Bone marrow small pre-B cells (CD19⁺sIgM⁻CD43⁻) were assessed for Ki67 staining. Data in b-e from two independent experiments and are presented as mean ± SD. Significant differences were calculated using Student's t-test. *p ≤ 0.05, **p ≤ 0.01, or ***p ≤ 0.001.

a **b** **c**