The X-linked helicase DDX3X is required for lymphoid differentiation and MYC-driven lymphomagenesis

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Abstract

The X-linked gene *DDX3X* encodes an RNA helicase and is mutated at high frequencies in several types of human B cell lymphoma. More precisely, *DDX3X* mutations are found in 30% of Burkitt lymphoma tumors affecting almost exclusively male patients. Females have two active *DDX3X* alleles and males carry a *DDX3Y* homolog on the Y chromosome. Although mutations have been detected in human B cell lymphoma, the role of DDX3X in B cell physiology and malignant B cells is unknown.

The aim of this study is to characterize the impact of *Ddx3x*-depletion in murine hematopoietic cells, more particularly in murine B cells and lymphomagenesis. A conditional knockout murine model was generated to determine the impact of *Ddx3x* deletion in hematopoiesis, more precisely in B lymphopoiesis and in MYC-driven lymphomagenesis.

Results shown here demonstrate that pan-hematopoietic, homozygous *Ddx3x*-deletion in female mice perturbs erythropoiesis causing early developmental arrest. However, both hemizygous male and heterozygous female embryos develop normally, suggesting that one allele is sufficient for fetal hematopoietic development in females and that the *Ddx3y* allele can compensate for *Ddx3x* loss in males. In adult mice, loss of DDX3X affects hematopoietic progenitors, early lymphoid development, marginal zone and germinal center B cells and lymphomagenesis driven by an Eμ-*Myc* or λ-*Myc* transgene in a sex-dependent manner. Loss of both *Ddx3x* allele abrogates MYC-driven lymphomagenesis in females, while *Ddx3x*-deletion in males impairs differently the formation of B cell lymphoma in both mouse models. Moreover, tumors that appeared in male mice lacking DDX3X showed upregulated expression of DDX3Y indicating a critical requirement for DDX3 activity for lymphomagenesis.

Our data reveal sex-specific roles of DDX3X in erythro- and lymphopoiesis as well as in MYC-driven lymphomagenesis, which are important when considering inhibition of DDX3 as a treatment of B cell lymphoma.

Résumé

Le gène *DDX3X* code une ARN hélicase qui se trouve fréquemment mutée dans plusieurs types de lymphomes B chez l'humain, particulièrement dans 30% des lymphomes de Burkitt quasi exclusivement masculins. Les individus de sexe féminin ont deux allèles actifs de ce gène tandis que les individus de sexe masculin portent un homologue nommé DDX3Y sur leur chromosome Y. Malgré le nombre important de mutations détectées dans les lymphomes B humains, le rôle de DDX3X dans les lymphocytes B et les lymphomes est inconnu.

Notre but est de caractériser l'effet de l'absence de DDX3X dans le système hématopoïétique et dans la lymphomagenèse. Nous avons généré un modèle murin permettant la délétion du gène *Ddx3x*, et nous déterminons l'impact de cette délétion dans le système hématopoïétique, plus particulièrement dans les lymphocytes B; ainsi que dans la lymphomagenèse initiée par une dérégulation de MYC.

Nous démontrons ici qu'une délétion homozygote de ce gène dans les cellules hématopoïétiques de souris femelles perturbe l'érythropoïèse, causant ainsi un arrêt prématuré dans le développement embryonnaire. Cependant, les embryons hémizygotes males et hétérozygotes femelles se développent normalement, suggérant ainsi qu'un allèle du gène *Ddx3x* est suffisant pour le permettre un développement embryonnaire normal chez les femelles; et que le gène *Ddx3y* peut compenser la perte de *Ddx3x* chez les mâles. Chez les souris adultes, une déficience de DDX3X altère l'érythropoïèse, les progéniteurs hématopoïétiques ainsi que le développement des lymphocytes B et T notamment les cellules B de la zone marginale et les cellules des centres germinatifs; de façon sexe-dépendante. Chez les souris femelles, une perte des deux allèles de *Ddx3x* empêche le développement de lymphomes B murins initié par une dérégulation de MYC dans les modèles transgéniques Eμ-*Myc* et λ-*Myc*, tandis que la délétion de *Ddx3x* chez les souris

mâles impacte différemment la formation des lymphomes B. De plus, les tumeurs des souris mâles ayant une déficience de DDX3X présentent aussi une surexpression de DDX3Y, ce qui indique un rôle critique de DDX3 pour la lymphomagenèse.

Ces données révèlent un rôle sexe-spécifique de DDX3X dans l'érythro- et la lymphopoïèse ainsi que dans le développement des lymphomes B murins initiés par une dérégulation de MYC.

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Contribution to original knowledge

This thesis contains data regarding the *Ddx3x* gene encoding an RNA helicase. I demonstrate that deletion of the *Ddx3x* gene in various murine models impairs several steps of hematopoiesis: embryonic and adult erythropoiesis, hematopoietic progenitors and lymphopoiesis. I show that these impairments can be compensated by one intact *Ddx3x* allele or the male specific *Ddx3y* paralog causing a sexual dimorphism. I show that germinal center B cells, where many human B lymphoma are initiated, are particularly affected by DDX3X deficiency. I finally demonstrate that loss of *Ddx3x* prevents tumorigenesis in two murine models of MYC-driven B lymphomagenesis; but this protective effect is reversed in male mice overexpressing DDX3Y protein. I argue that DDX3X or DDX3Y may represent relevant targets to investigate in the context of B lymphoma therapies.

Experimental results are presented in the "Traditional thesis" format. The majority of these results have been published in the following manuscript:

> **Lacroix M**, Beauchemin H, Fraszczak J, Ross J, Shooshtarizadeh P, Chen R, Möröy T. The X-Linked Helicase DDX3X Is Required for Lymphoid Differentiation and MYC-Driven Lymphomagenesis. Cancer Res. 2022 Sep 2;82(17):3172-3186. doi: 10.1158/0008-5472.CAN-21-2454. PMID: 35815807.

During my doctoral studies, I was also involved in the following publications:

> **Lacroix M**, Beauchemin H, Möröy T. The RNA helicase DDX3 and its role in c-MYC driven germinal center-derived B cell lymphoma Front. Oncol. 2023, 13:1148936 doi: 10.3389/fonc.2023.1148936

Lacroix M, Beauchemin H, Möröy T. DDX3: a relevant therapeutic target for lymphoma? Expert Opin Ther Targets. 2022 Dec;26(12):1037-1040. doi: 10.1080/14728222.2022.2166830. Epub 2023 Jan 10. PMID: 36620925.

Fraszczak J, Arman KM, **Lacroix M**, Vadnais C, Gaboury L, Möröy T. Severe Inflammatory Reactions in Mice Expressing a GFI1^{P2A} Mutant Defective in Binding to the Histone Demethylase KDM1A (LSD1). J Immunol. 2021 Sep 15;207(6):1599-1615. doi: 10.4049/jimmunol.2001146. Epub 2021 Aug 18. PMID: 34408010.

Contribution of Authors

Dr. Hugues Beauchemin was involved in the conceptualization, supervision, validation, investigation, visualization, methodology and writing–original draft.

Dr. Jennifer Fraszczak was involved in formal analysis, investigation, and the methodology.

Dr. Julie Ross was involved in formal analysis, investigation, and the methodology.

Dr. Peiman Shooshtarizadeh was involved in the conceptualization, data curation, supervision, investigation, and methodology.

Dr. Riyan Chen was involved in the resources and methodology.

Dr. Tarik Möröy was involved in the conceptualization, resources, supervision, funding acquisition, validation, investigation, visualization, methodology, writing–original draft, project administration, writing–review and editing.

I, **Marion Lacroix**, was involved in the conceptualization, resources, formal analysis, validation, investigation, visualization, methodology, writing–original draft, writing–review and editing of the manuscripts.

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List of Abbreviations

ABC-DLBCL	activated B cell like DLBCL
AID	activation induced deaminase
ALL	acute lymphoblastic leukemia
ANKL	aggressive natural killer leukemia
APE1	apurinic/apyrimidinic endonuclease 1
ARF	alternate open reading frame
ATP	adenosine triphosphate
BasoE	basophilic erythroblast
BCL2	B cell lymphoma 2
BCL6	B cell lymphoma 6
BCR	B cell receptor
BIM	BCL2 interacting mediator of cell death
BL	Burkitt lymphoma
BM	bone marrow
BRWD1	bromodomain and WD repeat containing 1
BTK	Bruton's tyrosine kinase
CHOP	cyclophosphamide doxorubicin vincristine and prednisone
CLL	chronic lymphocytic leukemia
CLP	common lymphoid progenitors
CML	chronic myeloid leukemia
CMP	common myeloid progenitor
CRM1	chromosome region maintenance 1
CSR	class switch recombination
CTE	C-terminal extension
DDX3	DEAD box helicase 3
DDX3X	DEAD box helicase 3 X-linked
DDX3Y	DEAD box helicase 3 Y-linked
DHL	double-hit lymphoma
DLBCL	diffuse large B cell lymphoma
DN	double negative
DNA	deoxyribonucleic acid
DP	double positive
DSB	double strand break
DZ	dark zone
EBV	Epstein-Barr virus
eIF4	eukaryotic translation initiation factor 4
FACS	fluorescence-activated cell sorting

FDC	follicular dendritic cell
FL	follicular lymphoma
FO	follicular B cell
GC	germinal center
GCB-DLBCL	germinal center-like DLBCL
GFP	green fluorescent protein
GMP	granulocytes/monocytes progenitors
HIV	human immunodeficiency virus
HL	Hodgkin lymphoma
HSC	hematopoietic stem cell
ID	intellectual disability
IDR	intrinsically disordered region
IFN	interferon
Ig	immunoglobulin
IgH	immunoglobulin heavy chain
IgL	immunoglobulin light chain
iGB	induced germinal center B cell
IL-4	Interleukine-4
ILC	innate lymphoid cell
KO	knockout
KRAS	Kirsten rat sarcoma virus
LK	$Lin^{-}c\text{-}kit^{+}$
LMPP	lymphoid-primed multipotent progenitor
LSK	$Lin^{-}c\text{-}kit^{+}sca1^{+}$
LT-HSC	long-term hematopoietic stem cell
LZ	light zone
MAX	myc associated factor X
MBC	memory B cell
MCL1	myeloid cell leukemia 1
MDM2	mouse double minute 2
MDP	macrophage/dendritic cell progenitor
MHCII	class II histocompatibility complex
MK	megakaryocyte
MKP	megakaryocyte progenitors
MLLT10	histone lysine methyltransferase DOT1L cofactor
Mo+Mph	monocyte/macrophage
MPP	multipotent progenitor
MYC	myelocytomatosis viral oncogene homolog
MZ	marginal zone
NES	nuclear export signal

NF	newly formed
NF-kB	nuclear factor kappa B
NHEJ	nonhomologous end joining
NHL	non-Hodgkin lymphoma
NK	natural killer
NKTCL	NK T cell lymphoma
NLRP3	NACHT LRR and PYD domains-containing protein 3
NLS	nuclear localization sequence
Notch	neurogenic locus notch homolog protein
NTE	N-terminal extension
OrthoE	orthochromatic erythroblast
PB	plasmablast
PC	plasmacell
PCR	polymerase chain reaction
PI3K	phosphoinositide 3-kinase
PMN	polymorphonuclear
PolyE	polychromatophilic erythroblast
PreCFUe	early erythroid progenitor
PreMegE	pre-megakaryocyte/erythroid
ProE	proerythroblast
PUMA	p53 upregulated modulator of apoptosis
RAG1/2	recombination-activating genes 1 and 2
RBC	red blood cell
R-CHOP	rituximab and CHOP
RNA	ribonucleid acid
RSS	recombination signal sequence
Scap	SREBP cleavage-activating protein
SHM	somatic hypermutation
SP	single positive
SP1	specificity protein 1
SRC	Proto-oncogene SRC, Rous sarcoma
TAP	Tip-associated protein
TCR	T cell receptor
TdT	terminal deoxynucleotidyl transferase
Tfh	follicular helper T cell
THL	triple hit lymphoma
TNF-alpha	tumor necrosis factor
TP53	tumor suppressor p53
UNG	uracil N glycosylase
WBC	white blood cell

WHO	world health organization
WNT	wingless type family member
WT	wildtype
YY1	yin yang transcription factor 1

1. CHAPTER I: Introduction

1.1. Hematopoiesis

Hematopoiesis originates from the Greek words haima- (blood) and -poiesis (generation) and is the biological process giving rise to hematopoietic cells composing the blood. Hematopoietic cells are divided into three main groups: first, erythrocytes or red blood cells (RBC); second, leucocytes or white blood cells (WBC); and third, thrombocytes or platelets. These differentiated cells ensure several vital functions: erythrocytes transport oxygen through the body, leucocytes compose the immune system essential for protecting the body, and thrombocytes are necessary for blood coagulation and wound repair. Hematopoietic cells are the major component of the immune system defined as the system defending the body against infectious agents and foreign antigens. Immunity, the state of protection by the immune system, is a combination of innate immunity and adaptive immunity. Innate immunity represents a first, rapid response to an infectious agent or substance and is supported by cells and mechanisms in place before any stimulation. In contrast, adaptive immunity represents a slower immune response but has the advantage of being specific, more complex, and efficient. Innate and adaptive immunity are connected and coordinated to ensure an appropriate and efficient overall immunity.

WBCs, composed of lymphocytes and myelocytes, as well as RBCs and platelets represent the final stages of differentiation and result from many processes of cellular division and

differentiation that start with hematopoietic stem cells (HSC) (Pinho & Frenette, 2019) (**Figure 1-1**).

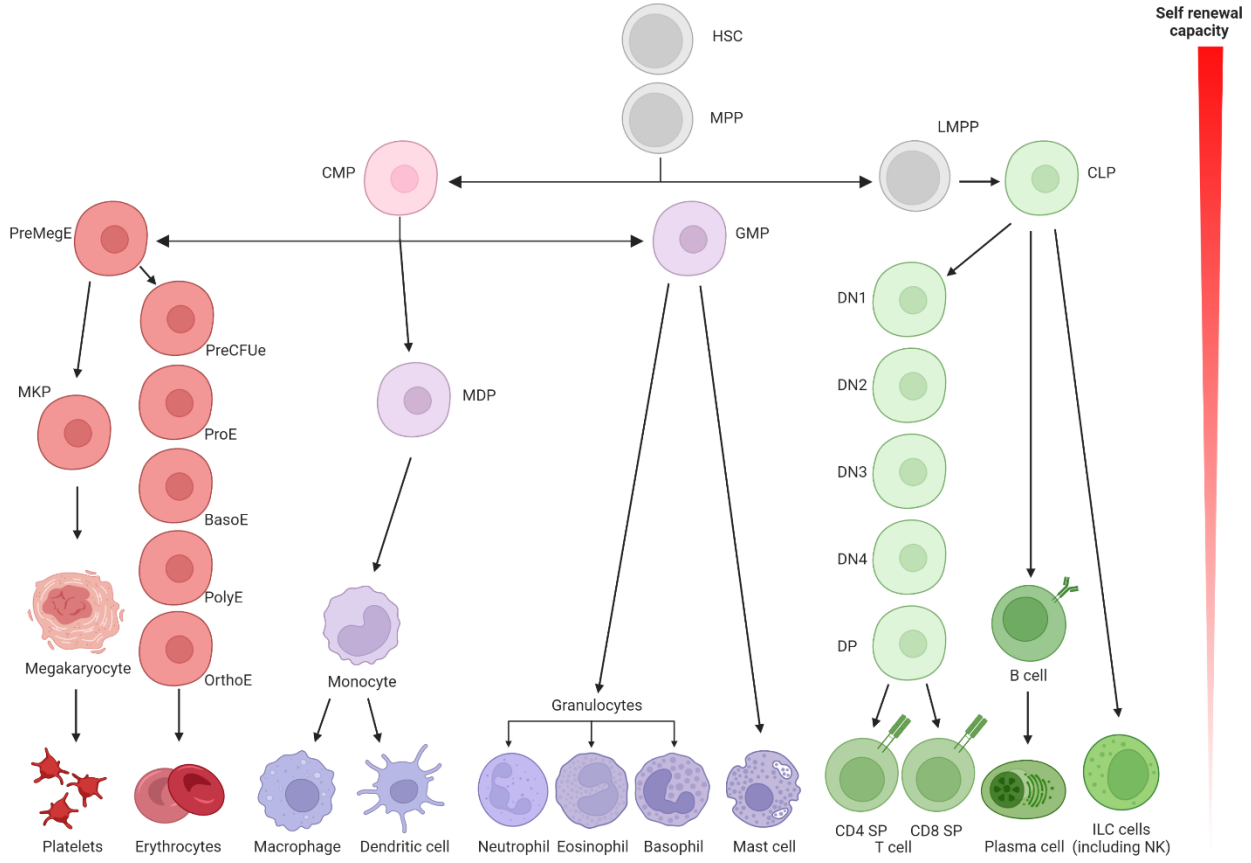


Figure 1-1: Schematic representation of hematopoiesis

Pyramidal representation of the hematopoiesis process. On top of the pyramid are hematopoietic progenitors, and at the bottom are differentiated cells. Red cells represent the erythroid lineage, purple cells the myeloid lineage and green cells represent the lymphoid lineage. Grey cells represent hematopoietic progenitors. HSC: Hematopoietic Stem Cell; MPP: Multipotent Progenitor; CMP: Common Myeloid Progenitor; LMPP: Lymphoid-primed Multipotent Progenitor; CLP: Common Myeloid Progenitor; PreMegE: pre-megakaryocyte/erythroid progenitor, Megakaryocyte/Erythroid Progenitor = PreMegE; GMP: Granulocyte/Macrophage Progenitor; MKP: Megakaryocyte Progenitor; PreCFUe: Early Erythroid Progenitor; ProE: ProErythroblast; BasoE: Basophilic Erythroblast; PolyE: Polychromatophilic Erythroblast; OrthoE: Orthochromatic Erythroblast; MDP: macrophage/dendritic cell progenitor; PMN: PolyMorphoNuclear also named granulocyte; DN: Double Negative 1, 2, 3 and 4; DP: Double Positive; SP: Single Positive; ILC: Innate Lymphoid Cell; NK: Natural Killer. Created with BioRender.com

HSCs are crucial for maintaining homeostasis and are niched in the bone marrow (BM). The BM is a semi-solid tissue localized at the center of bones constituting a particular microenvironment to maintain HSCs (Frobel et al., 2021). HSCs are a mostly quiescent cell population balancing between self-renewal or differentiation into hematopoietic progenitors

(Bryder et al., 2006). Even if they do not have a precise function in the body like differentiated cells do, they have a regenerative capacity and the potential to reconstitute an entire hematopoietic system.

1.1.1. Embryonic hematopoiesis

Hematopoiesis starts to take place early in embryonic development. Emerging from mesoderm and endoderm, the yolk sac is the first site where embryonic hematopoiesis is observed. The yolk sac generates a first wave of hematopoietic cells, mostly erythroblasts qualified as "primitive" erythroblasts, nucleated large RBCs (Palis, 2014; Yumine et al., 2017). Primitive hematopoiesis mostly generates those transient erythrocytes absent in the adult organism. Hematopoiesis is then supported by the fetal liver in mice, a process with numerous similarities compared to humans (Lewis et al., 2021; Yoder, 2002). Definitive hematopoiesis occurs mostly in the fetal liver generating adult erythrocytes, small and enucleated RBCs; as well as multilineage progenitor cells and HSCs (Moras et al., 2017; Sugiyama & Tsuji, 2006). Shortly before birth, definitive hematopoiesis occurs in the BM delivering hematopoietic cells continuously.

1.1.2. Adult hematopoiesis

Hematopoietic progenitors

HSCs differentiate into several types of progenitors that become more and more committed to a specific lineage, which is a characteristic trait named "potency" (Hofer & Rodewald, 2018). HSCs, described as pluripotent, differentiate into multipotent progenitors (MPP) capable of generating erythroid, lymphoid, and myeloid lineages. Those MPPs differentiate either into common myeloid progenitors (CMP) that can generate pre-megakaryocyte/erythroid (PreMegE) progenitors, granulocyte/macrophage progenitors (GMP); or either into lymphoid-primed

multipotent progenitors (LMPP) having the potential to differentiate into GMPs or common lymphoid progenitors (CLP) (**Figure 1-1**). All these progenitors then differentiate into unipotent cells to generate fully differentiated cells. In mouse, hematopoietic progenitors are often grouped under the terms "Lin$^-$c-kit$^+$sca1$^+$" (LSK) and "Lin$^-$c-kit$^+$" (LK) populations based on markers used to distinguish them by flow cytometry. The LSK subset contains HSC, MPP, LMPP and CLP populations and the LK subset contains LSK in addition to CMP, PreMegE and GMP populations.

Erythropoiesis and thrombopoiesis

In the BM, PreMegE progenitors differentiate into early erythroid (PreCFUe) progenitors, which differentiate into proerythroblasts (ProE), basophilic erythroblasts (BasoE), polychromatophilic erythroblasts (PolyE), and orthochromatic erythroblasts (OrthoE), the latter expelling their nuclei and organelles at the reticulocyte stage to finally terminating their differentiation into mature biconcave erythrocyte (Dzierzak & Philipsen, 2013) (**Figure 1-1**). PreMegE can also differentiate into megakaryocyte progenitors (MKP) and then megakaryocytes (MK) releasing thrombocytes into the blood flow, a process called megakaryocytopoiesis (Deutsch & Tomer, 2006, 2013). Thrombopoiesis is defined as the process of thrombocyte formation from MKs.

Myelopoiesis

Myelopoiesis is the process that generates myeloid cells from CMPs in the BM. CMPs can differentiate into GMPs giving rise to granulocytes, monocytes, and mast cells (**Figure 1-1**). Granulocytes, also called polymorphonuclear (PMN) leucocytes, are composed of neutrophils, eosinophils, and basophils (Geering et al., 2013). CMPs can also differentiate into macrophage/dendritic cell progenitors (MDP) generating monocytes, macrophages, and dendritic cells (Geissmann et al., 2010). Mast cells emerge from mast cell progenitors in the BM and

peripheral tissues (Dahlin & Hallgren, 2015; Krystel-Whittemore et al., 2015). All those differentiated myeloid cells are found in a multitude of tissues and are ready to exert their immune functions. Myelopoiesis occurs in homeostasis but can also be induced when the immune system is stimulated (Mitroulis et al., 2018; Schultze et al., 2019).

Lymphopoiesis

Lymphopoiesis is the process that generates lymphoid cells from CLPs in the BM. Several types of lymphoid cells are distinguished: T lymphocytes, B lymphocytes, and innate lymphoid cells (ILC), this last group including natural killer (NK) cells. ILCs have many similarities with T cells although there are considered part of innate immunity due to the absence of a specific antigen receptor (Lim & Di Santo, 2019). In contrast, T and B lymphocytes express a repertoire of specific antigen receptors and are part of the adaptive immune response.

T cell differentiation starts in the BM from CLPs but continues in the thymus where CLPs settle and generate early thymic progenitors that are called double negative (DN) cells since they lack the co-receptors CD4 and CD8, differentiating from DN1 to DN4 stages (**Figure 1-1**). Cells become entirely committed to the T cell lineage at the DN2 stage (Moore & Zlotnik, 1995), where they also start the process of variable diversity and joining recombination (V(D)J recombination), a process of T cell receptor (TCR) β (or δ to a much lesser extent) chain gene rearrangement (developed in later sections). This process is associated with an elevated expression of recombination-activating genes 1 and 2 (RAG1/2) and Notch1 signaling, both essential for this V(D)J recombination process (Dutta et al., 2021; Wolfer et al., 2002). DN3 cells that successfully rearrange their TCR β chain (also called β-selection checkpoint) express a surface pre-TCR, composed of the TCR β chain in addition to a pre-T cell receptor α chain. This pre-TCR functions in a ligand-independent manner sending survival and proliferation signals, which leads to the

expansion of the DN3 cells and their differentiation into DN4 cells. Another V(D)J recombination step leads to a rearrangement of the TCR α locus (or γ to a much lesser extent) and the upregulation of both CD4 and CD8 co-receptors driving the differentiation of DN4 cells into mature double positive (DP) CD4 and CD8 T cells in the thymus cortex. These DP cells undergo major histocompatibility complex (MHC) mediated positive and negative selection to finally give rise to single positive (SP) CD4 or CD8 T cells, which exit the thymus medulla to populate peripheral tissues (Irla, 2022; Rothenberg et al., 2008).

B cell lymphopoiesis including the V(D)J recombination process is described in more detail in the following section.

1.2. B lymphopoiesis

B lymphopoiesis is the part of hematopoiesis giving rise to B lymphocytes also called B cells. The main function of this specific type of leucocyte is to produce a humoral immune response – in other words to secrete antibodies - as part of the adaptive immune response. Their differentiation starts in the BM, where HSCs generate CLPs having the potential to generate T and B lymphocytes. CLPs differentiate into pre-pro B cells and then further into pro B cells, pre-B cells, immature B cells and mature B cells (**Figure 1-2**).

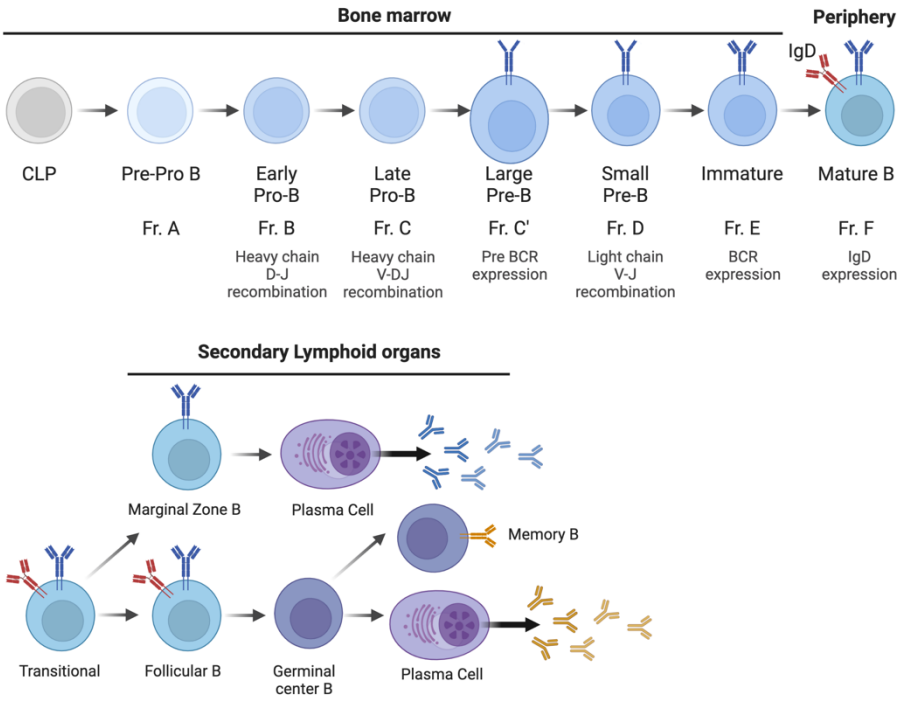


Figure 1-2: B lymphopoiesis

Process of B cell differentiation in the BM (top) and in secondary lymphoid organs (bottom). The B cell stages are also named according to the Hardy fractions. Blue antibodies represent IgM while red antibodies represent IgD. Yellow antibodies represent another isotype generated after class switch recombination. Fr.: Fraction; BCR: B cell Receptor; Ig: Immunoglobulin. Created with BioRender.com

The different stages of BM B cell maturation are also named Hardy fractions (from A to F) after Dr. Hardy who identified those populations (Hardy et al., 1982; Hardy et al., 1983). The various steps of B cell maturation allow the generation of cells that each carry a specific membrane-bound antibody, which acts as a surface antigen receptor and is therefore called B cell receptor (BCR), resulting from the recombination of the immunoglobulin (*Ig*) genes during the BM B cell lymphopoiesis. These "naïve" B cells reach the peripheral lymphoid organs where they become activated, i.e. when an antigen recognizes and binds the BCR; and differentiate into memory and plasma B cells (PC) able to secrete specific antibodies. To ensure the generation of B cells that can efficiently respond to any antigen, three primordial processes occur through B cell differentiation: V(D)J recombination to ensure the formation of a proper BCR; and class switch recombination (CSR) and somatic hypermutation (SHM) both occurring in peripheral lymphoid

organs to ensure the formation of antibodies capable of eliminating the detected antigen (Chi et al., 2020).

1.2.1. V(D)J recombination

V(D)J recombination is a process unique to B and T cell types involving random DNA recombination events to generate a large variety of antigen receptors (Clark et al., 2014; Johnson et al., 2009; Jung & Alt, 2004). In human, the B cell surface antigen receptor is built from three different genetic loci: chromosome 14 encoding the heavy chain (*Ig*H), and two other loci encoding the light κ chain (chromosome 2) or the light λ chain (chromosome 22) (Hesslein & Schatz, 2001; Tonegawa, 1983) (**Figure 1-3A**).

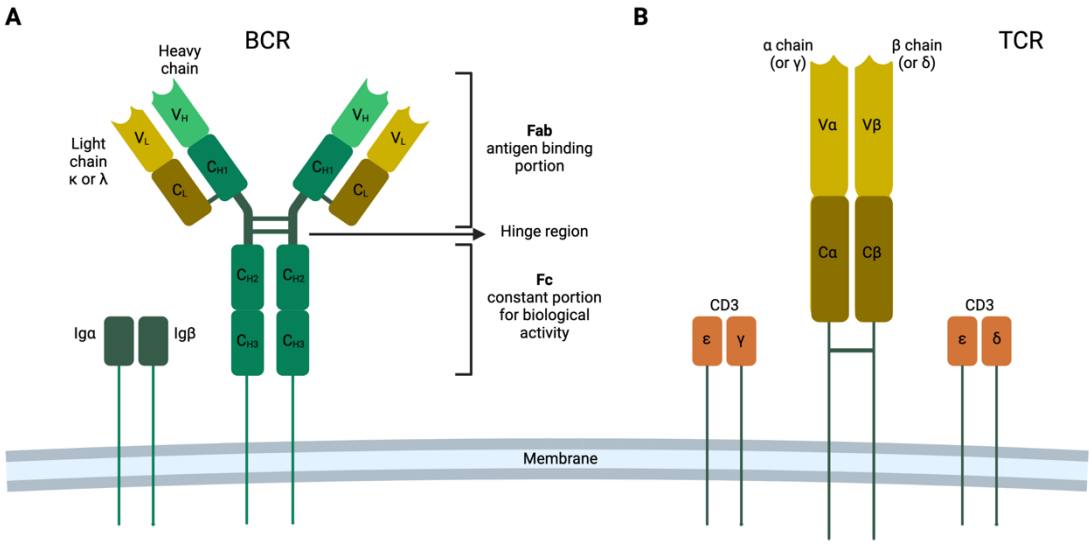


Figure 1-3: Lymphocytes antigen-binding receptors

Representation of **A,** B cell receptor (BCR) and **B,** T cell receptor (TCR). Variable regions are generated through the V(D)J recombination process to diversify the possibilities of antigen receptors. V: variable; C: constant; H: heavy chain; L: light chain; Ig: Immunoglobulin. Created with BioRender.com

Each of these loci contains many variable genes (V) followed by many joining genes (J) followed by many constant regions (C) forming the Ig germline sequences. Specific to the *Ig*H locus, there are also many diversity genes (D) in between the V and J segments causing two steps of DNA recombination: the first joining a D and a J segment, the second one joining the V to the fused DJ

segment (Alt et al., 1984). The first step of recombination is bringing the two selected gene segments closer by forming a DNA loop at recombination signal sequences (RSS) of the selected segments. Then, the lymphoid-specific RAG1/RAG2 complex recognizes the RSS where it introduces blunt DNA double strand breaks (DSB) harboring hairpins (Oettinger et al., 1990; Schatz et al., 1989; Teng & Schatz, 2015). The DNA loop is discarded while Artemis endonuclease opens the hairpins localized at the selected V and J segments, and the terminal deoxynucleotidyl transferase (TdT) enzyme adds nucleotides further increasing diversity of the V(D)J recombination process (Ma et al., 2002). Finally, the nonhomologous end joining (NHEJ) repair machinery fuses both V and J segments together generating a recombined DNA sequence. This recombined DNA sequence becomes transcriptionally active and is transcribed into an RNA that is then spliced to generate a mRNA with a unique sequence and a unique protein. This complex process allows even with a limited amount of coding DNA, to obtain a very large number of possibilities of antigen receptors (called "repertoire") to react to a variety of foreign antigens (Gauss & Lieber, 1996). The same mechanism occurs in T cells: β chain (or δ) genes are recombined at the DN2 (D-J recombination) and DN3 stage (V-DJ recombination) where cells express a pre-TCR (complex of β chain in addition to pre-T cell receptor α chain, CD3 and ζ proteins). Then, the α chain (or γ) genes are recombined before the DP stage (V-J recombination) generating together with the β chain a αβ TCR (**Figure 1-3B**). Additional proteins having a role of signal transduction at the surface of B and T cells complete the antigen binding part and form together, respectively the BCR and TCR.

B lymphopoiesis in the BM starts when the pre-pro B cells emerge from CLP progenitors (LeBien & Tedder, 2008). Pre-pro B cell is not yet B lineage committed but express the B220 marker although carrying an unrearranged *Ig*H locus (Hardy et al., 1991). Then, pre-pro B cells

differentiate into early pro-B cells recombining their D and J segments of the Ig heavy chain. During the late pro-B cell stage, the V segment is recombined to the fused DJ segment. After splicing, regions between the VDJ and the Cμ segment are removed and the mRNA containing the Cμ segment is translated into *Ig*H protein expressed at the pre-B cell surface. This *Ig*H protein in addition to the surrogate light chains and other surface proteins (Igα and Igβ) form the pre-BCR complex (T. H. Winkler & Martensson, 2018). If this pre-BCR is successfully formed, it transduces signaling for cell survival and differentiation; if not, the survival signal cannot be transduced, causing cell death (Martensson et al., 2010). In the next step, small pre-B cells start to rearrange their light chain (either κ or λ) genes by joining one V and one J gene segment thus generating a light chain protein that replaces the surrogate light chains and associates to the *Ig* heavy chain to form an entire IgM molecule at the surface of immature B cells (**Figure 1-3A**). These cells suppress *RAG* expression and receive survival signals from the BCR if successfully formed. These cells are called circulating mature B cells and express, in addition to an IgM, an IgD surface receptor through a splicing event of the recombined V(D)J DNA with Cδ sequence instead of the Cμ segment. Circulating mature B cells are released into the bloodstream and the cells carrying a functional and complete BCR terminate their maturation in secondary lymphoid organs such as the spleen, lymph nodes, Peyer patches, and tonsils.

Circulating B cells leave the BM, differentiate into transitional B cell stages and start to express the IgD (Zhou et al., 2020). Transitional B cells differentiate into mature B cells residing in various localizations where they acquire different phenotypes. In the spleen for example, they compose the follicular (FO) B cell subset residing in the follicular zone; and the marginal zone (MZ) B cell subset expressing the CD21 surface marker and residing in the marginal zone. At this

stage, mature B cells are considered “naïve”: they have a complete BCR but have never met an antigen. The following steps of B lymphopoiesis are induced by an antigenic stimulus, at the opposite to BM B lymphopoiesis and V(D)J recombination processes that are antigenic-independent. When an antigen binds to the BCR antigen receptor expressed at the surface of naïve mature B cells, these cells become activated resulting in further differentiation steps and antibody production against the antigen. The following differentiation steps depend on the nature of the antigen: some antigens induce a T cell-independent response while others induce a T cell-dependent response (Roulland et al., 2008).

B cells recognize a soluble or membrane associated antigen when it is presented by another immune cell (e.g. macrophage, dendritic cell) (Batista & Harwood, 2009). When an antigen is presented to naïve B cells, a T cell-independent response can be generated if the antigen requires a rapid but not complex answer (IgM producing response, short lived PCs). This type of response involves MZ B cells (Martin & Kearney, 2000a; Martin et al., 2001). If the detected antigen activates a T cell-dependent response, the B cell presents the antigen to $CD4^+$ follicular helper T cell (Tfh) via the histocompatibility complex class II (MHCII) at the B cell surface and the TCR interaction at the T cell surface. Then, the Tfh transmits activation signals, one of them going through the T cell CD40L binding to the CD40 receptor expressed at the B cell surface (Garside et al., 1998). This initiates a T cell-dependent response where B cells harboring low affinity antibodies induce a first rapid response producing short-lived plasmablasts (PB) (De Silva & Klein, 2015; Jacob & Kelsoe, 1992). In addition, a longer but more complex response is induced when B cells undergo the germinal center (GC) reaction to produce high affinity antibodies against the detected pathogen or antigen (T. A. Shih et al., 2002). GCs are defined as specific environments formed in secondary lymphoid organs by the Tfh signals, where several mechanisms occur to

generate high affinity antibodies to respond to the antigenic stimulus (termed GC reaction). GC structures are divided into two compartments: the dark zone (DZ) where B cells are termed centroblasts, and the light zone (LZ) where B cells are termed centrocytes (Victora et al., 2012). When B cells are activated, they undergo CSR and somatic hypermutation (SHM) to further refine the affinity of their BCR and generate an appropriate immune response against the detected antigen (**Figure 1-4**).

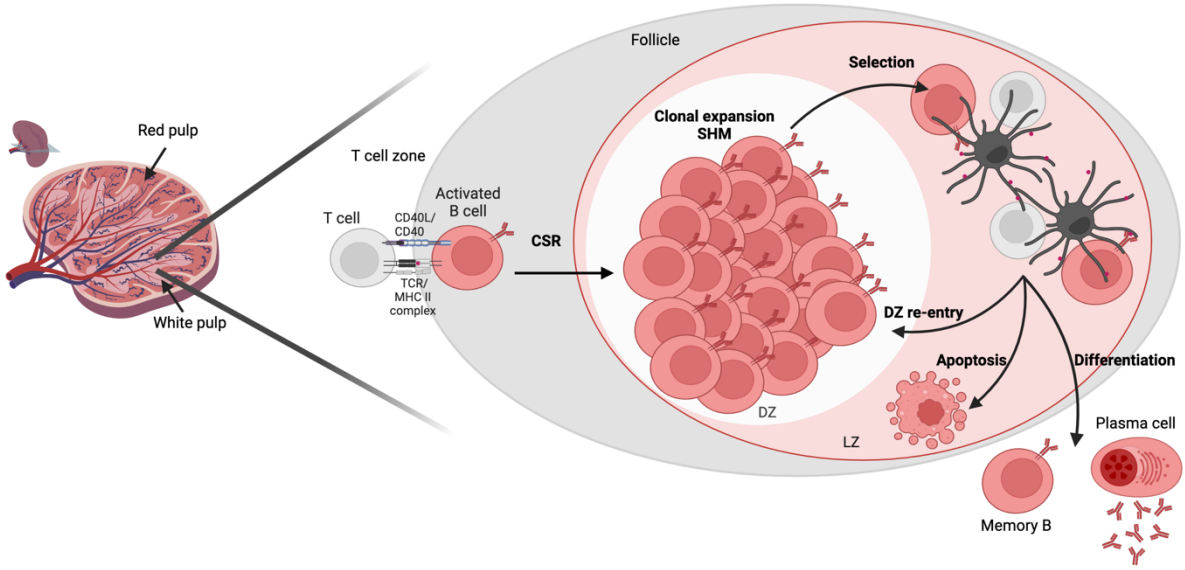


Figure 1-4: T cell-dependent B cell activation and GC reaction

T cells present an antigen to B cells that become activated and initiate the CSR process while entering the splenic follicle (or follicles of any secondary lymphoid organ). A massive clonal expansion and SHM allow the generation of a variety of clonal B cells with different antigen receptors in the DZ. These B cells are then selected in the LZ and re-enter the DZ, initiate apoptosis or undergo cell differentiation. White cells represent T cells; black cells represent follicular dendritic cells. CSR: Class Switch Recombination; SHM: Somatic Hypermutation; DZ: Dark zone (centroblasts); LZ: Light Zone (centrocytes). Created with BioRender.com

1.2.2. Class Switch recombination

When B cells interact with T cells at the extrafollicular area, it initiates the CSR process. CSR is defined as the process of DNA recombination generating a switch of the constant effector region of the antibody triggering different effector functions (Stavnezer et al., 2008) (**Figure 1-3**). In other words, CSR generates other antibody isotypes (other than IgM and IgD) which are defined

as antibodies with a similar variable region or antigenic binding region but a different constant region (i.e. IgE, IgA or IgG). CSR was commonly associated to the LZ of the GC stage (Klein & Dalla-Favera, 2008; Vinuesa et al., 2009), but it was recently demonstrated that it is initiated before the GC reaction outside the follicles at T cell/B cell border, so before the SHM process (Roco et al., 2019) confirming older reports (Jacob, Kassir, et al., 1991; Pape et al., 2003; Toellner et al., 1996). CSR starts with signals from the T cell interaction that allow the selection of an appropriate switch (S) region localized between the J and C gene segments (for example, in case of an allergic response, B cell switches their IgM to IgE; their Cμ region to Cε). Transcription at the selected switch segment (example: Sμ associated to the Cμ region and Sε associated to the Cε region) is activated generating a germline transcript and initiating an R-loop structure (RNA:DNA template duplex in addition to an open single strand DNA). These R-loop structures are critical to separate both DNA strands, a pre-requisite for the activity of activation induced cytidine deaminase (AID) enzyme, an essential enzyme for CSR (and SHM too, as discussed in the next section) (Muramatsu et al., 2000). AID, the expression of which is induced by CD40 signaling, catalyses the deamination of cytosines to uracil residues on both DNA single strands now that they are separated in the R-loop structure (Dedeoglu et al., 2004). Then, the uracil N glycosylase enzyme (UNG) removes uracil residues causing abasic sites; and the apurinic/apyrimidinic endonuclease 1 (APE1) nuclease cleaves those sites causing nicks in the DNA strands. At this stage, there are DNA breaks on both strands at both the Sμ and the selected switch region (Sε in the example), which are then ligated by the NHEJ machinery. All of this results in the same initial VDJ sequence being recombined with a Cε segment instead of a Cμ segment. Then, this sequence is transcribed and translated into an IgE surface receptor with the same variable region as the previous IgM isotype. CSR recombination occurs very early after an antigenic stimulation (the peak of germline

transcripts is detected between 1,5 to 3,5 days post antigenic stimulation) and is followed by the GC formation from 3,5 to 8 days post-stimulation in mice (Roco et al., 2019). Activated and eventually switched B cells then undergo the SHM process to further improve their surface receptor's antigen-binding region.

1.2.3. Somatic Hypermutation

Activated B cells follow their maturation and GC reaction in the DZ, where cells proliferate massively and undergo SHM (Victora et al., 2012; Victora et al., 2010). SHM is defined as the process inducing mutations in the *Ig* variable genes encoding both heavy and light chains (Berek et al., 1991; Jacob, Kelsoe, et al., 1991). Again, it begins with transcription of the VDJ locus generating a separation of both DNA strands to allow AID activity. AID catalyses the deamination of cytosines to uracil residues generating genetic alterations, particularly at the variable region of antibodies (Petersen-Mahrt et al., 2002). Then, uracil residues are either changed in thymine residues through the replication, or excised by UNG followed by repair mechanisms, or processed by the mismatch repair machinery (Di Noia & Neuberger, 2007). SHM allows increasing the number of possibilities of variable regions. SHM is thus essential to diversify the antibody repertoire and generate high affinity surface antibodies to respond to the presented antigen.

After SHM, various B cell clones are generated, all containing diverse mutations (point mutations, indels) that can induce either a decline or an improvement in the antigen affinity. The number of combinations to obtain a high affinity antibody is very high, justifying the massive proliferation undergoing in the DZ, and the majority of genetic modifications generate low affinity surface antibodies. Therefore, high affinity B cells, i.e. the most competent or fittest B cells, need to be selected (Nakagawa & Calado, 2021). This positive clonal selection occurs in the LZ of the

GC, an area rich in follicular dendritic cells (FDC) presenting to the B cells the antigen at the origin of the GC reaction (Suzuki et al., 2009; X. Wang et al., 2011). B cells expressing a specific BCR at their surface compete to interact with the FDCs presenting the antigen. B cells interacting with FDCs internalize and process the antigen to present an antigenic peptide through their MHCII to the Tfh via the MHCII-TCR interaction. This selection is highly competitive for B cells due to the limited numbers of FDC and Tfh, forcing the selection of B cells with high affinity antigen receptors (Allen et al., 2007; Schwickert et al., 2011; Victora & Nussenzweig, 2012). The majority of B cells carrying inappropriate BCRs do not receive any survival signaling and undergo apoptosis by default, whereas clones that are positively selected receive survival signaling from their BCR and their interaction with the CD40L of Tfh (Y. J. Liu et al., 1989; Victora & Nussenzweig, 2022). The synergy of BCR and CD40 signaling induces the expression of the *MYC* transcription factor in B cells with high affinity (Luo et al., 2018; Nowosad et al., 2016), a key molecular event of positive selection (Calado et al., 2012; Dominguez-Sola et al., 2012; Nakagawa et al., 2021; Victora et al., 2010).

Following this positive selection step, B cells can achieve distinct fates (Nakagawa & Calado, 2021; Victora & Nussenzweig, 2022) (**Figure 1-4**). First, a proportion of positively selected cells can re-enter into the DZ to proliferate, and be submitted to additional rounds of SHM, a process also known as cyclic re-entry (Dominguez-Sola et al., 2012; Kepler & Perelson, 1993; Long et al., 2022; Meyer-Hermann et al., 2012; Victora et al., 2010). Second, high affinity B cells differentiate into antibody-secreting cell types – i.e. PBs and PCs (Krautler et al., 2017; T. G. Phan et al., 2006). Short-lived and dividing PBs leave the GC toward the BM where they mature into long-lived and non-dividing PCs (Nutt et al., 2015). In case of a T cell-independent response, PBs/PCs also emerge from MZ B cells (Cerutti et al., 2013). Antibody-secreting cells represent

the ultimate stage of B cell differentiation ensuring a humoral immune response against the immunizing antigen by the production and secretion of high affinity antibodies. Third, the low affinity B cells that received weak help from T cells (not positively selected cells) are favored to differentiate into memory B cells (MBC) (Shinnakasu et al., 2016; Suan et al., 2017; Y. Wang et al., 2017). MBCs have the capacity to rapidly differentiate into PC in case of an antigen re-exposure.

The many steps of B lymphopoiesis, particularly the V(D)J recombination and GC reactions, are indispensable for an efficient humoral response. A tight regulation of these processes is critical and any unsteadiness in those mechanisms can have dramatic consequences such as immunodeficiencies or tumorigenesis.

1.3. B cell lymphomagenesis

It is estimated that 43% of Canadians will develop cancer in their lifetime and 1 out of 4 will die from this disease (Canadian Cancer Statistics 2021). Lymphoma is the third most common type of cancer diagnosed in children (0-14 years old) and the first one in adolescents and young adults (15-29 years) (Canadian Cancer Statistics 2021). Lymphoma is a specific type of cancer defined as an uncontrolled proliferation of mature cancerous lymphocytes originating from the lymphatic system. Lymphoma is included in the group of blood cancers also named “liquid tumors”, in addition to myeloma and leukemia. Leukemia tumors share many features with lymphoma since they are defined as an uncontrolled proliferation of mature cancerous lymphocytes or myelocytes although they originate from the BM (and thus, may also originate from progenitor cells) instead of the lymphatic system. It is estimated that every 3 minutes in the

US, one person is diagnosed with blood cancer (American Cancer Society, Cancer statistics 2023). Lymphomas are divided into two main groups: Hodgkin lymphoma (HL) and non-Hodgkin lymphoma (NHL), respectively representing 10% and 90% of all lymphomas. NHL accounts for 4% of all cancers in the US and represents the sixth most common cancer worldwide (American Cancer Society, Cancer statistics 2023).

85-90% of NHL are derived from B cells, the rest being derived from T of NK cells (Armitage et al., 2017). Overall, over 95% of all lymphomas are estimated to originate from B cells undergoing pathological changes in favor of malignant transformation and lymphoma development, a process named B cell lymphomagenesis. Consequently, many efforts have been made to understand the causes of B cell lymphomagenesis. One of the suspected causes is thought to be linked to chromosomal translocation: a rearrangement of the chromosomes induced by nonhomologous chromosomal pairing, i.e. chromosome breaks followed by joining to a different pair of chromosomes. Chromosomal translocations constitute severe genetic modifications with a considerable pathological impact. These translocations result in the modification of genetic sequences or modification in the localization of genes essential for cellular homeostasis thus affecting their regulation. Indeed, translocations can result in the inactivation of a tumor suppressor, or the activation of an oncogene through juxtaposing a proto-oncogene with an activating DNA sequence, or through the generation of a fusion protein with a new or enhanced function (Nambiar et al., 2008). Chromosomal translocations are a common event in tumorigenesis: they are reported in many tumors and are detected at a particularly high frequency in hematopoietic malignancies (Lieber, 2016; D. Liu & Lieber, 2022; Nambiar et al., 2008). Historically, one of the first translocations was discovered in chronic myeloid leukemia (CML) patients in 1972 (Rowley, 1973). This translocation involves an interchange between

chromosomes 9 and 22: t(9:22), known as Philadelphia or Ph chromosome. It was later shown that the t(9:22) causes the production of a BCR-ABL fusion protein having an increased tyrosine kinase activity driving tumorigenesis (Rowley, 2001). The development of Imatinib (Gleevec®), a tyrosine kinase inhibitor blocking the enzymatic activity of BCR-ABL fusion protein revolutionized CML treatment and is considered a landmark case in the area of targeted therapies and personalized medicine (Druker et al., 2001; N. Iqbal & Iqbal, 2014). Other well-known translocations involve the juxtaposition of the *MYC* proto-oncogene (a physiological form of a gene having the potential to become an oncogene when accidentally activated) with the *Ig* regulatory sequences resulting in MYC overexpression (developed in later sections) (Rowley, 2001).

Lymphomas originating from B cells often involve translocations of the *Ig* genes, recombined through various steps of B cell differentiation. Moreover, chromosomal translocations involve DSBs and DNA repair, two physiological mechanisms required for V(D)J recombination, CSR and SHM. Consequently, since B cells naturally encounter DSBs and DNA repair during the formation, diversification and expression of antigen receptors, chromosomal translocations found in lymphomas are associated with aberrations occurring during the V(D)J recombination, CSR or SHM processes (Jankovic et al., 2007; Kuppers, 2005; Kuppers & Dalla-Favera, 2001; Nussenzweig & Nussenzweig, 2010). Indeed, since DSBs and DNA repair are a requirement for antigen receptor formation and diversification, B cells have several mechanisms to shut down apoptotic pathways during those specific steps, making them escape cell death even in cases of mistakes in DSBs and DNA repair. Consequently, B cells are a cell type particularly sensitive to chromosomal translocations which can be caused by mistakes in those specific processes. The main actors of the V(D)J recombination, CSR and SHM, like RAG1/2, NHEJ or AID can thus

generate pathological genetic modifications. It is indeed thought that most of the translocations found in lymphomas result from the incorrect junction of an *Ig* locus to a proto-oncogene DNA sequence previously cut or modified by RAG1/2 and/or AID (Lieber, 2016; D. Liu & Lieber, 2022).

Errors linked to V(D)J defects can induce chromosomal translocation and cancers in both T and B lymphocytes (Christie et al., 2022; Lieber et al., 2006; Marculescu et al., 2006; Onozawa & Aplan, 2012). The RAG1/2 recombinase, the complex causing DSBs at recombination signal sequences (RSS) for antigen receptor formation at the immature B cell stage, can cause translocations through its transposase activity or by targeting RSSs localized in sites other than the *Ig* loci (RAG1/2 off-target effect); in proto-oncogenes for example (Christie et al., 2022; Marculescu et al., 2006; Nussenzweig & Nussenzweig, 2010). Defects in the NHEJ pathway, in addition to the inactivation of the apoptotic signaling can also generate translocations, although in a RAG1/2 dependent way (Difilippantonio et al., 2002; Zhu et al., 2002).

AID, through its cytidine deaminase activity (causing the replacement of cytosine into uracil nucleotides) provokes DNA mutations and allows the APE1 enzyme to introduce DNA breaks: mistakes in both mechanisms can induce lymphomagenesis. AID can indeed mutate non-*Ig* genes causing point mutations in oncogenes, a process known as aberrant SHM (Nussenzweig & Nussenzweig, 2010; Pasqualucci et al., 2001). This type of mutation in oncogenes already has the potential to generate severe damage, but the uracil nucleotides introduced by AID can also be processed by the UNG enzyme opening the DNA and potentially provoking translocations (Ramiro et al., 2006; Ramiro et al., 2004). A combination of AID deregulation and apoptosis impairment, like a p53 deficiency also causes translocations and eventually a lymphoma (Robbiani

et al., 2009). Finally, the actions of RAG1/2 and AID can also be combined to generate genetic translocations (Nussenzweig & Nussenzweig, 2010).

Since AID, which has the potential to cause translocation and lymphomagenesis, is expressed at its highest level in GC B cells (Muramatsu et al., 1999), the GC reaction is a particularly sensible step where pathological genetic rearrangements can be accidentally acquired (Pasqualucci et al., 2008). Moreover, many GC features correlate with the hallmark of malignant transformation (Basso, 2021; Mlynarczyk et al., 2019). Therefore, the majority of B lymphomas originate from the GC and are even named GC-derived B cell lymphoma (Kuppers et al., 1999; Stevenson et al., 1998). (Note: the cell of origin can be even more precise than "GC" thanks to recent single-cell analysis (Holmes et al., 2020)). More precisely, all B cell NHLs (95% of NHLs), except the rare lymphoblastic and mantle-cell lymphomas, are derived from GC or post-GC cells and contain chromosomal translocations and mutations resulting from aberrant CSR or SHM (Klein & Dalla-Favera, 2008; Kuppers & Dalla-Favera, 2001). In B-NHL, translocations involving two non-*Ig* genes are rare but still exist; and are also caused by AID producing DSBs in genes other than *Ig* (off-target effect) (D. Liu & Lieber, 2022; Robbiani et al., 2009; Tsai et al., 2008). On the opposite, the majority of these lymphomas harbor translocations juxtaposing the *Ig* genes with proto-oncogenes (Basso & Dalla-Favera, 2015).

GC-derived B cell lymphomas represent the majority of NHL and are subdivided into four main subtypes: Burkitt lymphoma (BL), diffuse large B cell lymphoma (DLBCL), follicular lymphoma (FL), and other rarer tumors (Alaggio et al., 2022; Loeffler-Wirth et al., 2022). Each subtype is characterized by specific genetic abnormalities (Basso & Dalla-Favera, 2015).

1.3.1. Burkitt Lymphoma (BL)

BL is a malignancy discovered in 1958 by Denis Burkitt giving his name to this disease (Burkitt, 1958). In the history of biology, BL investigations are important since they led to major discoveries in oncology (discovery of the *MYC* oncogene and chromosomal translocation), epidemiology (due to BL association with viruses like EBV or HIV), clinical oncology (one of the first cancer that became treatable and curable in some cases by chemotherapy, particularly in children) and immunology (Molyneux et al., 2012). BL tumors are conventionally classified into three subgroups: endemic, sporadic, and immunodeficiency associated. Endemic BLs are associated with EBV infection (>90% of cases) and malaria and account for 50% of childhood cancer in Africa (Magrath, 2012; Mawson & Majumdar, 2017), while sporadic BLs occur in worldwide populations (North America, Europe, Asia); in other words, in geographical areas not specifically associated with EBV or malaria. The immunodeficiency associated BL is observed in HIV infected patients. The latest 2022 WHO classification recommends now using two subtypes based on EBV status (positive or negative-BL) (Alaggio et al., 2022). All subtypes combined, BL mostly affects children, adolescents, and young adults: it is estimated that half of BL patients are younger than 40 years (Dozzo et al., 2017; Kalisz et al., 2019). A translocation of the *MYC* oncogene with an *Ig* locus deregulates its expression and is a common feature of >95% of BL tumors, whatever their subtype (Swerdlow et al., 2016).

BL is an aggressive tumor and one of the fastest-growing malignancies with cell doubling occurring within 24h to 48h. The main treatment for BL is a combination of chemotherapies: cyclophosphamide, doxorubicin, vincristine, and prednisone known as the CHOP regimen, particularly used in pediatric patients (Graham & Lynch, 2023). Other chemotherapeutic agents like etoposide, methotrexate, ifosfamide, cytarabine, or alkylators can also be used in combination,

particularly in advanced cases and in adult patients (Dunleavy et al., 2013; Jacobson & LaCasce, 2014; Rizzieri et al., 2014; D. A. Thomas et al., 1999; D. A. Thomas et al., 2006). At the beginning of the 2000's the rituximab monoclonal antibody targeting the CD20 B cell surface marker was approved and is since used in addition to chemotherapies (R-CHOP). Compared to chemotherapies alone, it improves the survival for both pediatric (Minard-Colin et al., 2020) and adult patients (Ribrag et al., 2016). The overall survival is relatively high, around 85-90% survival for young patients but declines in older adults and the elderly (Dozzo et al., 2017). However, BL treatment causes significant toxicity and life-threatening complications (Casulo & Friedberg, 2018; Choi et al., 2009). Moreover, refractory and relapsing patients have a poor prognosis and very few treatment options.

1.3.2. Diffuse Large B cell Lymphoma (DLBCL)

DLBCL is the most common type of NHL, accounting for 40% of all lymphoma cases worldwide. It is traditionally divided into three main subtypes: activated B cell-like (ABC-DLBCL), GC-like (GCB-DLBCL) and unclassified DLBCL (Alizadeh et al., 2000; Swerdlow et al., 2016). GCB-DLBCL originates from GC LZ cells while ABC-DLBCL originates from later stages of B cell maturation and is clinically more aggressive with an unfavorable outcome (Basso & Dalla-Favera, 2015). However, more recent studies have proposed more complex classifications based on genetic information highlighting some proximity between BL and DLCBL tumors (N. Thomas et al., 2023). Patient profiles are highly heterogenous although the median diagnosis age (66 years) is higher compared to BL. Moreover, older patients have a worse prognosis and are less likely to support chemotherapy regimens.

DLBCL treatments are very similar to BL treatments (and other types of NHL): they are composed of rituximab in addition to various chemotherapies (Coiffier et al., 2002; Pfreundschuh

et al., 2006; Sehn et al., 2005; Tilly et al., 2003). Similar to BL, refractory and relapsing patients have a poor prognosis and very few treatment options and it is estimated that up to 40% of patients relapse (Sarkozy & Sehn, 2018). A particular subset of patients carrying *MYC* and B cell lymphoma 2 (*BCL2)* and/or B cell lymphoma 6 (*BCL6)* rearrangement is poorly responsive to chemotherapies (Landsburg et al., 2016; Petrich et al., 2014). Few other treatments have shown encouraging results such as Ibrutinib, inhibiting the Bruton's tyrosine kinase (BTK) involved in BCR signaling; however, this option is most efficient in non-GCB subtype tumors and younger patients (Wilson et al., 2021; Wilson et al., 2015). A combination of R-CHOP with bortezomib, a proteasome inhibitor, has recently been shown to increase the progression-free survival of DLBCL patients (Davies et al., 2019). Glofitamab, a monoclonal bi-specific antibody binding to B and T cells through CD20 and CD3 receptors (Dickinson et al., 2022; Minson & Dickinson, 2021), or CAR-T cell therapies are now offering additional options even though there are still patients not sensitive to these treatments (Flowers & Odejide, 2022). Overall, DLBCL is still a life-threatening disease requiring the development of alternative therapies.

1.3.3. Follicular Lymphoma (FL)

FL is an indolent but incurable B lymphoma affecting the elderly (median age at diagnosis: 65 years). FL is driven by the t(14:18) translocation provoking *Bcl2* overexpression in 85-90% of cases (Nann et al., 2020). However, in 30-40% of cases, these tumors can transform into more aggressive lymphomas, very often into DLBCL (Fischer et al., 2018; Lossos & Levy, 2003). These transformations occur through the acquisition of additional genetic rearrangements, one of the most frequent being a *MYC* translocation causing an aggressive double-hit lymphoma (DHL) (Fischer et al., 2018; Kridel et al., 2015; Pasqualucci et al., 2014).

1.3.4. Oncogenes involved in GC-derived B cell lymphomas

The majority of GC-derived B cell lymphomas harbor translocations juxtaposing the *Ig* genes with proto-oncogenes (Basso & Dalla-Favera, 2015). Several proto-oncogenes are accidentally hyperactivated through these translocations and/or other types of dysregulation: among them, *BCL2*, *BCL6* and *MYC* (Willis & Dyer, 2000). These proto-oncogenes have relevant physiological roles in normal GC B cells, where CSR and SHM take place and their dysregulation represents major steps in the development of GC-derived malignancies.

The *BCL2* oncogene encodes an anti-apoptotic factor, differentially expressed through B cell maturation to either promote apoptosis when absent or inhibit apoptosis when expressed to tolerate DNA breaks during the GC reactions (Slomp & Peperzak, 2018). Therefore, the t(14:18) translocation, which is a juxtaposition of the *Ig*H enhancer elements with *BCL2* over activates this anti-apoptotic factor contributing to cell death resistance favoring B cell transformation (Sungalee et al., 2014; Tsujimoto et al., 1984; Vaux et al., 1988). The t(14:18) translocation is the hallmark of FL and is present in 85-90% of cases (Nann et al., 2020); but is also detected in 20-45% of GCB-DLBCL subtype (J. Iqbal et al., 2004; Lenz et al., 2008; Rosenwald et al., 2002; Vega & Medeiros, 2003), sometimes in addition to *BCL2* mutations (Schuetz et al., 2012). BCL2 is also found overexpressed in ABC-DLBCL through other mechanisms than translocations: either through the gain or amplification of the 18q chromosome (Lenz et al., 2008), or through the overactivation of the NF-κB pathway positively regulating BCL2 expression (Davis et al., 2001); or through somatic mutations in the *BCL2* promoter promoting its expression (M. Saito et al., 2009).

The BCL6 protein is a hallmark of GC B cells (Basso & Dalla-Favera, 2012, 2015; Dent et al., 1997; Ye et al., 1997). This transcription factor prevents the expression of the anti-apoptotic

BCL2 gene in the DZ (M. Saito et al., 2009) and the proliferation factor *c-MYC* driving B cell proliferation (Basso et al., 2010; Ci et al., 2009; Dominguez-Sola et al., 2012). *BCL6* also allows tolerance to DSBs in GC B cells, thus permitting the physiological SHM process to take place by transcriptionally repressing essential regulators of apoptosis, among them *TP53* (R. T. Phan & Dalla-Favera, 2004; Ranuncolo et al., 2007; Ranuncolo et al., 2008). Dysregulation of BCL6 expression induces lymphomagenesis (Basso & Dalla-Favera, 2012; Cattoretti et al., 2005). The t(3:14) translocation positively regulates BCL6 expression due to a juxtaposition of the *BCL6* gene with the regulatory sequences of the *Ig*H locus (Ye et al., 1995). This translocation is detected in 10-40% of DLBCL tumors (more frequent in the ABC subtype) and 15% of FL, and *BCL6* mutations are detected in all GC-derived B cell lymphoma subtypes (Aukema et al., 2011; J. Iqbal et al., 2007; Wlodarska et al., 2003). Other mechanisms can deregulate BCL6 expression, notably through impairment of *BCL6* positive regulators or epigenetic modifications (Basso & Dalla-Favera, 2015).

Another oncogene involved in GC-derived B cell lymphoma is *MYC* often associated with an aggressive phenotype (Ott et al., 2013). *MYC*'s role in GC physiology and malignant transformation is described more precisely in the next section.

1.4. The transcription factor *c-MYC*

MYC (used to describe c-MYC in this thesis) is a member of the MYC transcription factor family and is involved in a variety of cellular processes like proliferation, differentiation, apoptosis and metabolism (Carroll et al., 2018; Dang et al., 1999; Hoffman & Liebermann, 2008; Stine et al., 2015). It binds to E-box DNA sequences via its helix-loop-helix domain and heterodimerizes with MAX modulating the transcription of target genes (Dang, 2012; N. Meyer & Penn, 2008).

MYC is particularly important and involved in B cell differentiation and their malignant transformation (Ahmadi et al., 2021; de Barrios et al., 2020; Delgado & Leon, 2010; Klapproth & Wirth, 2010; Nguyen et al., 2017).

1.4.1. Role in physiological B cell differentiation

MYC allows pro-B to pre-B cell differentiation in the BM (Habib et al., 2007; Huang et al., 2008) and is repressed by BCL6 in quiescent pre-B cells (Nahar et al., 2011), but its expression is activated when the pre-B cell BCR is stimulated (Klemsz et al., 1989; Larsson et al., 1991).

In secondary lymphoid organs, MYC is essential for the maintenance of GCs (Calado et al., 2012; Dominguez-Sola et al., 2012) where expression is induced by both BCR and CD40 signaling repressing BCL6 expression at the same time (Luo et al., 2018). MYC is thus required to initiate the GC reaction and the first rounds of cell division (Calado et al., 2012; Dominguez-Sola et al., 2012) but is quickly repressed by BCL6 in the dividing DZ B cells (Calado et al., 2012; Dominguez-Sola et al., 2012; Klein et al., 2003; Victora et al., 2010). However, MYC expression is again induced in the LZ: the site where B cells are selected based on their antibody affinity (Calado et al., 2012; Dominguez-Sola et al., 2012). When B cells with high antibody affinity are selected, they exit the light zone, express BLIMP-1 suppressing *MYC* expression and differentiate into PB (Y. Lin et al., 1997) or in MBC by unknown mechanisms. However, when the BCR affinity is inappropriate, B cells re-express MYC and re-enter the DZ for additional rounds of SHM (Dominguez-Sola et al., 2012; Ersching et al., 2017). MYC is defined as the GC B cell division timer since the level of MYC protein expression in the LZ is proportional to the number of divisions in the DZ (Finkin et al., 2019).

1.4.2. Role in B cell lymphomagenesis

MYC is one of the first oncogenes discovered and largely contributed to defining the term "oncogene". The history of oncogenes starts in 1910 with a famous experiment realized by Dr. Peyton Rous. He made a cell-free extract of a chicken tumor and injected this extract into healthy animals which then developed cancer thus suggesting that a virus was at the origin of the tumor growth. This experiment later led to the discovery of the Rous sarcoma virus and the identification of the *SRC* proto-oncogene by Temin and Rubin (Temin & Rubin, 1958). Dr. Rous was awarded the 1966 Nobel Prize in Physiology or Medicine for "his discovery of tumor-inducing viruses". This experiment ultimately led to the discovery of viral oncogenes and the idea that cancer mechanisms could be explained by the study of viruses. Later, it was discovered that the avian acute leukemia virus MC29 isolated by Ivanov and colleagues in 1964 contains a *v-myc* sequence that was identified as the sequence causing myelocytes expansion and leukemia in animal models (Sheiness et al., 1978). The mammalian *c-myc* sequence was then identified as a homolog of the MC29 *v-myc* sequence (Roussel et al., 1979; Sheiness & Bishop, 1979). These discoveries largely contributed to the understanding of oncogenes and basic concepts of oncology.

Today *MYC* is one of the most studied proto-oncogene defined as a gene having the potential to initiate cellular growth and malignant transformation when activated (and then called oncogene once activated). In opposition to oncogenes, tumors suppressors are defined as genes negatively regulating cellular growth and their inactivation support an incontrollable cellular growth and malignant transformation. *MYC* is found dysregulated in more than 70% of human cancers (Beroukhim et al., 2010; Dang, 2012; N. Meyer & Penn, 2008; Spencer & Groudine, 1991), particularly in blood malignancies (Ahmadi et al., 2021; Ott et al., 2013). *MYC* translocations are found in GC-derived B cell lymphoma, particularly with *Ig* genes. In BL

patients, *MYC* (chromosome 8) is translocated with chromosome 14 encoding the *Ig* heavy chain genes, or to a lesser extent, translocated with chromosome 2 or 22 harboring the *Ig* κ and λ light chain genes respectively (respectively translocations t(8:14), t(2;8), t(8;22)), causing a constitutive MYC expression and thus supporting the process of malignant proliferation (Dalla-Favera et al., 1982; Neel et al., 1982; Taub et al., 1982). About 70 to 80% of BL patients harbor a t(8:14) translocation, whereas translocations t(2;8) and t(8;22) are found in 10 to 15% of BL patients (Bernheim et al., 1981; Bertrand et al., 1981; Kaiser-McCaw et al., 1977). *MYC* genetic rearrangement is also observed in approximately 10% of DLBCL cases as well as in other types of B cell lymphoma, but not in FL where it is considered rare (Karube & Campo, 2015; Niitsu et al., 2009; Xia & Zhang, 2020). In DLBCL, *MYC* duplication is also observed (Stasik et al., 2010; Valera et al., 2013), as well as *MYC* mutations that are enriched in *MYC*-rearranged cases and increase MYC protein stability (Pasqualucci et al., 2001). Even if DLBCL tumors most commonly present *BCL6* or *BCL2* translocations (respectively 30-40% and 20-30% of cases), *MYC* alterations can also be found in tumors harboring translocations in *BCL2* or/and *BCL6* genes commonly named DHL or triple hit lymphoma (THL) and associated with poor overall survival (Ott et al., 2013; Valera et al., 2013). DHL tumors harbor translocations in *MYC* and *BCL2*, or *MYC* and *BCL6* to a lesser extent, representing 10% of DLBCL cases (Dunleavy, 2014). The DHL and THL are now classified as high-grade B cell lymphoma (Alaggio et al., 2022; Cucco et al., 2020; Grimm & O'Malley, 2019; Kunstner et al., 2022; Sesques & Johnson, 2017). In addition, a considerable amount of DLBCL tumors harbor a MYC protein overexpression without any *MYC* genetic abnormalities (Karube & Campo, 2015; Ott et al., 2013).

1.4.3. Modelling *MYC* dysregulation in murine B lymphoma

Due to the important amount of *MYC* abnormalities observed in lymphoma, several biological models were generated to better understand *MYC*'s role in B cell lymphoma. Notably, several transgenic mice mimicking the *MYC* genetic rearrangements found in patients have been created (Adams et al., 1985; Kovalchuk et al., 2000; S. N. Meyer et al., 2021; S. S. Park et al., 2005; Pasqualucci & Klein, 2021; Sander et al., 2012). These transgenic mice with an elevated level of MYC protein develop various types of lymphoma and contributed significantly to a better understanding of MYC oncogenic roles (Ferrad et al., 2020; S. N. Meyer et al., 2021; Mossadegh-Keller et al., 2021; Vecchio et al., 2020; R. Winkler et al., 2022), even in cancers other than lymphoma (Gabay et al., 2014; Morton & Sansom, 2013).

Among these models, the Eμ-*Myc* transgenic mice reproduce the t(8:14) translocation juxtaposing *MYC* with the enhancer sequence of the *Ig*H carried by 70-80% of BL tumors (Adams et al., 1985). These mice develop spontaneously a B lymphoma considered immature and are a transgenic tool widely used since its generation in 1985 (R. Winkler et al., 2022). Another model named λ-*Myc* (or IgL-*Myc* or Igλ-*Myc*) mimics the t(8;22) translocation juxtaposing *MYC* with the regulatory sequences of the *Ig*λ locus found in a smaller proportion of BL patients compared to the t(8:14) translocation (Kovalchuk et al., 2000). These λ-*Myc* mice also spontaneously develop B lymphomas of more mature phenotype in opposition to the immature B lymphomas that emerge in Eμ-*Myc* mice.

1.4.4. Synergy of MYC activation with other genetic events in lymphomagenesis

Although MYC involvement in lymphomagenesis and its oncogenic role is better understood, several studies using murine models highlight that a *MYC* aberration alone is not sufficient for lymphomagenesis and a full malignant transformation relies on additional mutations and/or genetic dysregulations (Klapproth & Wirth, 2010; Schuster et al., 2011; Vecchio et al., 2020; R. Winkler et al., 2022). One compelling argument for this view is that lymphomas that emerge in *Myc* transgenic mice are monoclonal, i.e. they develop from a single cell. If activation of MYC alone were sufficient for malignant transformation, the development of many different oligoclonal tumors would be expected in *Myc* transgenic mice.

One of the best examples is the cooperation between MYC and PI3K signaling to contribute to mouse BL pathogenesis (Sander et al., 2012). This murine model clearly shows that over-expressing MYC or a constitutively activated PI3K mutant into GC B cells using lox-stop-lox insertions into the *Rosa*26 locus and the Cγ1-*cre* deleter does not induce lymphoma development in mice, while a combination of both MYC overexpression and expression of a constitutively activated PI3K mutant induces murine BL.

Interestingly, MYC is known as a transcription factor inducing cell proliferation whereas on the opposite, high MYC expression also triggers apoptosis (N. Meyer et al., 2006; Vecchio et al., 2020; Wasylishen & Penn, 2010). At first sight, the duality between cellular proliferation and apoptotic functions of MYC appears contradictory although the apoptotic function can be inhibited by external factors and is then considered as a safety mechanism to prevent abnormal proliferation in case of *MYC* pathological activation. Therefore, the apoptotic function caused by a high level of MYC is thought to be a primordial event in tumorigenesis and could explain why a high level

of MYC can cooperate with the loss or impairment of many apoptotic factors. Indeed, MYC cooperates with 1) either mutations activating an anti-apoptotic factor for example *BCL2* (Eischen et al., 2001; Strasser et al., 1990), - as observed in DHL and double expressor lymphoma patients (Cai et al., 2020; Dunleavy, 2014) – or *MCL1* (Grabow et al., 2016); or 2) or inactivation of a pro-apoptotic factor such as p53 (L. Yu et al., 2019), or its targets or regulators (e.g. *PUMA* (Garrison et al., 2008; Michalak et al., 2009), *ARF-MDM2*, *BIM*) (Vecchio et al., 2020); or 3) a combination of several impairments (e.g. the THL).

Moreover, other essential cellular functions collaborate with MYC activation to the benefit of B lymphomagenesis (R. Winkler et al., 2022). In this regard, MYC-induced apoptosis can also be an indirect effect caused by the dysregulation of essential cellular functions, like cell cycle arrest, transcriptional or metabolic stress, DNA damage or others (R. Winkler et al., 2022).

Several reasons have directed research toward discovering genes altered in MYC-driven lymphoma. First, since MYC activation synergizes with secondary hits to initiate lymphomagenesis, particular attention has been drawn to genes frequently altered in MYC-activated tumors. Second, the increase of large-scale sequencing studies has allowed the unveiling of the genes altered in MYC-driven B lymphoma. Third, MYC is traditionally described as an interesting but "undruggable" target, thus leading the research to focus on co-mutated genes that could be relevant targets in view of future therapies. Consequently, recent studies reported that the *DDX3X* gene encoding the DEAD box helicase 3 X-linked is frequently mutated in MYC-driven B lymphoma and could possibly represent a relevant target for these cancers.

1.5. DEAD box RNA helicases

Helicases are defined as enzymes remodeling DNA and/or RNA to separate or unwind double stranded molecules. Helicases constitute a specific group of ATPases since adenosine triphosphate (ATP) hydrolysis generates the source of energy allowing their motor action. They are highly conserved from bacteria, viruses to humans suggesting a fundamental role and vital function for biological organisms. Helicases are classified into six superfamilies based on their sequences (Singleton et al., 2007). The superfamily 2, the largest of the six, contains several clusters of helicases, among them the DEAD box cluster (Fairman-Williams et al., 2010; Gorbalenya & Koonin, 1993; Jankowsky, 2011).

DEAD box RNA helicases are named after the Asp-Glu-Ala-Asp (DEAD) amino acid sequence common to all the members of this family. This motif was first found in the eukaryotic translation initiation factor 4 A (eIF4A; also named DDX2) helicase and its homologs and was suspected to be linked to the enzymatic activity (Linder et al., 1989). This DEAD motif, also named motif II, became the denominative motif of the family and was later proved to be essential for the ATPase activity (Iost et al., 1999; Pause & Sonenberg, 1992). Eleven additional motifs characterize the DEAD box family which is exclusively composed of RNA helicases (Linder & Fuller-Pace, 2013). Today, around 37 to 42 DEAD box (DDX) proteins have been identified in human (Linder, 2006; Umate et al., 2011). The molecular mechanisms and basic functions of DEAD box RNA helicases started to be understood in the 1990's (Tanner & Linder, 2001). Their involvement in several cellular processes started to be highlighted in the 2000's: DEAD-box helicases were mostly linked to the regulation of many steps of RNA metabolism due to their RNA binding capacity (Cordin et al., 2006; Linder, 2006; Linder & Jankowsky, 2011).

1.6. *DDX3X* and *DDX3Y* genes

1.6.1. Sexual genetic disparity

The DEAD box helicase 3 (*DDX3*) genes encode members of the DEAD box RNA helicases. *DDX3* is the annotation used to qualify two genes named DEAD box helicase 3 X-linked (*DDX3X*) and DEAD box helicase 3 Y-linked (*DDX3Y*), respectively localized on the X- and Y-chromosome in both mouse and human. These specific chromosomes determine biologically the sex of most mammals: the general rule is that females have two X-chromosomes (XX) while males carry one X- and one Y-chromosome (XY). In human, *DDX3X* is localized on Xp11.3-11.23 (S. H. Park et al., 1998) and *DDX3Y* in the AZFa region (Lahn & Page, 1997). Because of their genetic localization, *DDX3* genes are subject to a sex-specific regulation detailed here.

The *DDX3X* gene is the X-linked homolog, female mammals (XX) carry two alleles while males (XY) carry only one allele. In females, one of the two X-chromosomes is epigenetically silenced to equalize the dosage of X-linked gene products between XX females and XY males, a process known as X-chromosome inactivation (Avner & Heard, 2001; Lyon, 1961; Ohno & Hauschka, 1960). X-chromosome inactivation causes the majority of X-linked genes to have only one active allele. However, some genes escape X-chromosome inactivation and conserve two active alleles (Carrel & Willard, 2005; F. Yang et al., 2010). Those “escapees” represent 12-20% of human and 3-7% of mouse X-linked genes (Balaton & Brown, 2016). *DDX3X* is characterized as an escapee in both species and therefore, females carry two active alleles.

The *DDX3Y* gene is the Y-linked homolog and thus, only carried by males. It is not rare that X-linked escapees have an active, closely related Y-linked homolog to supposedly maintain a balanced dosage between both sexes (Bellott et al., 2014; Pessia et al., 2014).

DDX3X has been much more studied compared to its Y paralog. One of the reasons is that the DDX3Y protein is thought not to be expressed in humans except in the testis (as detailed later). Consequently, the "*DDX3*" term is often used in the literature to define *DDX3X*, particularly when the model is a human cell line other than male germ cells. However here, *DDX3* refers to both *DDX3X* and *DDX3Y* genes without any specification.

1.6.2. Structural features

DDX3X and *DDX3Y* nucleic acid and amino acid sequences respectively share 88% and 91% of homology in human (84% and 90% in mouse). Their differences are not equally distributed among their sequences: approximately half of their differences are carried by their N-terminal domains (**Figure 1-5**). Human and mouse *DDX3X* nucleic acid and amino acid sequences respectively share 92% and 99% of homology, and human and mouse *DDX3Y* nucleic acid and amino acid sequences respectively share 89% and 89% of homology.

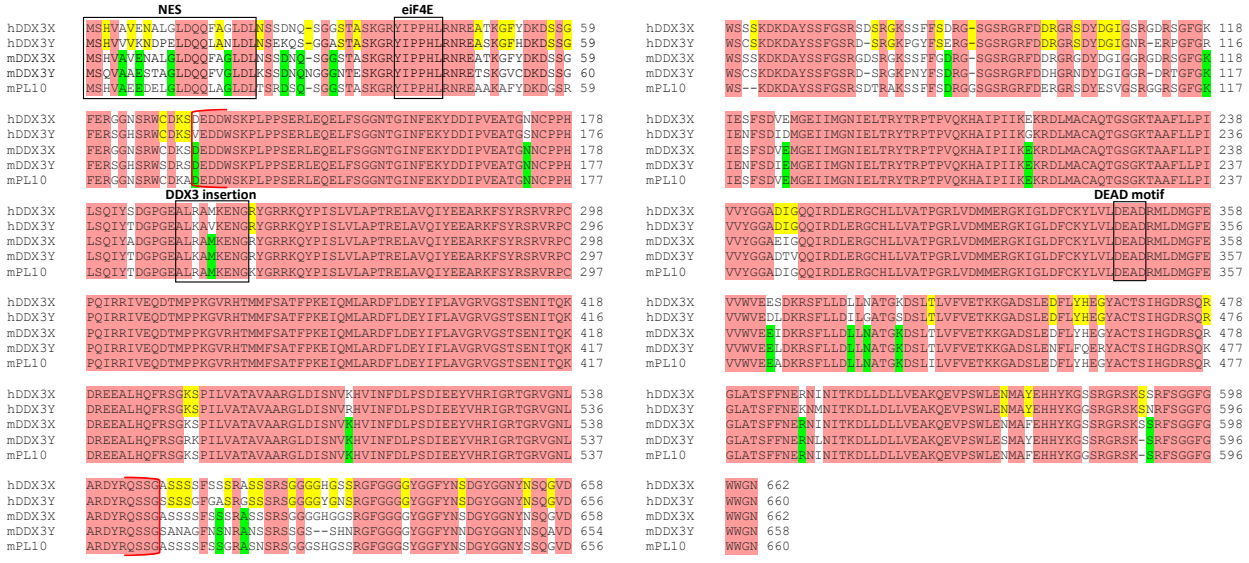


Figure 1-5: Alignment of DDX3 amino acid sequences from human and mouse

Alignment of amino acid sequences of human DDX3X, human DDX3Y, mouse DDX3X, mouse DDX3Y and mouse PL10. "h" and "m" letters indicate the species, respectively human and mouse; the numbers correspond to the amino acid positions. Sequences highlighted in red are common to all protein sequences; the yellow ones correspond to common sequences between the human DDX3X and DDX3Y paralogs; the green ones correspond to common sequences between the three murine DDX3 homologs. Black boxes indicate specific sequences such as the NES,

eIF4E binding, DDX3 insertion and DEAD motifs. The red brackets correspond to the minimal functional core for enzymatic activity. NES: Nuclear Export Signal. The alignment was done with Cluster Omega (Goujon et al., 2010; Sievers et al., 2011).

The structural features of DDX3 proteins were mostly studied for the human DDX3X. However, sequence homologies and similarities suggest that several of these features are also shared with DDX3Y and murine homologs (**Figures 1-5 and 1-6**).

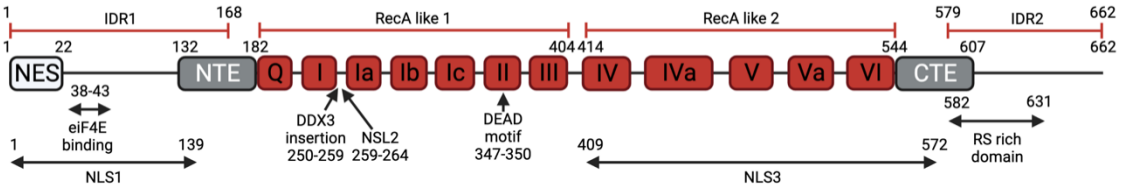


Figure 1-6: Structural organization of the human DDX3X protein

Amino acid sequences of human DDX3X and localization of the main features. Numbers correspond to the amino acid positions. RecA like 1 and 2 domains in addition to the NTE and CTE represent the minimal core for DDX3X enzymatic activity. NES: Nuclear Export Signal; NSL: Nuclear Localization Sequence; NTE: N-terminal extension; CTE: C-terminal extension; IDR: Intrinsically Disordered Region. Created with BioRender.com

Twelve conserved motifs defining the DEAD box family are found in DDX3X domains and are distributed among the helicase core composed of two RecA-like domains named after the RecA bacterial protein (Cordin et al., 2006; Fairman-Williams et al., 2010; Linder & Jankowsky, 2011; Soto-Rifo & Ohlmann, 2013) (**Figures 1-6**). The two RecA-like domains are bound by a cleft also named “linker” (aa404-414). The first RecA-like domain contains the following motifs: Q motif, I (Walker A or P-loop), Ia, Ib, Ic, II (Walker B or DEAD) and III (SAT motif S382). The second RecA-like domain contains the motifs IV, IVa, V Va, and VI. Some of the motifs are presumably essential for the ATPase activity (Q, I, II, VI), others for RNA-binding capacity (Ia, Ib, Ic, VI), and others for the communication between ATP and RNA binding sites (III, Va). Initially, the minimal functional core of DDX3 was described as aa168-582 although it was later found not to have any ATPase activity (Epling et al., 2015; Floor et al., 2016; Hogbom et al., 2007). Today, the minimal helicase core is defined as the region spanning from aa182 to aa544 in addition to the two N-terminal extension (NTE) and C-terminal extension (CTE) domains

respectively essential for the ATP binding and RNA binding capacity (Epling et al., 2015; Floor et al., 2016).

Although the helicase core is highly conserved through DEAD box helicases, DDX3 (confirmed in both X and Y) helicase core contains a unique ten amino acids insertion (aa250-259), referred to as "DDX3 insertion", between its motifs I and Ia (Hogbom et al., 2007). This motif is also found in murine DDX3X (Garbelli et al., 2011) (**Figures 1-5 and 1-6**) and was initially presumed important for RNA binding (Hogbom et al., 2007) although it was later shown that a deletion of this sequence in human DDX3X induces a reduction in ATPase and unwinding activity (Garbelli et al., 2011). Therefore, the exact function of this sequence remains unclear. However, this region seems highly relevant for drug design because 1) this sequence is specific to the human DDX3 and not to other DEAD box helicases, 2) it is important for its enzymatic activity, and 3) it is slightly different between the X- and Y- human paralogs.

N-terminal and C-terminal domains contain several motifs associated with functions independent of the enzymatic activity of DDX3 proteins. First, there is a known nuclear export sequence (NES) (Brennan et al., 2018; Shen et al., 2022; Yedavalli et al., 2004) in the N-terminal domain (**Figure 1-6**). Mutation of L19 and L21 leucine residues causes an accumulation of DDX3X in the nucleus. Since these leucine residues are conserved in human DDX3Y and mouse DDX3, the NES is very likely present in those proteins too. Second, the region covering aa38-43 contains a YxxxxL motif allowing DDX3X binding to eIF4E (J. W. Shih et al., 2008) (**Figure 1-6**). This motif is also present in human DDX3Y as well as in mouse DDX3 (**Figure 1-5**). Third, an RS-like domain (aa582-631) was identified in the C-terminal region (Owsianka & Patel, 1999) and has been shown to be essential for Tip-associated protein (TAP), a nuclear export receptor (Lai et al., 2008) (**Figure 1-6**). In addition, three independent redundant nuclear localization

sequences (NLS) have also been identified along the sequence: one within the region covering aa1-139, another within aa259-264 and a predicted one within aa409-572 (Brennan et al., 2018). Also, two intrinsically disordered regions (IDR) have been identified: IDR1 in N-terminal and IDR2 in the C-terminal domain (Shen et al., 2022) (**Figure 1-6**).

1.6.3. Localization and post-translational regulation

DDX3 has a nucleocytoplasmic shuttling capacity (Brennan et al., 2018; Heerma van Voss et al., 2017; Owsianka & Patel, 1999; Yedavalli et al., 2004). DDX3 shuttles to the cytoplasm by binding to TAP through its RS-domain (Lai et al., 2008; C. S. Lee et al., 2008) and through the binding to the CRM1/Exportin 1 receptor (Heerma van Voss et al., 2017; Yedavalli et al., 2004). In the nucleus, DDX3 has been shown to be recruited to the E-cadherin promoter (Botlagunta et al., 2008) and the type-I IFN promoter in the context of an infection (Soulat et al., 2008). DDX3 can also localize to the centrosome (W. J. Chen et al., 2017) and the mitochondria (Heerma van Voss et al., 2018).

These studies did not account for potential differences between DDX3X and DDX3Y but by comparing both sequences, it is likely that both human proteins, if expressed, share similar intracellular localizations except maybe in cases where their localization depends on the N-terminus since this is the most divergent region between DDX3X and DDX3Y (**Figure 1-5**). However, one group generated a DDX3Y-specific antibody and showed that DDX3Y also shuttles between the nucleus and cytoplasm in human male germ cells (Gueler et al., 2012).

DDX3Y undergoes a specific translational regulation: it is widely accepted that human *DDX3Y* is transcribed in many cell types but not translated except in the testes (Ditton et al., 2004; Gueler et al., 2012). It was shown that a specific structure in the human *DDX3Y* 5'UTR allows its protein expression only in a specific type of spermatozoa progenitor (Gueler et al., 2012;

Jaroszynski et al., 2011; Rauschendorf et al., 2011). However, two reports suggest it could be expressed at the protein level in leukemic and lymphoma cells (Gong et al., 2021; Rosinski et al., 2008). In mice, this regulatory process is presumed not to occur (Rauschendorf et al., 2011), and the DDX3Y protein was indeed detected in cardiomyocytes and fibroblasts (Deschepper, 2020). Moreover, it is known that mice carry an additional DDX3 homolog on chromosome 1 named D1Pas1 or PL10, with a high degree of sequence similarity with both murine DDX3 (Vong et al., 2006) (**Figure 1-5**).

1.6.4. Functions

Transcription

As detailed previously, DDX3X can regulate the transcription by directly binding the promoter of E-cadherin and IFN (Botlagunta et al., 2008; Soulat et al., 2008). Moreover, through the regulation of the SP1 transcription factor, DDX3 can indirectly regulate the transcription of *KRAS* (Wu et al., 2016), *MDM2* (Wu et al., 2014), and *P21* (Chao et al., 2006; Wu et al., 2011). By transactivating the expression of the YY1 transcription factor, DDX3 indirectly regulates the transcription of genes associated with β-catenin activation (*WNT1*, *WNT3*, *AXIN2*, *FZD10*, *BMP4*) (F. Yang et al., 2019).

Translation initiation

DDX3 binds to eiF3 and the 40S ribosomal subunit suggesting that it is a component of the 43S pre-initiation complex (H. H. Chen et al., 2018; Geissler et al., 2012; C. S. Lee et al., 2008). In addition, DDX3 is also important for translation initiation supporting the formation of the 80S translation initiation complex independently of its enzymatic function (Geissler et al., 2012), which instead points to a chaperone function rather than a helicase role. DDX3 competes with eIF4G for eIF4E binding to repress translation (J. W. Shih et al., 2008) and also binds to PABP (Lai et al.,

2008; J. W. Shih et al., 2012; Soto-Rifo et al., 2012) as well as it binds to eIF4G and to mRNA 5'UTRs thus being part of the eIF4F complex (Adjibade et al., 2017; Soto-Rifo et al., 2012). Moreover, DDX3 can bind to the 5' 7-methylguanosine mRNA cap and substitute for eIF4E to promote translation (Soto-Rifo et al., 2013). Although there is no doubt that DDX3 is involved in translation, it is still unclear whether DDX3 activates and/or represses translation. Indeed, many contradictory results can be found in the literature, probably because of the different biological contexts and model cell lines used for these studies (Ryan & Schroder, 2022).

Indirect translation regulation

DDX3 also indirectly regulates the translation at various other levels. First DDX3 was found to be associated with spliced RNA via the exon junction complex (Merz et al., 2007). DDX3 is also involved in mRNA export (Lai et al., 2008; Yedavalli et al., 2004), the RNA interference pathway (Kasim et al., 2013; Pek & Kai, 2011), and microRNA biogenesis (Zhao et al., 2016). Moreover, DDX3 has the potential to isolate RNA into stress granules to prevent their translation under stress conditions (Oh et al., 2016; Samir et al., 2019; Shen et al., 2022; J. W. Shih et al., 2012; Valentin-Vega et al., 2016), but again, controversies exist in the literature about whether DDX3X is dispensable (Adjibade et al., 2017) or not (J. W. Shih et al., 2012) for stress granule induction. DDX3X is also a component of P-bodies, another type of cytoplasmic granules containing RNAs important for mRNA decay (Chahar et al., 2013). P-bodies and stress granules are both membraneless organelles but P-bodies regulate mRNA decay while stress granules store mRNA specifically during stress conditions (Stoecklin & Kedersha, 2013).

All of DDX3 functions in translation have been intensively reviewed (Ariumi, 2014; J. T. Park & Oh, 2022; Ryan & Schroder, 2022; Sharma & Jankowsky, 2014; Soto-Rifo & Ohlmann, 2013).

Other functions

By regulating the translation of many other mRNAs and through other mechanisms, DDX3 is linked to a variety of cellular processes such as cell cycle regulation, apoptosis, DNA damage (Cargill et al., 2021), hypoxia, stress response, Wnt signaling and embryogenesis (Ariumi, 2014; Bol et al., 2015; Mo et al., 2021; Sharma & Jankowsky, 2014). In addition, DDX3 is important for innate immunity through the regulation of NLRP3 inflammasome, its involvement in the NF-κB signaling pathway and modulation of viral infections (Hernandez-Diaz et al., 2021; Kwon et al., 2022; Samir & Kanneganti, 2022; Schroder, 2011; Valiente-Echeverria et al., 2015; Winnard et al., 2021).

Sex-specific functions

All the described DDX3 functions have been attributed to DDX3X because the DDX3Y protein is presumably not expressed elsewhere other than in human testes which were rarely used as an experimental model in those studies. However, since two reports have now shown that DDX3Y could be expressed in malignant cells (Gong et al., 2021; Rosinski et al., 2008), it is not excluded that the DDX3Y protein is expressed in some of the transformed cell lines used to study DDX3X. Therefore, it is possible that DDX3Y may have clouded some effects by compensating DDX3X loss, downregulation or inactivation in some models. It is indeed known that DDX3Y expression rescues DDX3X loss of function in a hamster cell line (Sekiguchi et al., 2004). Moreover, it was recently demonstrated that both DDX3Y and DDX3X have redundant functions in protein synthesis for example (Gong et al., 2021; Venkataramanan et al., 2021), an expected

result based on their sequence homology. However, around half of the differences between DDX3X and DDX3Y amino acid sequences resides in their N-terminal domain containing an IDR1 domain (Shen et al., 2022) (**Figure 1-5**). Shen and colleagues recently demonstrated that the IDR1 domain is involved in stress granules formation in both DDX3X and DDX3Y, but DDX3Y has a weaker enzymatic activity and thus, promotes less dynamic stress granules, causing higher translational repression compared to DDX3X (Shen et al., 2022). Consequently, it is expected that functions attributed to DDX3X (or efficacy of these functions) may slightly differ from DDX3Y functions, particularly when it involves their N-terminal domains.

In mice, the situation is different since DDX3Y is not post-translationally repressed (Deschepper, 2020; Rauschendorf et al., 2011). It is known that DDX3Y and D1Pas1 rescue DDX3X loss of function in hamster cells (Sekiguchi et al., 2004). Moreover, several studies recently suggested a compensatory effect by DDX3Y in *Ddx3x*-deleted male mice since these male KO showed different phenotypes compared to *Ddx3x*-deficient female mice in brain cells (Hoye et al., 2022; Patmore et al., 2020), hepatocytes (Chan et al., 2019) and bone marrow-derived macrophages (Szappanos et al., 2018). It was even shown that neurons without *Ddx3x* have a higher level of *Ddx3y* mRNA in favor of a male-specific compensatory effect, suggesting that both murine DDX3X and DDX3Y exert very similar functions (Hoye et al., 2022; Patmore et al., 2020). However, ubiquitous expression of DDX3Y protein in mice was never clearly demonstrated because of the lack of a DDX3Y-specific antibody. Consequently, it is not excluded that other male-specific factors also compensate for DDX3X loss in these mouse models.

1.6.5. Pathological role of DDX3

DDX3 is an essential gene in humans and is involved in major cellular functions indispensable for cell survival (H. Chen et al., 2020; T. Wang et al., 2015). DDX3 was also shown

to be indispensable to murine embryonic development (C. Y. Chen et al., 2016; Q. Li et al., 2014). Consequently, DDX3 dysregulation has been linked to many different diseases, again highlighting its importance for many cell types.

Intellectual deficiencies

Germline mutations in the human *DDX3X* gene are associated with 1-3% of neurodevelopmental and intellectual disabilities (ID) (Deciphering Developmental Disorders, 2015, 2017; Johnson-Kerner et al., 2020; Lennox et al., 2020; Scala et al., 2019; Snijders Blok et al., 2015; Y. Sun et al., 2022; X. Wang et al., 2018), including autism spectrum disorders (Iossifov et al., 2014; Ng-Cordell et al., 2022; Ruzzo et al., 2019; Stefaniak et al., 2022; Takata et al., 2018; Tang et al., 2021; Yuen et al., 2017) and Toriello-Carey syndrome (Dikow et al., 2017). Most of the patients affected by germline *DDX3X* mutations are females suggesting that males carrying germline mutations are not viable. However, a few males have been reported (Kellaris et al., 2018; Nicola et al., 2019; Snijders Blok et al., 2015; Tang et al., 2021); the majority inherited the mutation from their unaffected mother. Since *Ddx3x* hemizygous male mice are embryonic lethal, but not the female heterozygous (Boitnott et al., 2021; C. Y. Chen et al., 2016), it is thought that DDX3X mutants found in males are hypomorphic and retain some function.

Amyotrophic lateral sclerosis

Changes in DDX3X expression were observed in amyotrophic lateral sclerosis patients (Cheng et al., 2019) and also in an amyotrophic lateral sclerosis transgenic mouse model (Y. Chen et al., 2017). Interestingly, DDX3Y more strongly promotes FUS and TDP-43 aggregation in stress granules compared to DDX3X (Shen et al., 2022), two major pathological proteins involved in amyotrophic lateral sclerosis and other types of neurodegenerative diseases like frontotemporal dementia (Portz et al., 2021).

Fertility

In humans, it was shown that a deficiency of DDX3Y protein in patients leads to infertility (Foresta et al., 2000; Ramathal et al., 2015), whereas in mice, this function was not attributed to murine DDX3Y, dispensable for spermatogenesis (Matsumura et al., 2019; Mazeyrat et al., 2001). In mice, this function is rather attributed to the D1Pas1 homolog (Inoue et al., 2016; Mazeyrat et al., 2001) that is expressed in male germ cells (Session et al., 2001).

Cancers

DDX3X is known to be impaired in a variety of cancer types and was found somatically mutated, truncated, overexpressed, or even translocated or mislocalized, depending on the type of cancer (Ariumi, 2014; Bol et al., 2015; He et al., 2018; T. C. Lin, 2019; Mo et al., 2021; Secchi et al., 2022; Sharma & Jankowsky, 2014). The variability among the types of DDX3X alterations highlights that its functions may be highly context-dependent making it difficult to determine whether it acts as a tumor suppressor or an oncogene (He et al., 2018). In some cancer subtypes, DDX3X is even proposed as a relevant drug target and its enzymatic inhibition prevents cancer growth (Bol et al., 2015; Mo et al., 2021).

One study reported DDX3Y overexpression and nuclear localization in *in situ* testicular carcinoma (Gueler et al., 2012). Another study analysis concluded that high levels of *DDX3Y* RNA correlate with a poor prognosis in several cancer types (T. C. Lin, 2019). It was also shown that low levels of *DDX3Y* RNA increase the probability for males to develop a malignancy (Caceres et al., 2020). However, two reports demonstrated that the DDX3Y protein could be expressed in human cells other than male germ cells and is indeed ectopically expressed in leukemic and lymphoma cells (Gong et al., 2021; Rosinski et al., 2008). Another study has shown the presence of DDX3Y protein in the enteric nervous system of male patients affected by Hirschsprung disease

(Cardinal et al., 2020). To date, a clear picture of where and under which circumstances the DDX3Y protein is expressed in human cells remains unclear and a matter of debate.

DDX3X in hematopoietic malignancies

Since other genetic alterations are essential in addition to *MYC* activation in order to initiate a B cell lymphoma, particular attention was drawn to genes co-mutated in MYC rearranged lymphoma. *DDX3X* is one of them and was found mutated in GC-derived B cell lymphoma at a significant frequency (Arthur et al., 2018; Burkhardt et al., 2022; Grande et al., 2019; M. Kim et al., 2022; Richter et al., 2012; Schmitz et al., 2012; N. Thomas et al., 2023; Zhang et al., 2020). Many studies agree that 30% of BL tumors harbor a *DDX3X* mutation in addition to the hallmark *MYC* translocation making it one of the most mutated genes in this particular disease. *DDX3X* mutations were even recently used to characterize new clusters of BL and DLBCL tumors (N. Thomas et al., 2023). *DDX3X* is mutated to a lesser extent in DLBCL tumors (2-8%), although recent analyses have shown that the *DDX3X* mutation rate is higher (around 14%) in *MYC* dysregulated DLBCLs (Cucco et al., 2020; Gong et al., 2021). An additional study estimates that 28% of DLBCL associated with *MYC* dysregulation also harbor a *DDX3X* mutation, which is particularly enriched in tumors defined as single-hit lymphoma (*MYC* but no *BCL2* or *BCL6* rearrangement) and *MYC* cluster amplified subtypes (Miyaoka et al., 2022).

Independently of MYC, it was proposed that mutated *DDX3X* is associated with DLBCL patients having worse outcomes and chemoresistance (Kizhakeyil et al., 2021). It was also estimated that 14% of primary mediastinal B cell lymphoma, a specific subset of DLBCL, and 10% of HLs – tumors that also originate from the GC (Weniger & Kuppers, 2021), carry a *DDX3X* mutation although the link with *MYC* dysregulation was unclear (Moffitt & Dave, 2017).

Single as well as double or triple mutations, truncations, frameshift mutations and alterations of splice sites were found in the *DDX3X* gene in B lymphoma tumors, but no specific hotspot could be defined. The majority of these mutations are missense mutations generating a change of one amino acid (Figure 1F from (Gong et al., 2021)). In addition, a significant frequency of nonsense mutations introducing a STOP codon early in the sequence; and rarer frameshift mutations have been detected in both BL and DLBCL (Burkhardt et al., 2022; Gong et al., 2021). Some of these mutations, for instance those affecting aa residues R475 and R534 have also been detected in medulloblastoma and are known to impair DDX3X's enzymatic activity (Epling et al., 2015; Floor et al., 2016; Lennox et al., 2020). Similar missense mutants from NK T cell lymphoma (NKTCL) tumors have been shown to have a decreased helicase activity *in vitro* (Jiang et al., 2015). Therefore, missense mutations detected in B lymphoma presumably have an impaired helicase activity even if it has never been experimentally demonstrated. However, Gong and colleagues recently demonstrated that DDX3X regulates the translation of proteins involved in global protein synthesis in B cells and that the R475S mutation decreases global protein synthesis (Gong et al., 2021). Mutations detected in B cell lymphoma are therefore expected to be LOF mutations regarding the translational function of DDX3X. In addition, several groups recently pointed out that *DDX3X* mutations are almost exclusively found in male patients and rarely detected in female patients (Burkhardt et al., 2022; Gong et al., 2021; N. Thomas et al., 2023).

DDX3X is also extensively mutated in hematopoietic malignancies other than GC-derived B cell lymphoma and independently of *MYC* translocations, for instance in many subtypes of NHL originating from B cells (Moffitt & Dave, 2017). In 1-10% of chronic lymphocytic leukemia (CLL), truncation or missense mutations were found (Kanagal-Shamanna et al., 2019; J. A. Kim et al., 2016; Landau et al., 2013; Meier-Abt et al., 2021; Ojha et al., 2015; Puente et al., 2015;

Quesada et al., 2011; Trimech et al., 2021; Vollbrecht et al., 2015; L. Wang et al., 2011). *DDX3X* mutations were even associated with worse overall response and progress-free survival in lenalidomide-refractory CLL patients (Takahashi et al., 2018), as well as monoclonal B cell lymphocytosis patients (Ojha et al., 2014). In hairy cell leukemia patients, 2-3% of cases present *DDX3X* mutations (Durham et al., 2017; Waterfall et al., 2014). Moreover, *DDX3X* mutations have also been observed in canine lymphoma, an emerging animal model for those cancers (Coyle et al., 2022; Elvers et al., 2015).

Also, *DDX3X* mutations were discovered in NHL originating from T or NK cells. 20% of enteropathy-associated T-cell lymphoma samples harbor *DDX3X* mutations, a lymphoma associated with a severe complication of coeliac disease (Cording et al., 2022), as well as in 4% of hepatosplenic T cell lymphoma (McKinney et al., 2017; Moffitt & Dave, 2017) and other types of T cell lymphoma (Fan et al., 2022; Moffitt & Dave, 2017). In NKTCL, 12-20% of patients harbor truncations or missense mutations in the *DDX3X* gene (Dobashi et al., 2016; Jiang et al., 2015; Xiong et al., 2020). In NKTCL, *DDX3X* mutations are associated with poor outcome prediction (Jiang et al., 2015; J. J. Kim et al., 2023) and are found in approximately 29% of aggressive natural killer leukemia patients (ANKL) (Dufva et al., 2018).

Further, *DDX3X* mutations were reported in acute lymphoblastic leukemia (ALL) originating from B or T cells (B. Li et al., 2020; Y. Liu et al., 2017; Manchev et al., 2017; Oshima et al., 2016; Zhang et al., 2016), and *DDX3X:MLLT10* translocations were identified in ALL patients (Brandimarte et al., 2014; Brandimarte et al., 2013; Wong et al., 2020). These *DDX3X:MLLT10* translocations were also recently identified in acute myeloid leukemia tumors (B. Kim et al., 2019; Nilius-Eliliwi et al., 2022; Ries et al., 2019).

The numerous reports of *DDX3X* alterations in so many types of human cancers have made it a gene of interest, particularly in hematopoietic malignancies associated with *MYC* activation. Moreover, the variety of *DDX3X* alterations in those diseases highlights the possibility of various mechanisms across hematopoietic cell types that need to be discovered.

1.7. Hypothesis, rationale and objectives of the research

This present project was initiated at a time when many *DDX3X* mutations were reported in B cell lymphoma, with a particularly high frequency in BL (around 30% of BL patients). In addition, the high frequency of *DDX3X* mutations in pediatric BL tumors, which usually carry a limited number of somatic mutations compared to adult tumors (Lawrence et al., 2013; Vogelstein et al., 2013) may indicate its strong impact on lymphomagenesis. While reported in many cohorts, the consequences of such genetic alterations for B lymphoma were entirely unknown. Therefore, it was of great interest to elucidate the impact of DDX3X alterations, presumed to be LOF, for future therapeutic options.

However, elucidating the impact of DDX3X alterations in lymphoma require first understanding in which cells DDX3X is important and what its functions are in these specific cell types. When this project was initiated, DDX3X's role in B lymphopoiesis was completely unknown and even if several various functions were attributed to DDX3X, they were often contradictory and not demonstrated in the B cell context since they were discovered in *in vitro* models of various cell types that were not lymphoid models. Since DDX3X is an RNA binding protein, its functions very likely differ according to the cell type which does not have the same RNA content; hence the necessity to design more appropriate lymphoid models. In addition, there

was a lack of *in vivo* models to study DDX3X in the hematopoietic context. Therefore, this project was set up with two main objectives:

1) **To define DDX3X's role in hematopoiesis, more particularly in B lymphopoiesis.**

The lack of knowledge about DDX3X in B cells and in hematopoiesis in general, has led our laboratory to generate an *in vivo* conditional KO model. **I hypothesized that DDX3X is important for hematopoiesis, more particularly for B lymphopoiesis**. The strategy was to delete the *Ddx3x* gene in hematopoietic cells and assess which populations are sensitive to its deletion in order to gain information about the biological role of DDX3X in these cells.

2) **To assess the impact of DDX3X LOF in B lymphomagenesis.**

Numerous reports of DDX3X mutations in B lymphoma tumors have raised interest in understanding their role and effects on tumor development. One question was whether deleting the *Ddx3x* gene *in vivo* would accelerate or decelerate the lymphomagenesis. **I hypothesized that *DDX3X* LOF is involved in lymphomagenesis**. To test this hypothesis, the strategy was to use the conditional KO mice to delete the *Ddx3x* gene in mouse models that mimic B lymphomagenesis *in vivo*.

2. CHAPTER II: Methodology

2.1. Animal models

Ddx3x-conditional knockout mice were generated by Ingenious Targeting Laboratory. *Vav-cre* (Georgiades et al., 2002), *Cd21-cre* (Kraus et al., 2004), *Cd19-cre* (Rickert et al., 1997), *R26-cre*^{ER} (Badea et al., 2003), Cγ1-*cre* (Casola et al., 2006), CD45.1, *R26*$^{mT/mG}$ (Muzumdar et al., 2007), *Trp53* (Jacks et al., 1994), and Eμ-*Myc* mice were purchased from the Jackson Laboratory (Adams et al., 1985). λ-*Myc* mice were a gift from Dr. Siegfried Janz (Medical College of Wisconsin, Milwaukee, Wisconsin) (Kovalchuk et al., 2000). Mice were held in a C57BL/6 genetic background in a Specific-Pathogen-Free+ environment at the animal facility of the Montreal Clinical Research Institute (Institut de recherches cliniques de Montréal – IRCM). Experimental procedures and mouse maintenance were approved by the Animal Care Committee (ACC#2013-04) of the Montreal Clinical Research Institute in compliance with the Canadian Council on Animal Care guidelines (www.ccac.ca).

2.2. Embryo analysis

Embryos were prepared from euthanized pregnant females counted from the day of the plug (E0.5) and were fixed in formalin and stained with hematoxylin and eosin according to the manufacturer's protocol. For blood analysis, embryos were washed with PBS and blood cells were collected from the umbilical cord and cytocentrifuged onto slides (Thermo Fisher Scientific

Cytospin), stained with May-Grünwald-Giemsa (Sigma-Aldrich) and imaged with a DM4000b microscope (Leica) and CellSens Entry software (Olympus).

2.3. Flow cytometry

Spleens and thymi were extracted from euthanized mice and single-cell suspensions were obtained by mechanical dissociation and flushed from tibiae and femora to obtain BM single-cell suspension. Cell solutions were filtered and depleted of RBC by 10 minutes of incubation in RBC lysis buffer Hybri-Max (Sigma). Cells were incubated with indicated fluorochrome-labeled antibodies (**Table 2-1**). Intracellular staining was done with Cyto-Fast Fix/Perm kit (Biolegend) according to the manufacturer's instructions with DDX3X (A300-474A, Bethyl) primary antibody and PE-donkey anti-rabbit IgG (406421 Cell Signaling) secondary antibody. Data were recorded on an SA3800 Spectral Cell Analyzer (Sony) or a BD LSRFortessa (BD Biosciences) and analyzed with the FlowJo software.

Antibody	Fluorochrome	Company	Cat Number	Antibody	Fluorochrome	Company	Cat Number
NK1.1	biotin	Biolegend	108704	CD43	PECy7	BD	562866
Gr1	biotin	BD	51-01212J	NK1.1	PECy7	Biolegend	108714
Ter119	biotin	BD	51-09082J	IL7R	PE	eBioscience	12-1271-83
CD3e	biotin	BD	51-01082J	BP-1	PE	BD	553735
CD4	biotin	Biolegend	100508	CD62L	PE	BD	553151
CD5	biotin	eBioscience	13-0051-85	CD41	PE	Biolegend	133906
CD8	biotin	Biolegend	100704	CD23	PE	BD	553139
IgM	biotin	BD	553406	CD71	PE	Biolegend	113808
B220	biotin	Biolegend	103204	CD38	PE	Biolegend	102708
Mac-1	biotin	BD	51-01712J	CD24	PE	BD	553262
CD43	biotin	BD	553269	CD43	PE	BD	553271
DX5	biotin	Biolegend	108904	CD4	PE	BD	553049
CD19	biotin	Biolegend	115504	Gr1	PE	BD	553128
Flt3	APC	Biolegend	135310	AnnexinV	FITC	Biolegend	640945
B220	APC	Biolegend	103212	CD34	FITC	eBioscience	11-0341-85

CD19	APC	BD	550992	CD24 (HSA)	FITC	eBioscience	11-0241-85
CD138	APC	Biolegend	142506	Streptavidin	FITC	Biolegend	405202
CD25	APC	BD	557192	CD21/35	FITC	BD	553818
CD3	APC	Biolegend	100312	sca-1	FITC	Biolegend	108106
CD16/32	APC.Cy7	Biolegend	101328	CD44	FITC	Biolegend	103006
CD8a	APC.Cy7	Biolegend	100714	CD95	FITC	BD	554257
CD19	APC.Cy7	Biolegend	115530	CD71	FITC	BD	553266
CD45.2	APC Cy7	Biolegend	109824	CD45.1	FITC	eBioscience	11-0453-85
CD38	APC Cy7	Biolegend	102728	CD105	AF647	Biolegend	120420
c-kit	BV421	Biolegend	105828	ter119	AF647	Biolegend	116218
IgM	BV421	Biolegend	406532	CD25	AF647	Biolegend	102020
CD19	BV421	Biolegend	115538	BP-1	AF647	Biolegend	108312
IgD	BV605	Biolegend	405727	CD9	AF700	Novus Bio	NBP1-44876
Streptavidin	BV605	Biolegend	405229	B220	AF700	Biolegend	103232
sca-1	PECy7	Biolegend	108114	IL7R	AF700	Invitrogen	56-1271-82
Streptavidin	PECy7	Biolegend	405206	CD150	Pacific Blue	Biolegend	115924
CD93	PECy7	eBiosience	25-5892-82	GL7	Pacific Blue	Biolegend	144614
CD95	PECy7	BD	557653	CD4	Pacific Blue	Biolegend	100531
				Mac1	Pacific Blue	Biolegend	101224

Table 2-1: List of antibodies used for flow cytometry

2.4. BM transplantation

CD45.1 mice were treated with trimethoprim and sulfamethoxazole (TNS, Chiron pharmaceuticals) 3 days pre-irradiation and 7 days post-irradiation. They received additional enrofloxacin (Baytril, CDMV) treatment on days 8 to 14 post-irradiation. Recipient mice (CD45.1 or CD45.2) were irradiated at 9,5 Gray and intravenously transplanted with 1 X 10^6 RBC-depleted BM cells from CD45.2 or CD45.1 mice. BM reconstitution was validated by flow cytometry analysis of blood samples 8 weeks post-transplantation. The *R26-cre*^{ER} was activated with two successive intraperitoneal injections of tamoxifen (Sigma) at 100mg/kg on day 0 and 50mg/kg on day 1.

2.5. BCR activation

Resting B cells were isolated from the spleen of *Cd19-cre/Ddx3x*-floxed mice with the Mojosort magnetic cell separation (Biolegend) and stimulated with soluble IgM ($F(ab')_2$ fragment goat anti-mouse IgM, Jackson Immuno Research) for 10min at 37°C. Total cell lysates were obtained with RIPA buffer following by a Western blot analysis with ERK and phospho-ERK primary antibodies.

2.6. Western blot

For total extracts, cells were lysed in RIPA buffer with complete protease inhibitor (Roche) for 15 minutes at 4°C followed by two rounds of sonication. For the nuclear extraction, cells were lysed in buffer I (10mM Hepes, 10mM KCl, 2mM EDTA, 0,5% NP-40, 10% Glycerol, complete protease inhibitor (Roche), pH 7.5) incubated at 4°C for 15 minutes and centrifuged at 13'000 rpm for 10 minutes. The supernatant was discarded and pellets were lysed in buffer II (50mM sodium phosphate, 300mM NaCl, 1mM β-mercaptoethanol, 10% glycerol, 0,5% NP-40, 0,5% Triton X-100, complete protease inhibitor (Roche), pH7.5) incubated at 4°C for 15 minutes after two rounds of sonication. Laemmli loading buffer was added to the lysates followed by 10min incubation at 95°C and separation by SDS-PAGE electrophoresis. Gels were transferred on PVDF membranes and immunoblotted with the following primary antibodies: β-Actin (Ac-15 A5441, Sigma), MYC (9402, Cell Signaling), DDX3X (A300-474A, Bethyl), TBP (D5C9H 44059, Cell Signaling), ERK (9107S, Cell Signaling), Phospho-ERK (9101S, Cell Signaling). DDX3Y antibody was generated in collaboration with Biomatik as indicated in the results section from chapter III.

2.7. Immunization

Sheep RBCs (Innovative Research) were washed with PBS and counted with trypan blue. 1 X 10^8 sheep RBCs were intravenously injected into mice with the indicated genotype at day 0. Splenic GCs were analyzed on days 3, 5, 7 or 10 post sheep RBC immunization. A solution of 4-hydroxy-3-nitrophenyl-acetyl (NP)(42) conjugated to chicken gamma globulin (CGG; Biosearch Technologies) was prepared at 1mg/mL in PBS. Mice with indicated genotype were immunized intraperitoneally with 200uL of the NP-CGG solution precipitated in alum (1:1). Control mice were immunized with 1:1 PBS in alum solution. NP^+ gate was assessed using mice that received alum without NP-CGG and an anti-NP-PE antibody (Biosearch Technologies).

2.8. Tumor analysis

Eμ-*Myc* and λ-*Myc* mice were crossed with *Vav-cre*/*Ddx3x*-floxed or *Cd19-cre*/*Ddx3x*-floxed animals and offspring was checked regularly until any endpoint was detected as defined by palpable tumor, respiratory discomfort, weight loss, impaired activity, hunched posture, or any other sign of suffering. Blood was collected by cardiac puncture and analyzed on an Advia 120 cell analyzer (Bayer) using the mouse archetype of multi-species software vv.2.2.06. Tumor masses were harvested for genotyping and analyzed by flow cytometry. λ-*Myc* tumors have been classified into three groups according to the intensity of the GL7 marker assessed by flow cytometry of tumor samples. Pre-tumor stage is defined as 6-week-old mice not presenting any sign of disease.

2.9. Induced GC B cells

40LB cells were obtained from Dr. Di Noia's laboratory in December 2021. 40LB cells tested negative for *Mycoplasma* by PCR and were not maintained in culture for more than a month. Confluent 40LB feeder cells were irradiated at 120 Gray and plated at 0.13 X 10^6 cells in 24 well-plates. One day post-plating (day 0), primary naïve B cells were isolated from the spleens of *R26*$^{mT/mG}$/Cγ1-*cre/Ddx3x*-floxed mice with the Mojosort magnetic cell separation (Biolegend) and cultured with 1ng/mL of murine IL-4 (Peprotech) on a 40LB feeder layer.

3. CHAPTER III: DDX3X's role in hematopoiesis

1. Loss of DDX3X impacts erythroid differentiation

To understand the potential DDX3X's role in hematopoiesis, a conditional knockout (KO) mouse model allowing a tissue-dependent *Ddx3x*-deletion was generated. Using gene targeting, two loxP sites flanking the exon 2 containing the translation initiation codon were inserted at the *Ddx3x* locus on the X chromosome (**Figure 3-1A**).

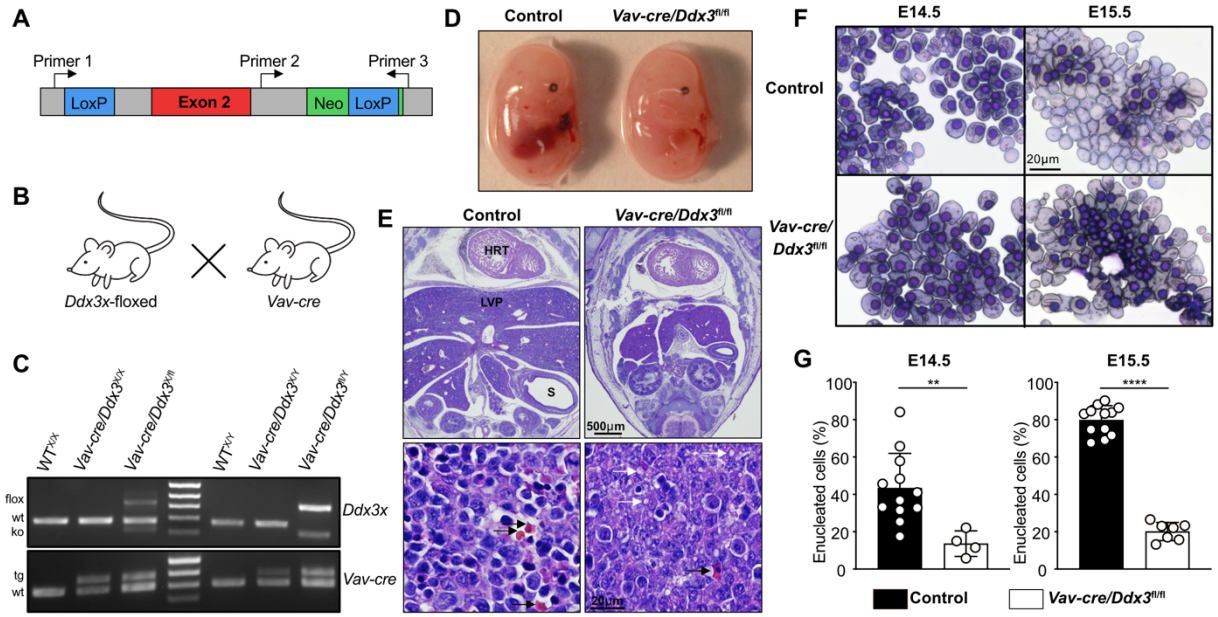


Figure 3-1: Female *Ddx3x*-KO mice have an impaired embryonic erythropoiesis

A, Schematic representation of the targeted *Ddx3x* allele and the genotyping strategy; primer1 = GGCTAGCACTCTACTAACTGAACTAAG; primer2 = CCGTGATCATGTCCTTGAATGGC; primer3 = TGAAGCTAGCTTTGTCAGCCAG. **B,** Schematic representation of the breeding strategy. **C,** Agarose gel of PCR results obtained with primers indicated in (A) and published strategy for *Vav-cre* genotyping. PCR products for *Ddx3x* are the following: flox = 407bp; WT = 277bp and KO = 207bp. **D,** Comparison of a control embryo and *Vav-cre/Ddx3*$^{fl/fl}$ at stage E14.5. **E,** H&E staining of an embryo at E14.4; black arrows show RBC and white arrows show abnormal cellular debris; HRT: heart; LVP: liver parenchyma; S: stomach. **F,** May-Grünwald-Giemsa staining of fetal blood from the embryo of the indicated genotypes and stages of development. **G,** Quantification of enucleated cells in fetal blood samples from embryos with the indicated ages and genotypes; *t*-test is used to determine significance.

These conditional KO mice were bred to the *Vav-cre* deleter specific to hematopoietic cells since the early stages of differentiation (Georgiades et al., 2002) (**Figure 3-1B**). The *cre*-mediated deletion of the floxed exon 2 leads to an excision of *Ddx3x* as detected by PCR analysis (**Figure 3-1C**). First, full KO mice which are, according to the X chromosome localization of *Ddx3x*, homozygous female *Vav-cre*/*Ddx3*$^{fl/fl}$ mice were analyzed. Female *Vav-cre*/*Ddx3*$^{fl/fl}$ pups harboring a full *Ddx3x*-KO were never obtained indicating that a developmental arrest must have occurred. However, *Vav-cre*/*Ddx3*$^{fl/fl}$ embryos could be analyzed at E14.5 (**Figure 3-1D**). Compared to control genotypes (referring here to all genotypes generating viable mice - i.e. all possible genotypes other than *Vav-cre*/*Ddx3*$^{fl/fl}$), *Vav-cre*/*Ddx3*$^{fl/fl}$ embryos were smaller and showed an abnormal fetal liver with a decreased cellularity (**Figure 3-1D, E**). Blood smears of *Vav-cre*/*Ddx3*$^{fl/fl}$ embryos demonstrated a severe decrease of enucleated erythrocytes suggesting a block of the definitive erythropoiesis (**Figure 3-1F, G**).

To investigate this phenotype further, *Vav-cre*/*Ddx3x*-floxed mice were bred to the *R26*$^{mT/mG}$ (membrane-tdTomato/membrane-Green) reporter strain (Muzumdar et al., 2007) (**Figure 3-2A**).

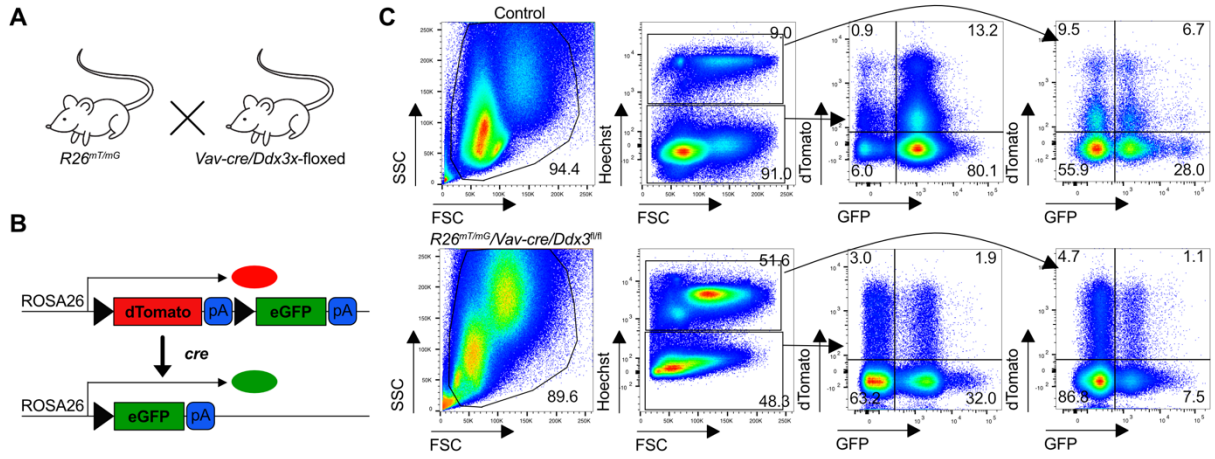


Figure 3-2: *Ddx3x* deletion impairs definitive erythropoiesis

A, Schematic representation of the breeding strategy. **B,** Representation of the transgenic *R26*$^{mT/mG}$ mouse model. Briefly, this transgene allows GFP expression in cells harboring a *cre* system while it allows the tdTomato expression

in the absence of *cre* activity. **C,** Blood was collected from embryos of *R26*$^{mT/mG}$/*Vav-cre/Ddx3x*-floxed mice and submitted to FACS analyses where GFP and tdTomato fluorescence was detected in nucleated (Hoechst^{+}) and enucleated (Hoechst^{-}) cells.

R26$^{mT/mG}$ mice were designed to express the tdTomato red fluorescent protein in all cell types. In cells harboring a *cre* (here, all hematopoietic cells when bred to the *Vav-cre*), homologous recombination led to an excision of the tdTomato sequence and activated GFP expression, allowing for tracking the cells with an active *cre* (**Figure 3-2B**). Blood was collected from the *R26*$^{mT/mG}$/*Vav-cre/Ddx3*$^{fl/fl}$ embryos and stained with Hoechst to discriminate nucleated cells (Hoechst^{+}) from enucleated cells (Hoechst^{-}). Hoechst staining confirmed the decrease of enucleated cells in *R26*$^{mT/mG}$/*Vav-cre/Ddx3*$^{fl/fl}$ embryos (**Figure 3-2C**). Moreover, both green and red fluorescence could be detected in nucleated and enucleated erythrocytes, albeit with a significant reduction of GFP^{+} cells in the enucleated cells from *R26*$^{mT/mG}$/*Vav-cre/Ddx3*$^{fl/fl}$ embryos compared to controls. These results suggest that enucleated cells with a *Ddx3x*-deletion died and are likely to be the cause of the developmental arrest. A *Ddx3x*-deletion in hematopoietic cells impaired fetal erythropoiesis by blocking the transition from primitive to definitive erythropoiesis. Interestingly, levels of GFP were higher in enucleated erythrocytes compared to nucleated erythrocytes from control mice suggesting that the *Vav-cre* is mostly deleting at the beginning of definitive erythropoiesis and the phenotype observed in KO mice probably correlates with *Vav-cre* activation.

Accordingly, it was impossible to analyze adult *Vav-cre/Ddx3*$^{fl/fl}$ mice representing the full *Ddx3x*-KO. However, the *Vav-cre/Ddx3*-floxed breeding strategy generated other genotypes: heterozygous female *Vav-cre/Ddx3*$^{X/fl}$ mice and hemizygous male *Vav-cre/Ddx3*$^{fl/Y}$ mice carrying a *Ddx3x*-floxed allele along with an intact *Ddx3y* allele. *Vav-cre/Ddx3*$^{X/fl}$ mice were obtained at a mendelian ratio indicating that one *Ddx3x* allele is sufficient to prevent the developmental arrest caused by a defect in fetal erythropoiesis. Similarly, male *Vav-cre/Ddx3*$^{fl/Y}$ mice also overcame

the erythroid developmental block and developed into adulthood, indicating some compensatory effects, very likely from the Y chromosome; and very likely from the highly similar *Ddx3y* gene. However, the presence of the murine male specific-DDX3Y protein was suspected (Deschepper, 2020; Rauschendorf et al., 2011), but was never clearly demonstrated due to the absence of a DDX3Y-specific antibody. Therefore, three peptides were designed (collaboration with Dr. Riyan Chen) based on the few differences observed between murine DDX3X and DDX3Y amino acid sequences (**Figure 1-5**). In collaboration with Biomatik, those three peptides were injected into rabbits to generate antibodies targeting specifically DDX3Y and not DDX3X (**Figure 3-3A**).

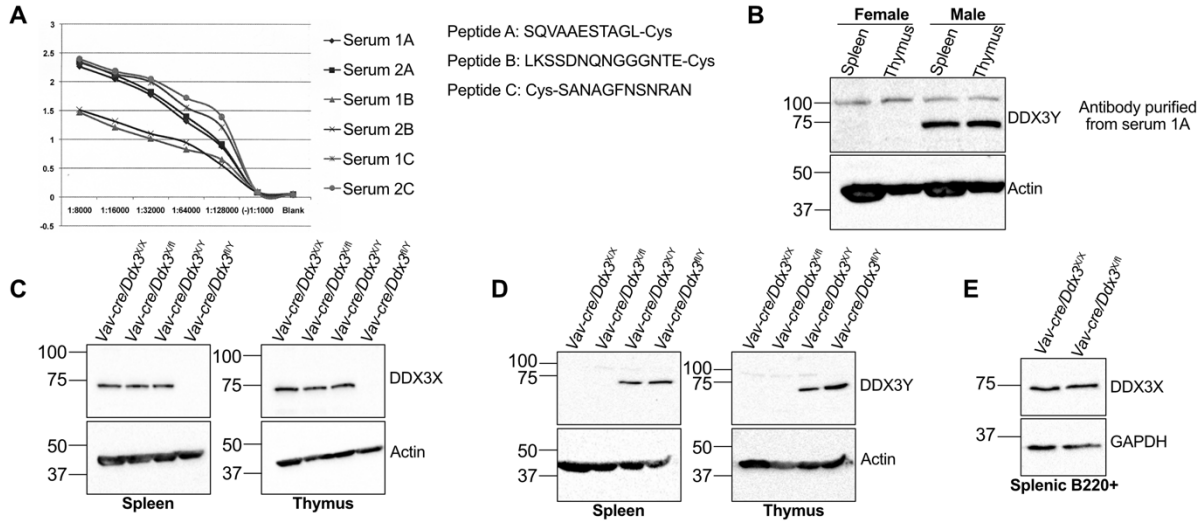


Figure 3-3: Analyses of DDX3X and DDX3Y protein expression in several murine tissues

A, Elisa experiment to determine antigen affinity of DDX3Y antibodies purified from 6 immunized rabbits. The three indicated peptides were injected each in 2 animals from which the sera were extracted and purified. Riyan Chen designed the peptides, and this experiment was realized by Biomatik. **B,** Western blot with samples extracted from WT male and female mice. Membranes were blotted with anti-DDX3Y purified from antiserum 1A. **C,** DDX3X Western Blot of splenocytes and thymocytes extracted from animals with indicated genotypes. **D,** DDX3Y Western Blot of splenocytes and thymocytes extracted from animals with indicated genotypes. **E,** Western Blot of $B220^{+}$ splenic cells isolated from animals with indicated genotypes.

A newly generated antibody was purified and demonstrated male specificity by Western blot analysis (**Figure 3-3B**). It was then confirmed that *Vav-cre*/*Ddx3*$^{fl/Y}$ adult mice lacked DDX3X but expressed DDX3Y in the spleen and thymus compared to *Vav-cre*/*Ddx3*$^{X/Y}$ control male mice with two DDX3X- and DDX3Y-specific antibodies (**Figure 3-3C, D**). *Vav-cre/Ddx3*$^{X/fl}$

heterozygous mice have a similar level of DDX3X protein in the spleen and thymus as well as in splenic B220^{+} cells compared to *Vav-cre/Ddx3*$^{X/X}$ control female mice (**Figure 3-3C-E**).

Since the full *Ddx3x*-KO mice provoked erythroid defects, erythroid differentiation of *Vav-cre/Ddx3*$^{fl/Y}$ and *Vav-cre/Ddx3*$^{X/fl}$ adult mice was analyzed by flow cytometry. PreMegE progenitors were reduced in *Vav-cre/Ddx3*$^{fl/Y}$ male mice although in the next stage of differentiation, the PreCFUe were unaffected (**Figure 3-4A, B**).

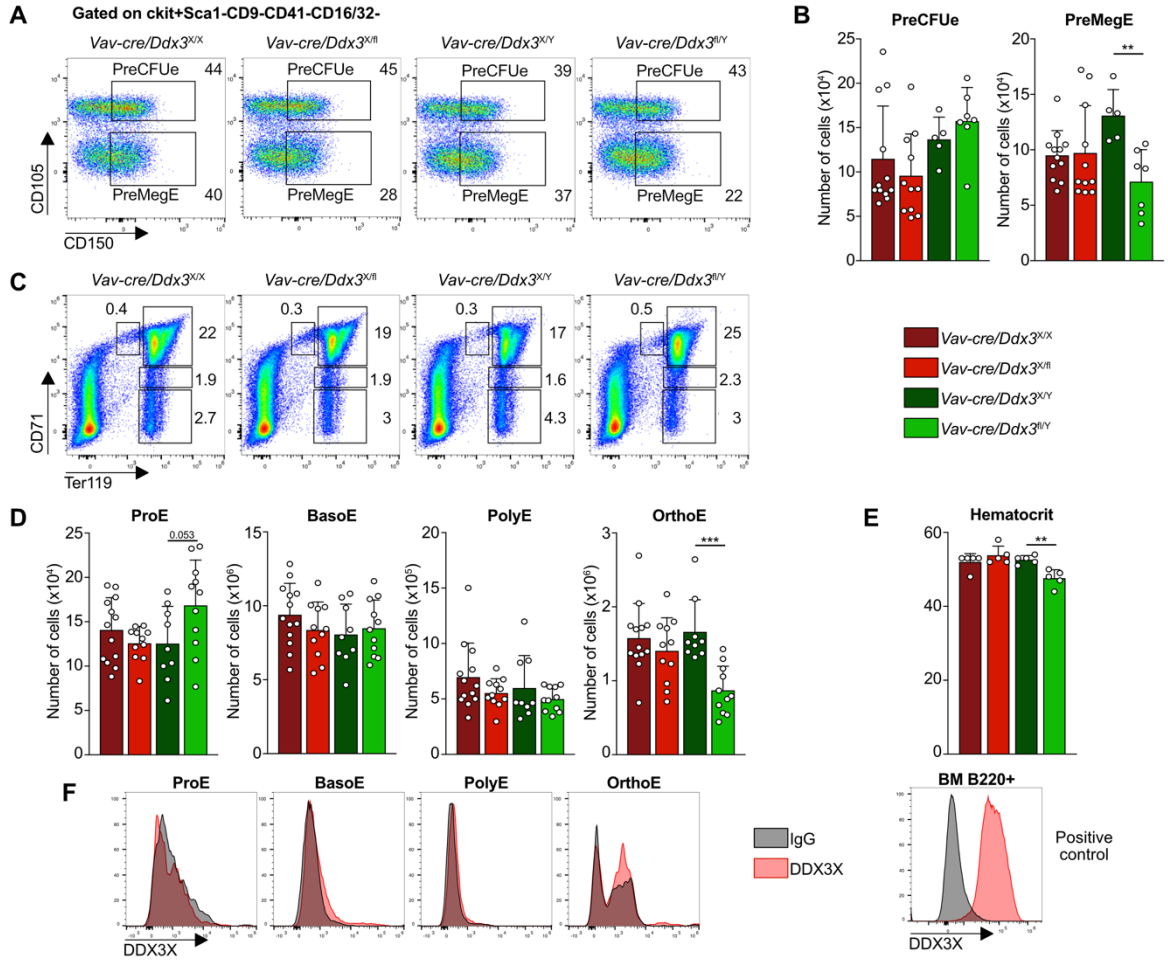


Figure 3-4: Erythroid differentiation is impaired in *Vav-cre/Ddx3*$^{fl/Y}$ adult males

A, Representative flow cytometry analyses and **B,** quantification of early erythroid (PreCFUe) and pre-megakaryocyte/erythroid (PreMegE) progenitors in adult BM. **C,** Representative flow cytometry analyses and **(D)** quantification of erythroid developmental stages from proerythroblasts (ProE) to basophilic (BasoE), polychromatic (PolyE) and orthochromatic (OrthoE) erythroblast stages in adult BM from animals with the indicated genotypes. Populations were defined as follows: ProE (CD71^{+}Ter119^{low}), BasoE (CD71^{+}Ter119^{+}), PolyE (CD71^{low}Ter119^{+}), OrthoE (CD71^{+}Ter119^{+}). **E,** Percentages of RBC in the blood of mice (i.e. hematocrit) with indicated genotypes. *t*-test with Welsh correction was used to compare female heterozygous with female controls and male KO with male controls. The p value is indicated as follows: *ns*, p>0.05, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. **F,** BM cells

from WT male animals were extracted and stained with CD71 and Ter119 extracellular markers to define the RBC differentiation steps. The cells were fixed and stained with DDX3X antibody or IgG control followed by incubation with a PE-secondary antibody. Fluorescence in different RBC populations was detected by flow cytometry. A positive control using BM-B220^{+} cells demonstrated the antibody's suitability to detect intracellular DDX3X by flow cytometry.

Vav-cre/Ddx3$^{fl/Y}$ males exhibited increased ProE along intact BasoE and PolyE stages and decreased OrthoE (**Figure 3-4C, D**). Moreover, *Vav-cre/Ddx3*$^{fl/Y}$ presented a decreased hematocrit -i.e. a decreased percentage of RBC in their peripheral blood even if this reduction was not life-threatening (**Figure 3-4E**). The lack of DDX3X protein expression in different subsets of the RBC differentiation suggested that defects observed in *Vav-cre/Ddx3*$^{fl/Y}$ were likely a consequence of defects occurring in earlier erythroid populations (**Figure 3-4F**). While the *Ddx3y* gene was supposedly compensating for erythroid defects seen in *Ddx3x*-KO embryo, this compensation was imperfect as several erythroid populations are affected by *Ddx3x*-KO in adult male mice. All of these mild erythropoietic defects observed in adult *Vav-cre/Ddx3*$^{fl/Y}$ were not seen in *Vav-cre/Ddx3*$^{X/fl}$ females (**Figure 3-4A-E**).

2. DDX3X's role in hematopoietic progenitors

The effect of *Ddx3x*-deletion on hematopoietic progenitors from adult mice was then assessed. The BM was extracted from adult *Vav-cre/Ddx3*$^{fl/Y}$ and *Vav-cre/Ddx3*$^{X/fl}$ mice as well as sex-matched controls and submitted to flow cytometry analyses. A decrease of LK and LSK groups of hematopoietic progenitors was detected specifically in male *Vav-cre/Ddx3*$^{fl/Y}$ mice (**Figure 3-5A**). More precisely, flow cytometry analyses revealed that MPPs, LMPPs and CLPs are diminished in male KO animals compared to sex-matched controls (**Figure 3-5B, C**).

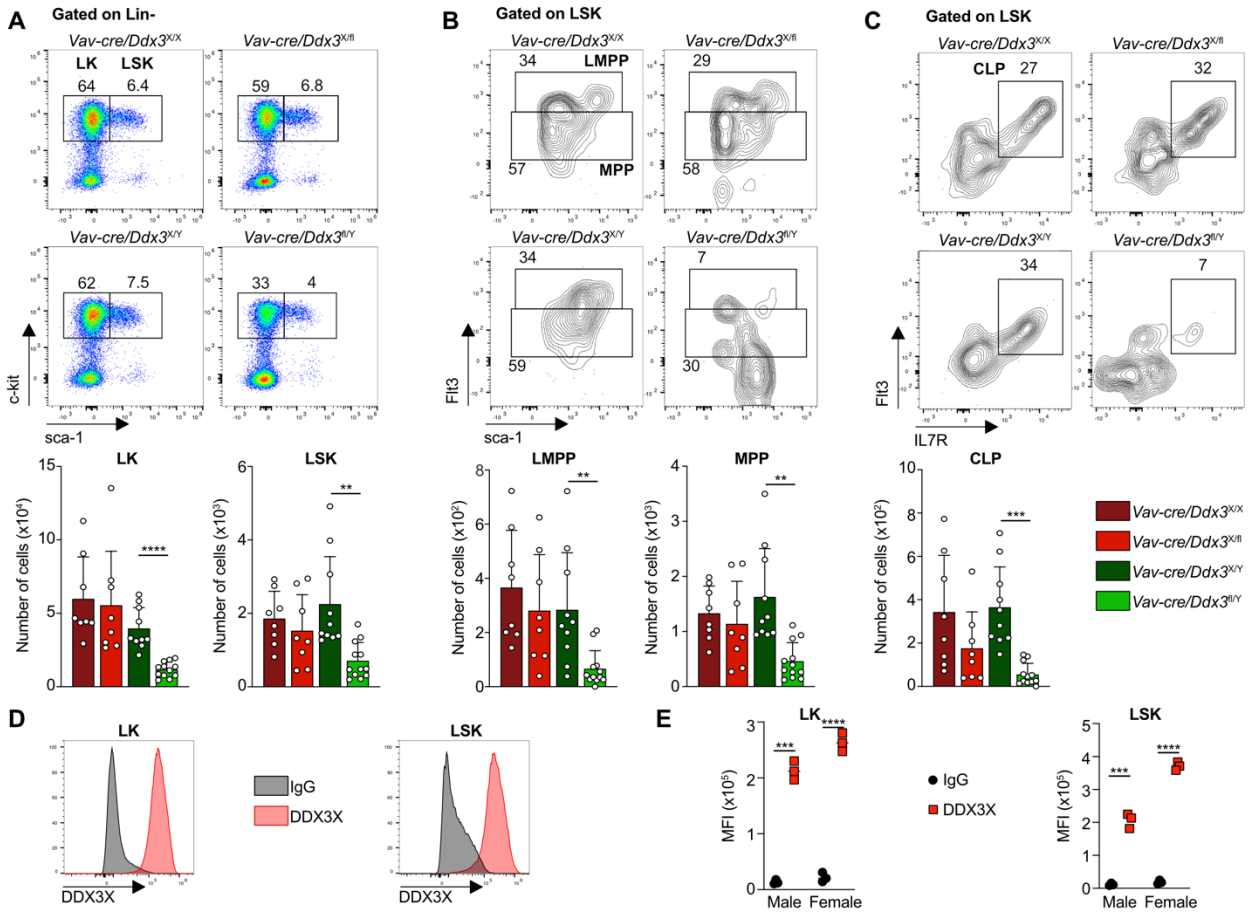


Figure 3-5: LK and LSK groups of hematopoietic progenitors are decreased in male *Ddx3x*-depleted

A, B, C, Flow cytometry analysis of BM from animals with the indicated genotype and quantification of the absolute number of cells. Populations were defined as follows: LK ($Lin^-c\text{-}kit^+sca\text{-}1^-$), LSK ($Lin^-c\text{-}kit^+sca\text{-}1^+$), MPP ($Lin^-c\text{-}kit^+sca\text{-}1^-Flt3^{low}$), LMPP ($Lin^-c\text{-}kit^+sca\text{-}1^-Flt3^+$), CLP ($Lin^-c\text{-}kit^+sca\text{-}1^-Flt3^+IL7R^+$). *t*-test with Welsh correction was used to compare female heterozygous with female controls and male KO mice with male controls. The p value is indicated as follows: *ns*, p>0.05, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. **D,** DDX3X protein expression was assessed by intracellular staining followed by flow cytometry in LK and LSK populations. **E,** Quantification of DDX3X protein expression in LK and LSK cells. Two-way ANOVA was used to compare IgG and DDX3X fluorescent signals. LK: $Lin^-c\text{-}kit^+$; LSK: $Lin^-Sca^+c\text{-}kit^+$; MPP: Multiple Pluripotent Progenitor; LMPP: Lymphoid-primed Multipotent Progenitor; CLP: Common Lymphoid Progenitor.

DDX3X protein expression was detected in LK and LSK cells of WT animals demonstrating that the absence of DDX3X protein provokes a diminution of hematopoietic progenitors and lymphoid progenitors in male mice (**Figure 3-5D, E**).

Since LK and LSK were affected in *Vav-cre/Ddx3*$^{fl/Y}$ mice, additional populations of hematopoietic progenitors were analyzed. The long-term HSCs (LT-HSC) were not impacted by DDX3X loss regardless of sex (**Figure 3-6A**).

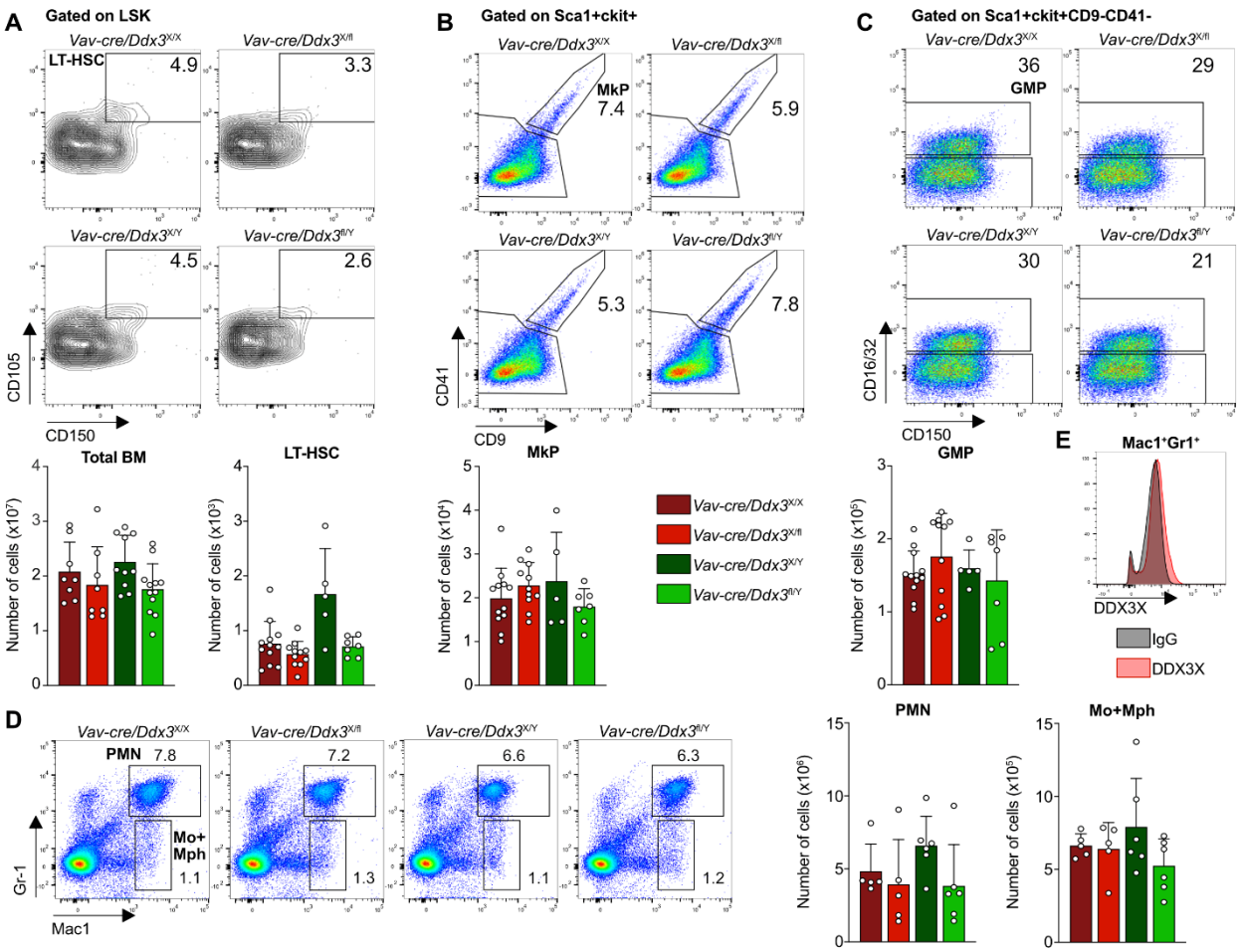


Figure 3-6: Hematopoietic stem cells, megakaryocytes and myeloid progenitors are not impacted by *Ddx3x*-deletion

Flow cytometry analysis of BM LT-HSC in **(A)**, MKP in **(B)** and GMP in **(C)** from *Ddx3x*-KO mice and quantification in absolute number. Populations were defined as follows: LT-HSC ($Lin^-c\text{-}kit^+sca\text{-}1^+CD105^+CD150^+$), MKP ($c\text{-}kit^+sca\text{-}1^+CD41^+CD9^+$), GMP ($c\text{-}kit^+sca\text{-}1^+CD9^-CD41^-CD16/32^+$). **D,** Flow cytometry analysis of myeloid cells in *Vav-cre*/*Ddx3x*-floxed mice. Populations were defined as: PMN ($Mac1^+Gr\text{-}1^+$), Mo+Mph ($Mac1^+Gr\text{-}1^-$). *t*-test with Welsh correction was used to compare female heterozygous mice with female controls and male KO mice with male controls. The p value is indicated as follows: *ns*, p>0.05, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. **E,** DDX3X protein expression was assessed by intracellular staining followed by flow cytometry $Mac1^+Gr1^+$ population representing myeloid cells. LT-HSC: Long-term Hematopoietic Stem cell; GMP: Granulocyte/Monocyte Progenitor; MKP: Megakaryocyte Progenitor; PMN: Polymorphonuclear leukocyte; Mo+Mph: Monocyte and macrophage.

MKPs were not affected by DDX3X loss in both male and female *Ddx3x*-deleted mice (**Figure 3-6B**), whereas PreMegE progenitors were diminished in adult *Vav-cre/Ddx3*$^{fl/Y}$ mice (**Figure 3-4A, B**). GMPs were not impaired by *Ddx3x*-deletion (**Figure 3-6C**); neither were the differentiated myeloid cells gated as monocytes/macrophages (Mo+Mph) and PMN cells also named granulocytes (**Figure 3-6D**). These data indicated that myelopoiesis does not rely on DDX3X most

likely because this protein is not expressed at readily detectable levels in this population (**Figure 3-6E**).

The differentiation and self-renewal capacities of DDX3X deficient hematopoietic progenitors were then investigated. LSK progenitors were sorted from the BM of *Vav-cre/Ddx3*$^{fl/Y}$ and *Vav-cre/Ddx3*$^{X/Y}$ control mice and analyzed *in vitro*. First, the differentiation capacity was tested by co-cultivating *Ddx3x*-deleted LSKs on OP9 or OP9-DL1 cells with cytokines to test respectively the capacity of B and T lymphoid differentiation. *Ddx3x*-deleted LSKs were unable to differentiate *in vitro* into B220^{+}CD19^{+} cells (**Figure 3-7A, B**), nor did they reach the T cell double negative (DN) stages of differentiation (**Figure 3-7C, D**).

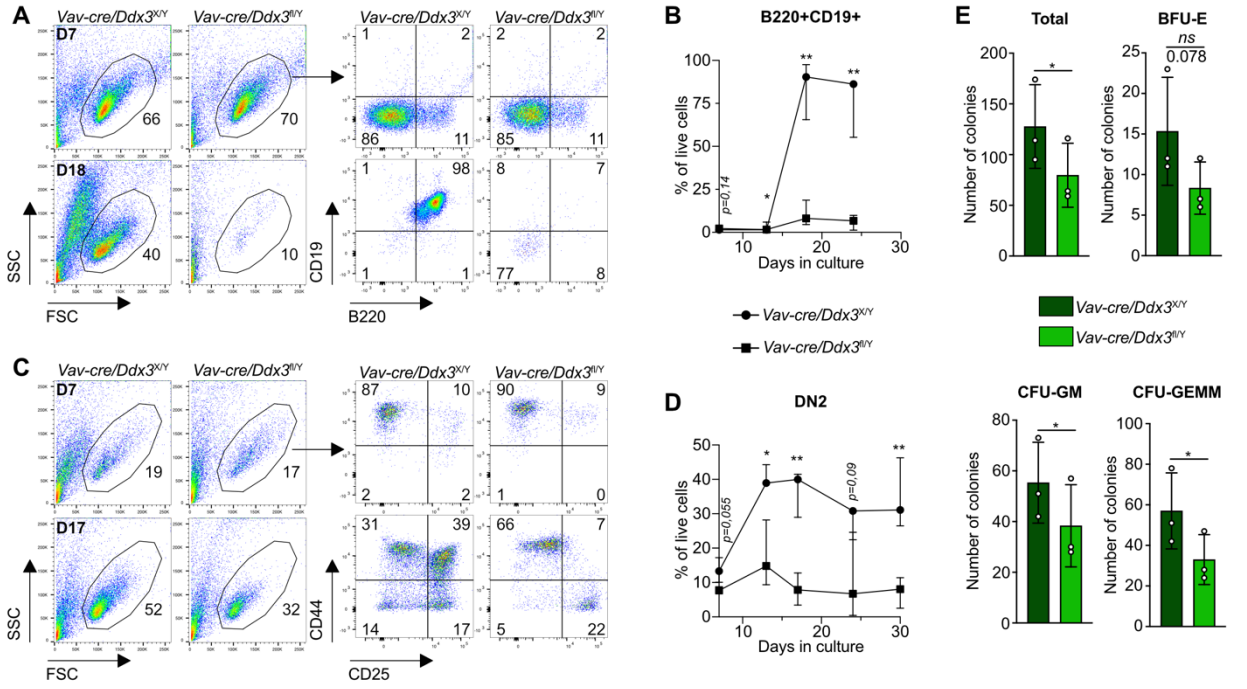


Figure 3-7: Loss of DDX3X impairs the lymphoid differentiation

A, LSKs were sorted from the BM of male KO and control mice and co-cultured on the OP9 layer with IL-7 and Flt3 cytokines for B cell differentiation. **B,** B220^{+}CD19^{+} cells are quantified relatively to live cells by flow cytometry through the differentiation. **C,** LSKs were sorted from the BM of male KO and control mice and co-cultured on the OP9-DL1 layer with SCF, Flt3 and IL-7 cytokines for T cell differentiation. **D,** DN2 cells (CD44^{+}CD25^{+}) were quantified relatively to live cells by flow cytometry through the differentiation. Two experiments were performed in triplicate for both B and T cell differentiation. **E,** Sorted LSK cells were grown in Methocult GF-M3434 media at 37°C and colonies were counted and identified 7 days later. This experiment was performed three times and paired *t*-test was used to assess statical significance. The p value is indicated as follows: *ns*, p>0.05, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. CFU: Colony Forming Unit; CFU-GM: CFU-Granulocyte, Monocyte; CFU-GEMM:

CFU-Granulocyte, Erythrocyte, Monocyte, Megakaryocyte; BFU-E: Burst Forming Unit, Erythrocyte; DN2: Double Negative stage 2.

Second, the self-renewal capacity was tested by culturing sorted progenitors in a methylcellulose-based media. *Ddx3x*-depleted LSKs produced fewer colonies *in vitro* compared to controls, without any lineage bias (**Figure 3-7E**). These data indicated that DDX3X is required to maintain the cellularity and lymphoid lineage potential of adult hematopoietic progenitor cells.

3. *Ddx3x*-deletion affects lymphoid differentiation

Due to the defects observed in the lymphoid differentiation capacity of *Ddx3x*-deficient LSKs as well as the decrease of CLP progenitors in *Vav-cre/Ddx3*$^{fl/Y}$ mice, the following steps of lymphoid differentiation were investigated. *Ddx3x*-deletion in *Vav-cre*/*Ddx3*$^{fl/Y}$ mice correlated with a significantly reduced number of cells at DN2 and DN3 stages of pre-T cell differentiation (**Figure 3-8A, B**). Interestingly, these same DN2 and DN3 stages were those impaired in *in vitro* T cell differentiation assay (**Figure 3-7C, D**). However, this defect did not impact the following differentiation steps since DP, CD4 and CD8 cells were intact in the thymus of DDX3X-KO mice (**Figure 3-8C, D**).

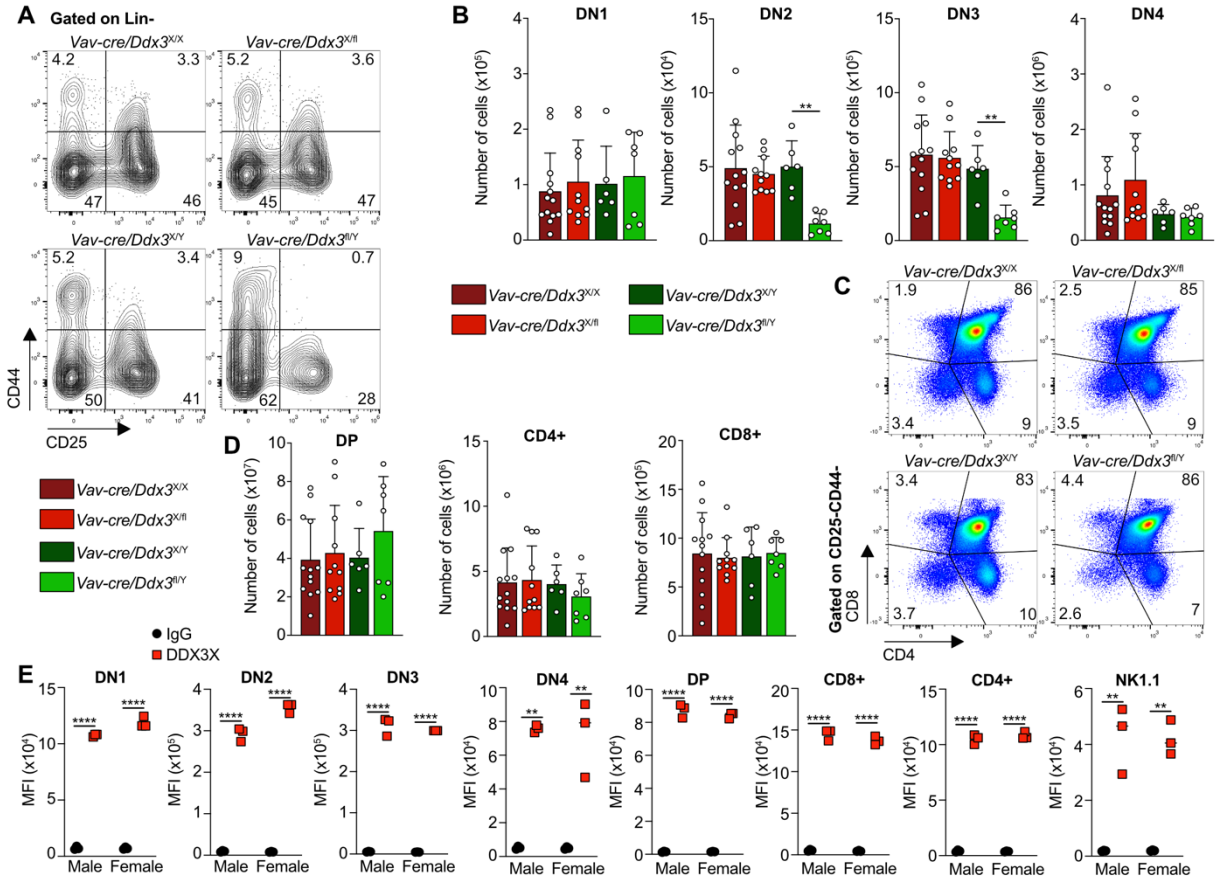


Figure 3-8: DN2 and DN3 stages of T-cell differentiation are impaired by *Ddx3x*-deletion

A, Thymi were extracted from *Vav-cre/Ddx3x*-floxed mice and analyzed by flow cytometry with extracellular markers for T cell progenitors (DN stages from 1 to 4). Populations were defined as followed: DN1 ($Lin^-CD44^+CD25^-$), DN2 ($Lin^-CD44^+CD25^+$), DN3 ($Lin^-CD44^-CD25^+$), DN4 ($Lin^-CD44^-CD25^-$). **B,** Quantification of T cell populations in absolute numbers. *t*-test with Welsh correction was used to compare female heterozygous mice with female controls and male KO mice with male controls. **C,** Flow cytometry analysis of thymic CD4 and CD8 T-cells extracted from *Vav-cre*/*Ddx3x*-floxed and absolute quantification in **(D)**. **E,** DDX3X protein expression was assessed by intracellular staining followed by flow cytometry in thymic T cell populations. Two-way ANOVA was used to compare IgG and DDX3X fluorescent signals. The p value is indicated as follows: *ns*, p>0.05, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. DN: Double Negatif; DP: Double Positif; NK: Natural Killer.

DDX3X protein expression was evident in all stages of T cell differentiation and NK cells and was particularly elevated in DN2 and DN3 cells (**Figure 3-8E**). Analysis of mature T cell differentiation in *Vav-cre/Ddx3x*-floxed mice lymph nodes revealed a CD4/CD8 ratio imbalance (**Figure 3-9A, B**).

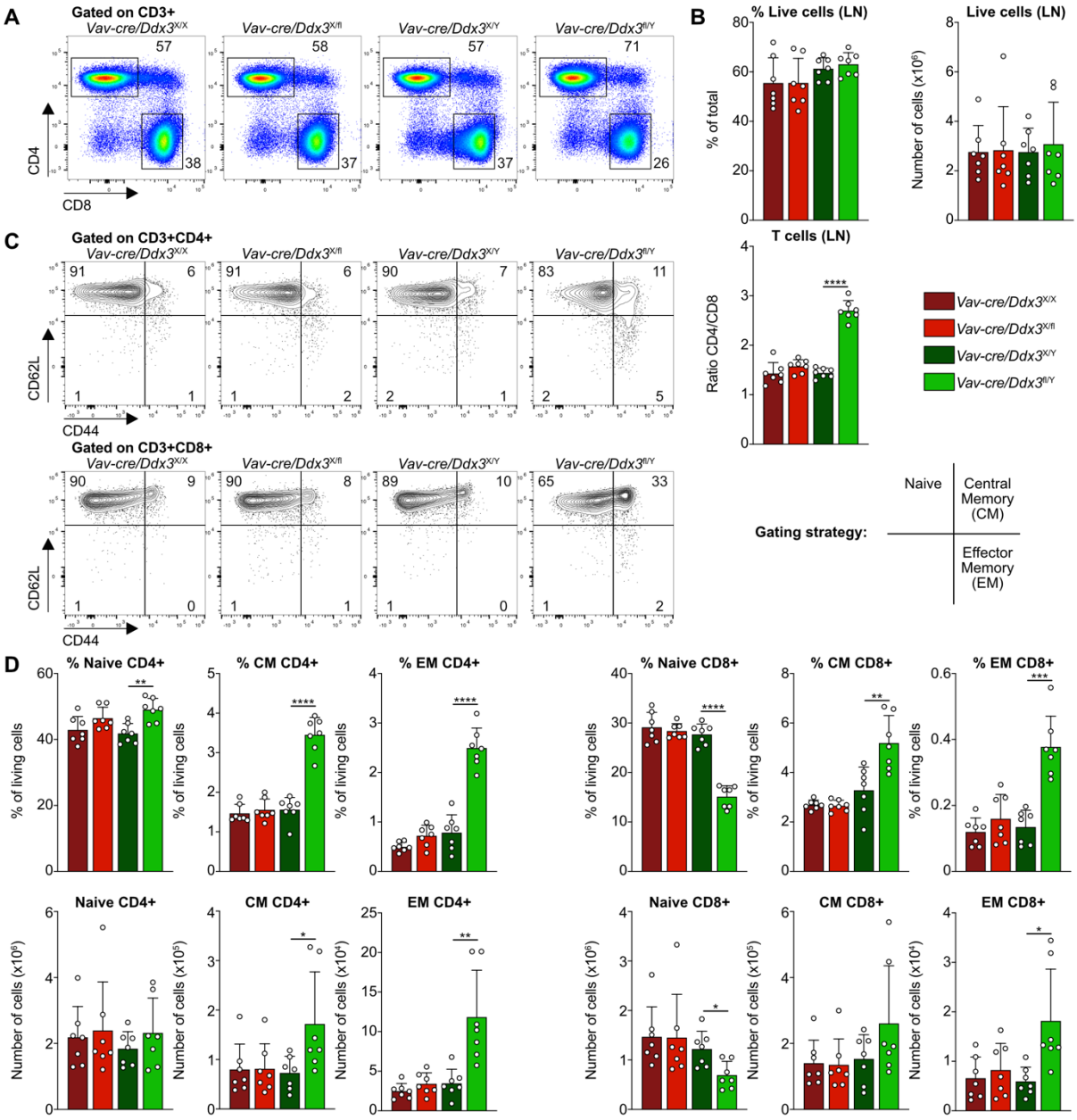


Figure 3-9: Mature T cell subsets are impaired by DDX3X depletion in lymph nodes of male KO mice

A, Flow cytometry analysis of SP CD4 and CD8 T cells extracted from the lymph nodes of *Vav-cre*/*Ddx3x*-floxed mice and quantification in **(B)**. **C,** Flow cytometry analysis of mature T cell subsets extracted from the lymph nodes of *Vav-cre*/*Ddx3x*-floxed mice and quantification in **(D)**. *t*-test with Welsh correction was used to compare female heterozygous mice with female controls and male KO mice with male controls. The p value is indicated as follows: *ns*, p>0.05, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. LN: lymph node.

An elevated quantity of central and effector memory T cells was detected in the lymph nodes of male KO mice (**Figure 3-9C, D**). Similar phenotypes were detected in splenic T cells from male KO mice (**Figure 3-10A-D**). However, these phenotypes were less striking in the spleen compared

to lymph nodes, an effect which very likely resulted from a decrease of live splenocytes (**Figure 3-10B**).

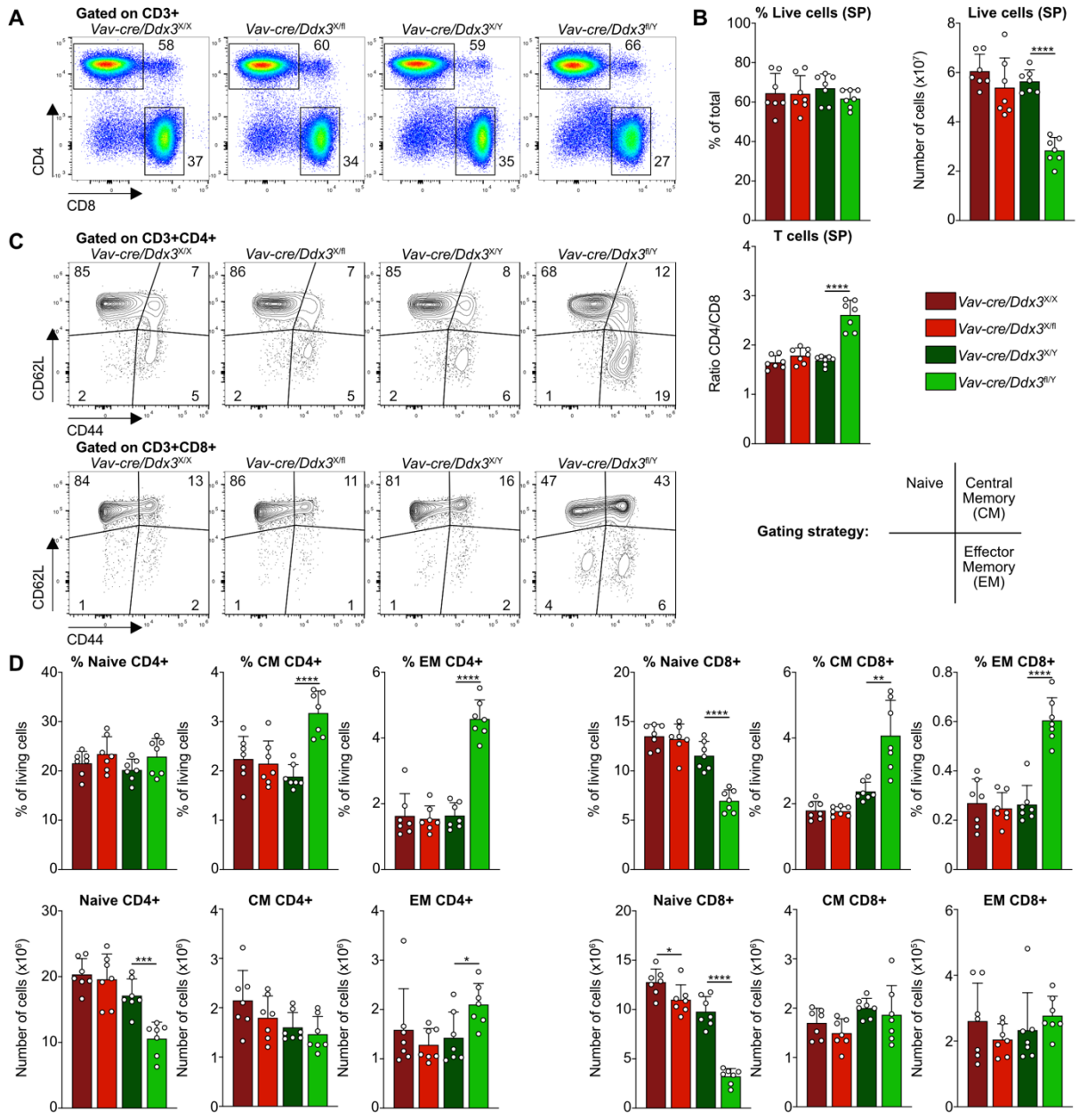


Figure 3-10: Splenic mature T cell subsets are impaired by DDX3X depletion in male KO mice

A, Flow cytometry analysis of SP CD4 and CD8 T cells extracted from spleens of *Vav-cre*/*Ddx3x*-floxed mice and quantification in **(B)**. **C,** Flow cytometry analysis of mature T cell subsets extracted from spleens of *Vav-cre*/*Ddx3x*-floxed mice and quantification in **(D)**. *t*-test with Welsh correction was used to compare female heterozygous mice with female controls and male KO mice with male controls. The p value is indicated as follows: *ns*, p>0.05, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. SP: spleen

Ddx3x-deletion in *Vav-cre*/*Ddx3*$^{fl/Y}$ mice also impaired several stages of B cell differentiation. Pro-B cells (Fraction B) were reduced in male KO mice (**Figure 3-11A, B**).

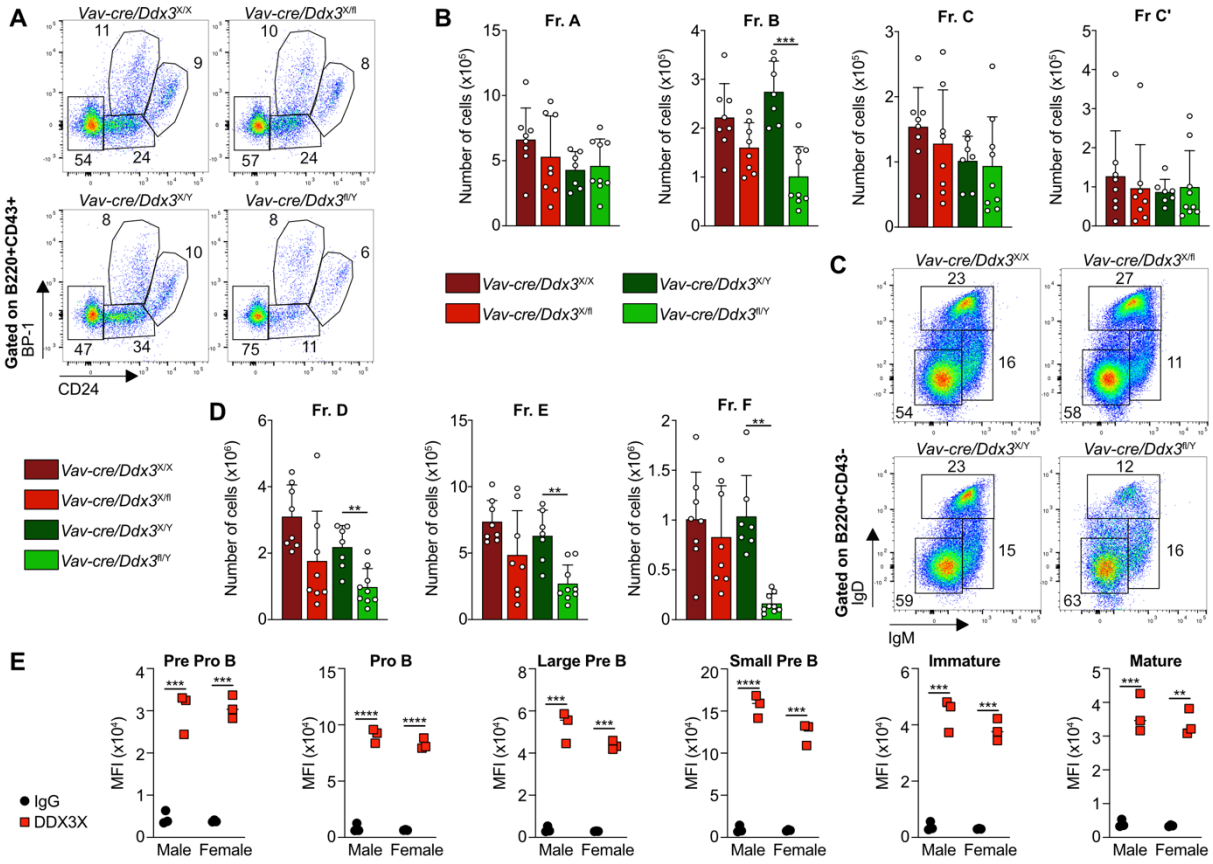


Figure 3-11: DDX3X is required for several steps of early B cell differentiation

A, Flow cytometry analysis of BM B cell progenitors (Hardy fractions) from A to C' and absolute quantification in **(B)**. **C,** Flow cytometry analysis of BM B cell progenitors (Hardy fractions) from D to F and absolute quantification in **(D)**. *t*-test with Welsh correction was used to compare female heterozygous mice with female controls and male KO mice with male controls. **E,** DDX3X protein expression was assessed by intracellular staining followed by flow cytometry in BM B cell progenitors. Two-way ANOVA was used to compare IgG and DDX3X fluorescent signals. The p value is indicated as follows: *ns*, p>0.05, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

In addition, pre-B cells (Fraction D), immature (Fraction E) and recirculating mature B cells (Fraction F) were decreased in male KO mice (**Figure 3-11C, D**). DDX3X protein was found broadly expressed among all subsets of B cell progenitors (**Figure 3-11E**). The total B cell population was diminished in the spleen of *Vav-cre*/*Ddx3*$^{fl/Y}$ mice, as well as total splenic cellularity (**Figure 3-12A, B**).

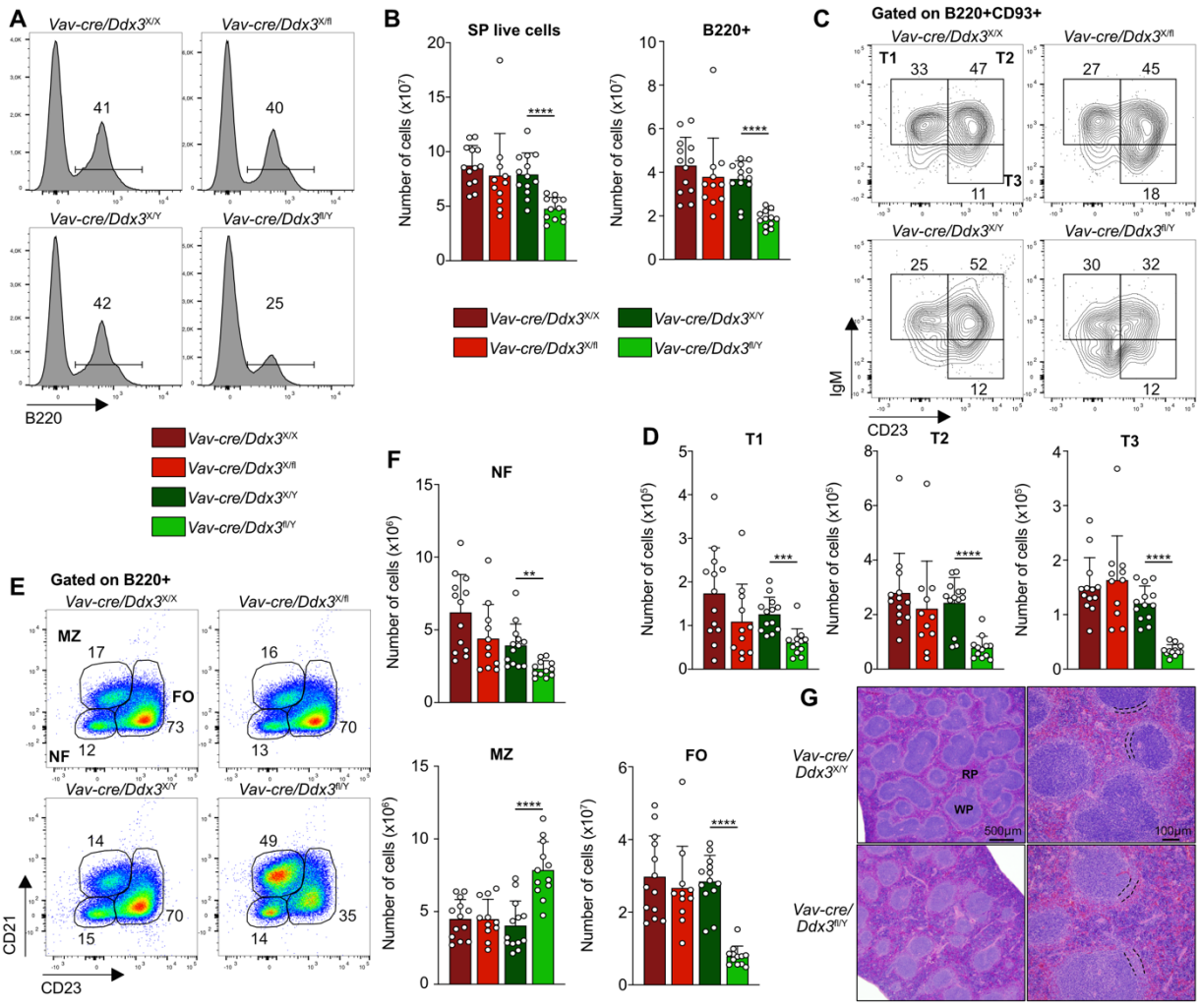


Figure 3-12: Mature B cells and splenic structure are impacted by *Ddx3x*-deletion

A, Flow cytometry analysis of splenic B220^{+} cells extracted from *Vav-cre*/*Ddx3x*-floxed mice. **B,** Quantification of the total number of splenic cells and absolute quantification of B220^{+} cells. **C,** Flow cytometry analysis of splenic transitional B cells extracted from *Vav-cre*/*Ddx3x*-floxed mice and absolute quantification in **(D)**. Populations were defined as follows: transitional B cells (B220^{+}CD93^{+}) divided in T1 (B220^{+}CD93^{+}IgM^{+}CD23^{-}), T2 (B220^{+}CD93^{+}IgM^{+}CD23^{+}) and T3 (B220^{+}CD93^{+}IgM^{-}CD23^{+}).) **E,** Flow cytometry analysis of spleens extracted from *Vav-cre*/*Ddx3x*-floxed mice and absolute quantification in **(F)**. Populations were defined as followed: NF (B220^{+}CD21^{-}CD23^{-}), FO (B220^{+}CD21^{-}CD23^{+}), MZ (B220^{+}CD21^{+}CD23^{-}). *t*-test with Welsh correction was used to compare female heterozygous mice with female controls and male KO mice with male controls. The p value is indicated as follows: *ns*, p>0.05, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. **G,** Splenic histological analysis of male mice with H&E staining. Dotted lines indicate MZ layer. MZ: NF: Newly formed; FO: Follicular; MZ: Marginal Zone; T1, T2, T3: Transitional B cell stage 1, 2 and 3, respectively.

More precisely, the cellularity of their transitional populations (**Figure 3-12C, D**) and FO B cell compartments were decreased, whereas their MZ B cell subset was increased (**Figure 3-12E, F**). Histological sections of spleens from *Vav-cre*/*Ddx3*$^{fl/Y}$ male mice demonstrated altered structures of follicles and MZs compared to control sections (**Figure 3-12G**). GC B cells were significantly

decreased in *Vav-cre*/*Ddx3*$^{fl/Y}$ male KO mice (**Figure 3-13A**), while B220^{+}IgD^{-}CD38^{+} B cells and PCs were unaffected (**Figure 3-13B, C**). DDX3X was found to be expressed in all subtypes of splenic B cells, and its protein expression was higher in GC B cells (**Figure 3-13D**).

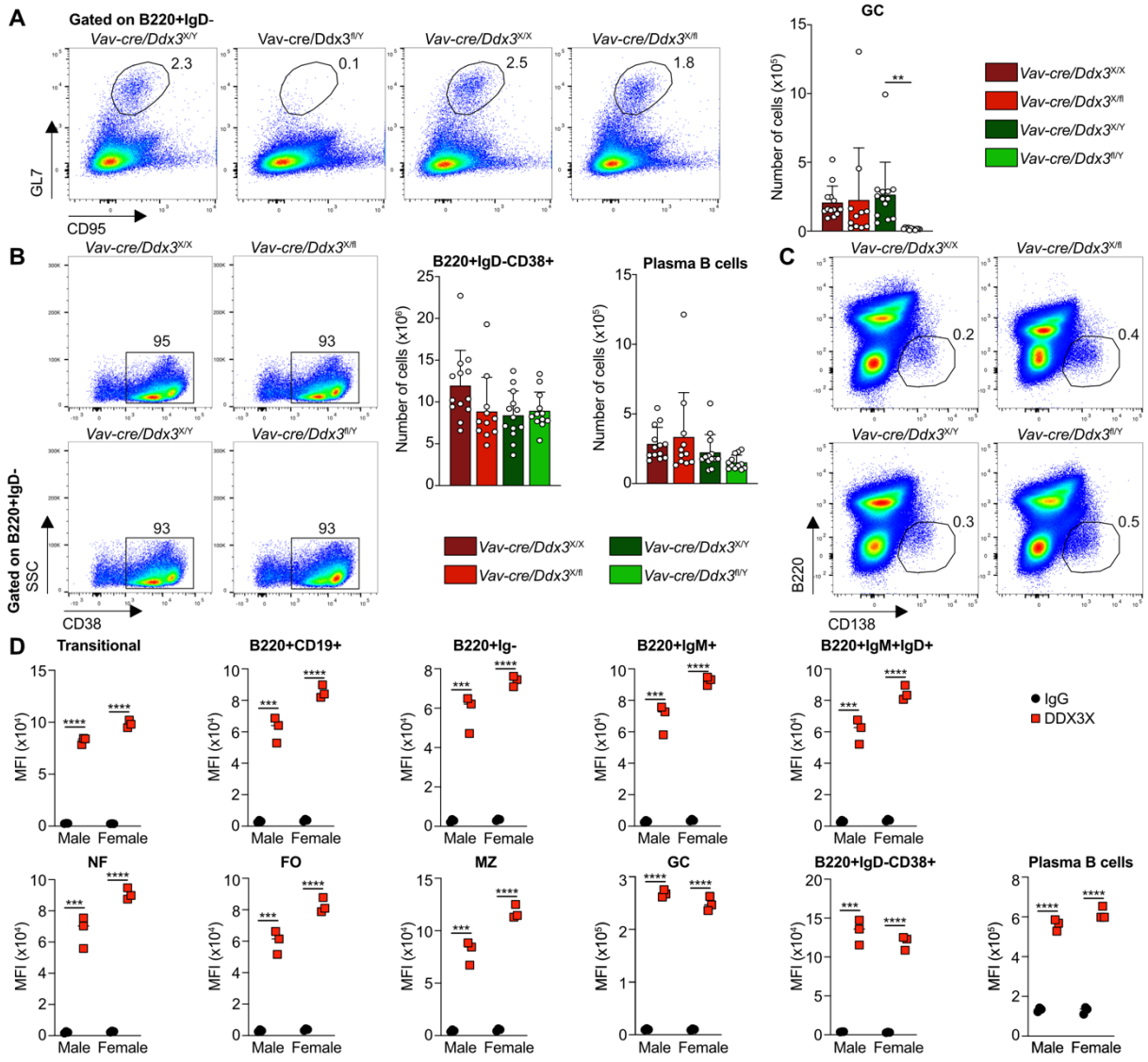


Figure 3-13: Non-immunized GC B cells are decreased in DDX3X KO male mice

A, Flow cytometry analysis of GC (B220^{+}IgD^{-}CD95^{+}GL7^{+}) in the spleen of *Vav-cre/Ddx3x*-floxed mice and absolute quantification. **B,** Flow cytometry analysis of B220^{+}IgD^{-}CD38^{+} B cells and PCs **(C)** from spleens of *Vav-cre*/*Ddx3x*-floxed mice and quantification of absolute cell numbers. *t*-test with Welsh correction was used to compare female heterozygous mice with female controls and male KO mice with male controls. **D,** DDX3X protein expression was assessed by intracellular staining followed by flow cytometry in splenic B cells. Two-way ANOVA was used to compare IgG and DDX3X fluorescent signals. The p value is indicated as follows: *ns*, p>0.05, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

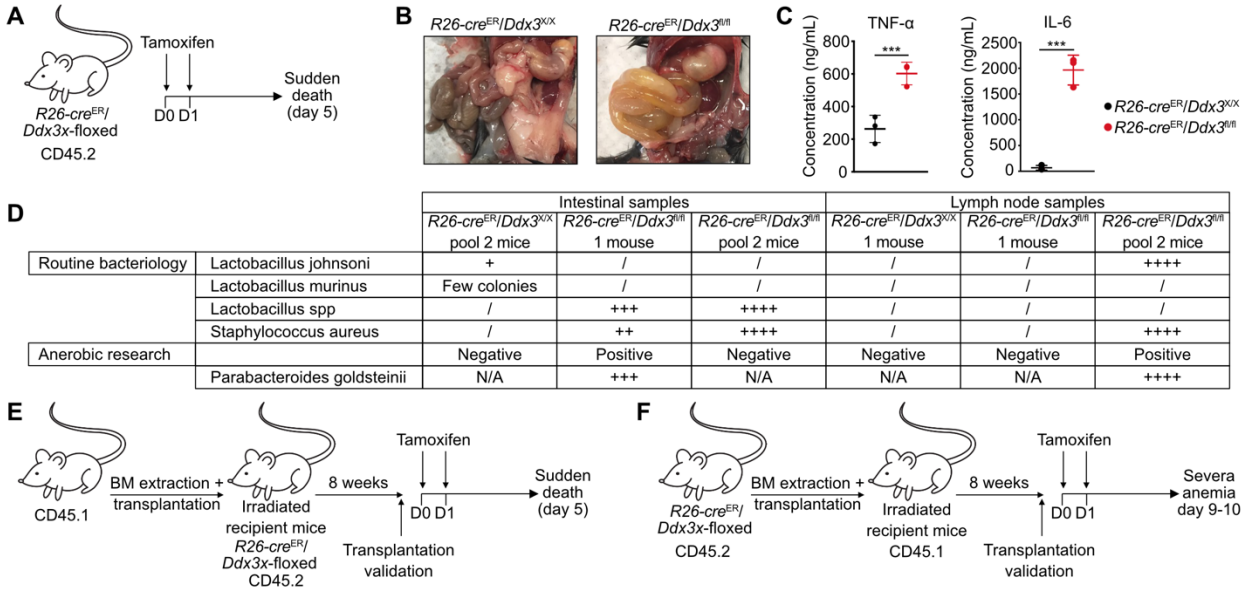
To summarize, several lymphoid populations were decreased in *Vav-cre*/*Ddx3*$^{fl/Y}$ mice: the DN2-DN3 thymic T cell stages; CD4/CD8 ratio and memory T cells in the spleen and lymph nodes, as well as pro-B cell, pre-B cell, immature and recirculating mature B cell BM populations; and transitional, FO and GC B cells. Surprisingly, MZ B cells were increased in the *Vav-cre*/*Ddx3*$^{fl/Y}$ mice. In addition, *Vav-cre*/*Ddx3*$^{fl/Y}$ mice harbored several defects in erythroid populations and hematopoietic progenitors. Therefore, I concluded that DDX3X is essential for murine hematopoiesis, more precisely for the maintenance and function of hematopoiesis progenitors, erythropoiesis and lymphopoiesis. All these phenotypes were detected in *Ddx3x*-deleted male mice while no phenotype was detected in *Vav-cre/Ddx3*$^{X/fl}$ heterozygous females, suggesting that one *Ddx3x* allele is sufficient to maintain proper hematopoiesis in mice.

4. Inducible *Ddx3x*-deletion in adult female mice causes BM failure

Even though analyses of hemizygous *Vav-cre*/*Ddx3*$^{fl/Y}$ and heterozygous *Vav-cre/Ddx3*$^{X/fl}$ mice have permitted the discovery of several hematopoietic phenotypes, both models did not constitute a perfect KO because either the *Ddx3y* allele or one intact *Ddx3x* allele was always present and most likely exert compensatory effects as also seen during embryonic erythropoiesis (**Figure 3-1**). Since this compensation may hide some effect(s) caused by *Ddx3x*-deletion, the generation of female full-KO mice was necessary. Therefore, *Ddx3x*-floxed mice were crossed with *R26-cre*^{ER} animals allowing *cre*-mediated deletion of floxed alleles upon tamoxifen administration (Badea et al., 2003). The fatal defect of embryonic erythropoiesis observed in full-KO mice was overcome in this model by activating *Ddx3x*-deletion in adult animals. However, the

R26-cre^{ER} deleter allele is not specific to the hematopoietic cells and is ubiquitously active; but this *cre* had the advantage of being inducible.

The first strategy was to inject tamoxifen in *R26-cre*^{ER}/*Ddx3x*-floxed adult mice and analyze the impact on hematopoietic cells (**Figure 3-14A**).



		Intestinal samples			Lymph node samples		
		R26-cre^{ER}/*Ddx3*$^{X/X}$ pool 2 mice	*R26-cre*^{ER}/*Ddx3*$^{fl/fl}$ 1 mouse	*R26-cre*^{ER}/*Ddx3*$^{fl/fl}$ pool 2 mice	*R26-cre*^{ER}/*Ddx3*$^{X/X}$ 1 mouse	*R26-cre*^{ER}/*Ddx3*$^{fl/fl}$ 1 mouse	*R26-cre*^{ER}/*Ddx3*$^{fl/fl}$ pool 2 mice
Routine bacteriology	Lactobacillus johnsoni	+	/	/	/	/	++++
	Lactobacillus murinus	Few colonies	/	/	/	/	/
	Lactobacillus spp	/	+++	++++	/	/	/
	Staphylococcus aureus	/	++	++++	/	/	++++
Anerobic research		Negative	Positive	Negative	Negative	Negative	Positive
	Parabacteroides goldsteinii	N/A	+++	N/A	N/A	N/A	++++

Figure 3-14: *Ddx3x*-acute deletion in adult female mice induces a sudden death within days

A, Schematic representation of the acute deletion in adult female mice. **B,** Pictures of the intestine 5 days post tamoxifen injection of mice with indicated genotypes. **C,** Concentration of TNF-α and IL-6 cytokines measured by ELISA from the sera of mice with the indicated genotype. *t*-test with Welsh correction was used to compare female heterozygous mice with female controls and male KO mice with male controls and the p value is indicated as follows: *ns*, p>0.05, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. **D,** Table representation of results from bacteriology tests; collaboration with Dr. Ovidiu Jumanca, IRCM veterinarian and director of animal facilities and animal experimentation at the IRCM. Samples were collected with a swab from the intestines and lymph nodes of mice with indicated genotypes 5 days post-tamoxifen injection. Collected samples were sent to the « Faculté de médecine vétérinaire de l'Université de Montréal » (Service de diagnostic, Saint-Hyacinthe) who realized the testing. **E,** Schematic representation of the model leading to the *Ddx3x*-deletion in all cell types except hematopoietic cells. **F,** Schematic representation of the model leading to the *Ddx3x*-deletion specifically in hematopoietic cells.

However, the effects of *Ddx3x*-acute deletion on hematopoietic cells could not be assessed in this model. An unexpected phenotype was detected a few days after the tamoxifen injection causing sudden death of *R26-cre*^{ER}/*Ddx3*$^{fl/fl}$ mice five days post-injection (**Figure 3-14A, B**). This sudden death did not occur in *R26-cre*^{ER}/*Ddx3*$^{X/fl}$ and *R26-cre*^{ER}/*Ddx3*$^{X/X}$ mice revealing a phenotype dependent on *Ddx3x*-deletion and not the *cre* system nor the tamoxifen injections. *R26-*

cre^{ER}/*Ddx3*$^{fl/fl}$ mice presented perturbations of the stomach and the intestines associated with a yellow coloration (**Figure 3-14B**). *R26-cre*^{ER}/*Ddx3*$^{fl/fl}$ mice exhibited a strong inflammatory reaction as seen by an elevated level of TNF-α and IL-6 cytokines (**Figure 3-14C**). A bacterial contamination of the intestine was confirmed by an analysis of the intestinal liquid collected from *R26-cre*^{ER}/*Ddx3x*-floxed mice 5 days after tamoxifen injections (**Figure 3-14D**). An abnormally high level of *Staphylococcus aureus*, *Lactobacillus spp* and *Parabacteroides goldsteinii* bacteria were present in KO mice intestines compared to *R26-cre*^{ER}/*Ddx3*$^{X/X}$ mice. Some KO mice even had strong levels of *Staphylococcus aureus*, *Lactobacillus johnsoni* and *Parabacteroides goldsteinii* in their lymph nodes, highlighting general bacterial contamination and dissemination possibly stemming from the intestinal flora (**Figure 3-14D**). Tamoxifen-induced *Ddx3x*-KO mice showed features reminiscent of septic shock: a strong inflammatory reaction and a high quantity of intestinal bacteria. I hypothesized that acute deletion of *Ddx3x* in adult females perturbs intestinal homeostasis and facilitates a bacterial infection and a strong inflammatory reaction lethal for mice. However, whether this phenotype was related to any defect caused by *Ddx3x*-deletion in hematopoietic cells remained unclear. Due to the large spectrum of deletions with the *R26-cre*^{ER} allele, further experiments were necessary to evaluate the potential involvement of hematopoietic cells in this phenotype.

A second strategy was therefore used to determine whether hematopoietic cells were involved in the life-threatening bacterial infection. BM from donor mice was transplanted into recipient mice in order to either delete *Ddx3x* in all cells except hematopoietic cells – CD45.1 BM cells transplanted into *R26-cre*^{ER}/*Ddx3x*-floxed recipients (CD45.2) (**Figure 3-14E**); or to either delete *Ddx3x* only in hematopoietic cells – *R26-cre*^{ER}/*Ddx3x*-floxed CD45.2 BM cells transplanted into CD45.1 recipients (**Figure 3-14F**). Transplantation of CD45.1 BM cells into *R26-*

cre^{ER}/*Ddx3x*-floxed recipients followed by tamoxifen injections caused the same phenotype in KO-mice as the one observed in *R26-cre*^{ER}/ *Ddx3*$^{fl/fl}$ mice. Mice with a *Ddx3x*-deletion in all cells except hematopoietic cells suddenly died five days after tamoxifen injection with similarly abnormal stomach and intestines as previously described (**Figure 3-14B**). This demonstrated that the lethality caused by *Ddx3x* acute deletion was independent of hematopoietic cells. I then hypothesized that acute deletion of *Ddx3x* in adult females perturbs intestinal homeostasis provoking perforations of the intestinal barrier at the origin of a bacterial infection and an inflammatory reaction causing death. Since CD45.1 recipient mice transplanted with *R26-cre*^{ER}/*Ddx3*$^{fl/fl}$ CD45.2 BM cells did not suddenly die nor did they develop a bacterial infection after tamoxifen injection, these mice were then used as a KO model for assessing DDX3X's role in hematopoietic cells.

Recipient animals that received *R26-cre*^{ER}/*Ddx3*-floxed BM cells were left for eight weeks to fully reconstitute the hematopoietic system and were then injected with tamoxifen activating the *R26-cre*^{ER} (**Figure 3-15A**). Animals that received *R26-cre*^{ER}/*Ddx3*$^{fl/fl}$ BM cells died very rapidly within 9 to 10 days after tamoxifen induction unlike recipients transplanted with BM cells from *R26-cre*^{ER}/*Ddx3*$^{X/X}$ or *R26-cre*^{ER}/*Ddx3*$^{X/fl}$ mice (**Figure 3-15B**). Transplanted mice that received *R26-cre*^{ER}/*Ddx3*$^{fl/fl}$ cells developed symptoms of anemia such as white paws, gray coats, as well as low body temperature and low hematocrit (**Figure 3-15C**).

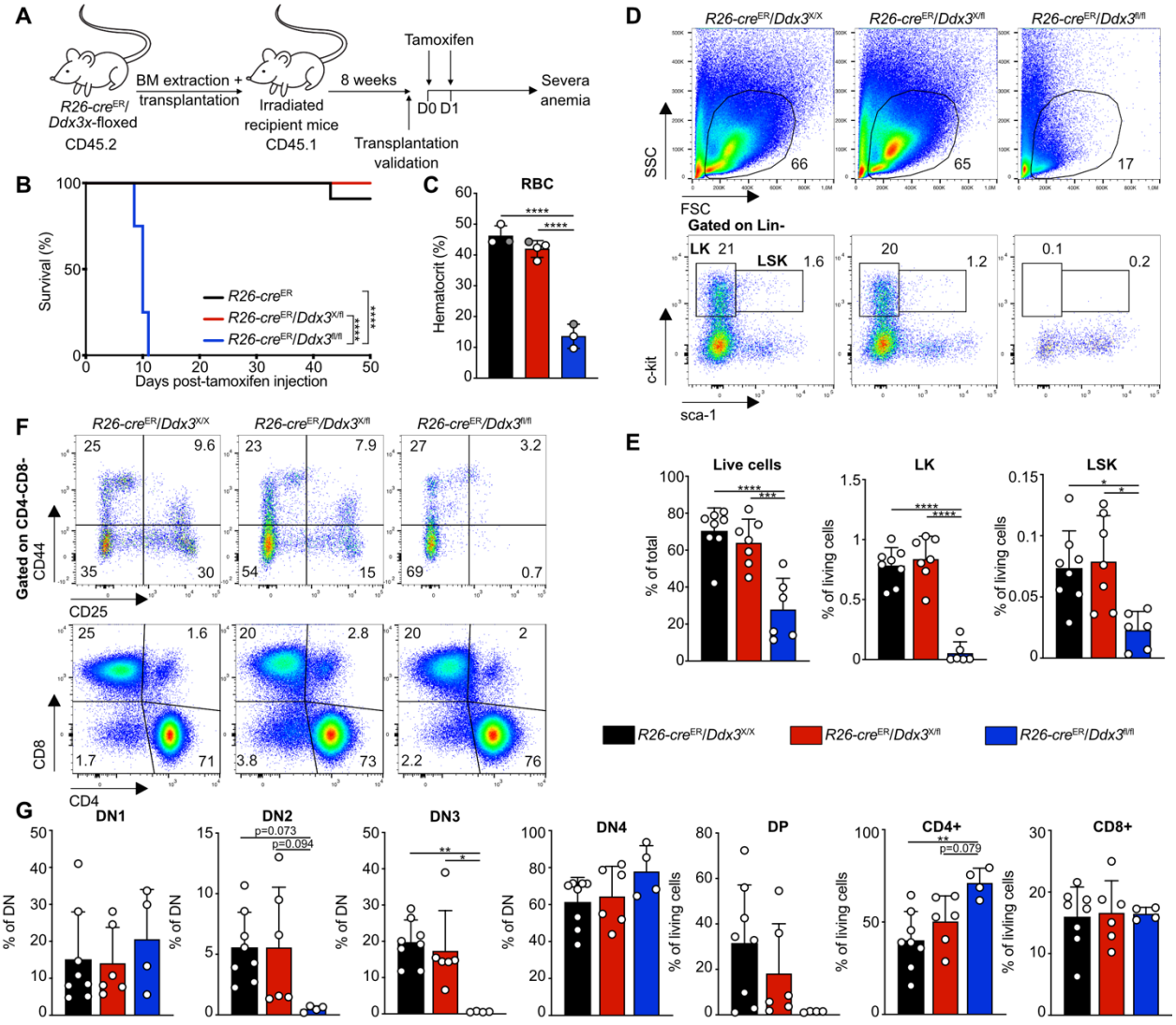


Figure 3-15: *Ddx3x*-acute deletion in hematopoietic cells of adult female mice induces death-related anemia and severe loss of hematopoietic progenitors

A, Schematic representation of the BM transplant experiment to delete *Ddx3x* specifically in hematopoietic cells. The BM transplantation efficiency was validated 8 weeks post-transplantation, the tamoxifen was injected at Day 0, (and the second dose at Day 1) and the survival was measured from the first injection. **B,** Survival curve of recipient CD45.1 mice transplanted with BM from CD45.2 mice: *R26-cre*^{ER}; *R26-cre*^{ER}/*Ddx3*$^{X/fl}$; or *R26-cre*^{ER}/*Ddx3*$^{fl/fl}$. **C,** Hematocrit was measured either on day 9 (grey dots) or day 10 (white dots) from the blood of moribund mice. **D,** Flow cytometry analysis of hematopoietic progenitors from the BM of transplanted mice at day 6 and relative quantification in (**E**). **F,** Flow cytometry analysis of T cell populations extracted from the thymus of transplanted mice at day 6 and relative quantification in (**G**). Statistical significance was measured by one-way ANOVA. The p value is indicated as follows: *ns*, p>0.05, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

In these mice, LK and LSK progenitors were almost completely lost, and the amount of live BM cells was significantly lower compared to control mice (**Figure 3-15D, E**). CD45.1 mice that had received *R26-cre*^{ER}/*Ddx3*$^{fl/fl}$ BM cells lost the DN2 and DN3 pre-T cells from the thymus, a

phenotype similar to the one observed in *Vav-cre*/*Ddx3*$^{fl/Y}$ mice (**Figures 3-7C, D and 3-8A, B**); whereas CD4^{+} cells were slightly increased and CD8^{+} remained unchanged (**Figure 3-15F, G**). The analysis of B cells revealed that B cell progenitors from the BM, transitional B cells as well as B220^{+}IgD^{-}CD38^{+} and PCs were unaffected by *Ddx3x*-deletion (**Figure 3-16A-F**). However, the MZ B cell population was increased while GC B cell numbers were decreased in mice with *Ddx3x* acute deletion compared to controls (**Figure 3-16G, H**).

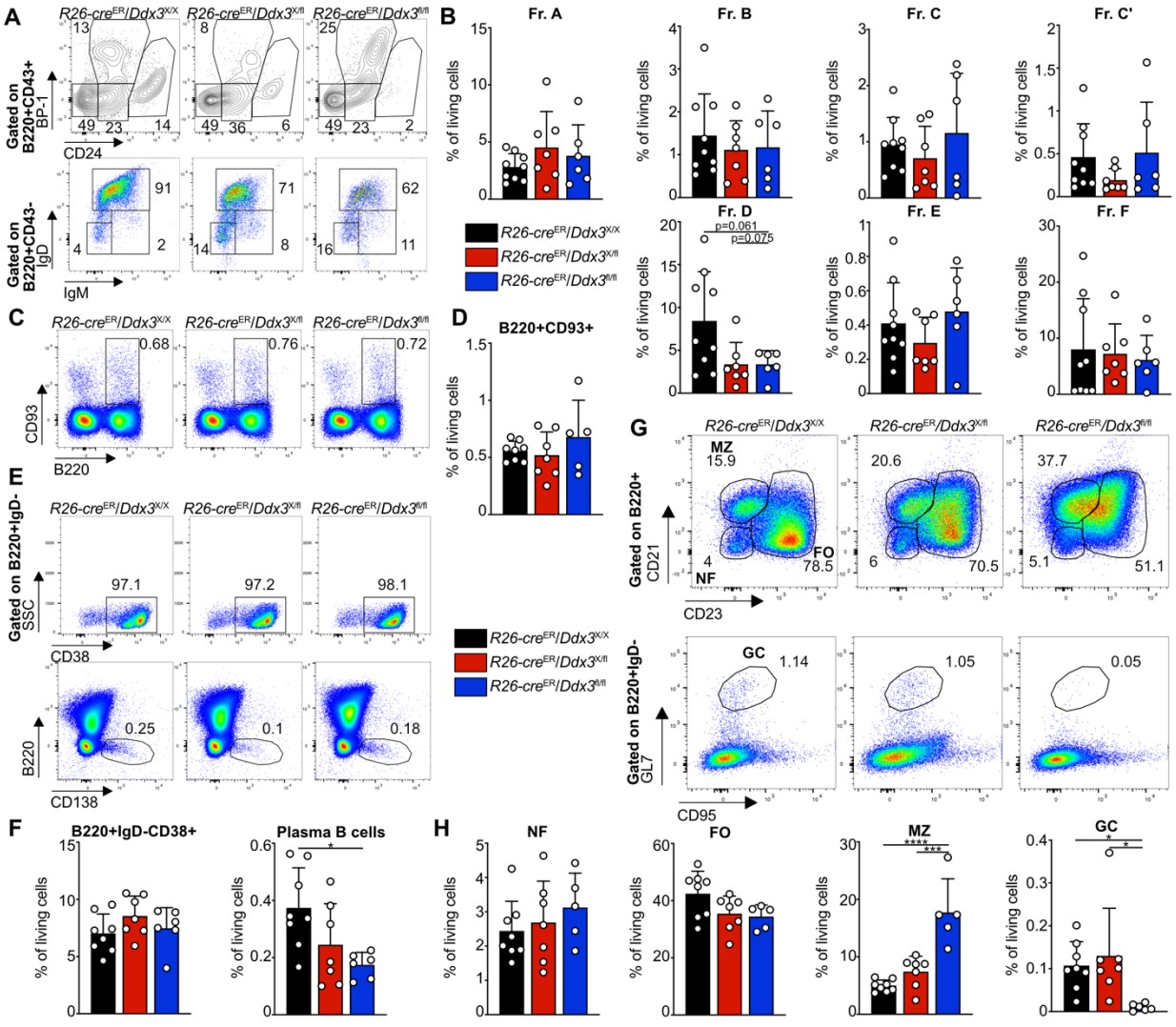


Figure 3-16: *Ddx3x*-acute deletion in hematopoietic cells provokes a loss of GC and an increase of MZ B cells

A, Flow cytometry analysis of BM B cell progenitors (Hardy fractions) from transplanted mice at day 6 after tamoxifen injection and relative quantification in (**B**). **C,** Flow cytometry analysis of transitional splenic B cell population from transplanted mice at day 6 after tamoxifen injection and (**D**) relative quantification. **E,** Flow cytometry analysis of

B220^{+}IgD^{-}CD38^{+} and PCs from spleens of transplanted mice at day 6 after tamoxifen injection and (**F**) relative quantification. **G,** Flow cytometry analysis of the indicated B cell populations from spleens of transplanted mice at day 6 after tamoxifen injection and (**H**) relative quantification. Statistical significance was measured by a one-way ANOVA. The p value is indicated as follows: *ns*, p>0.05, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Analyses of mice that did not receive any tamoxifen 8 weeks after receiving a BM transplant validated the BM transplantation efficacy and excluded the presence of any phenotypes before tamoxifen injections. Indeed, the blood of these mice contained CD45.2 differentiated lymphocytes and myelocytes, while CD45.1 cells were almost absent (**Figure 3-17A**).

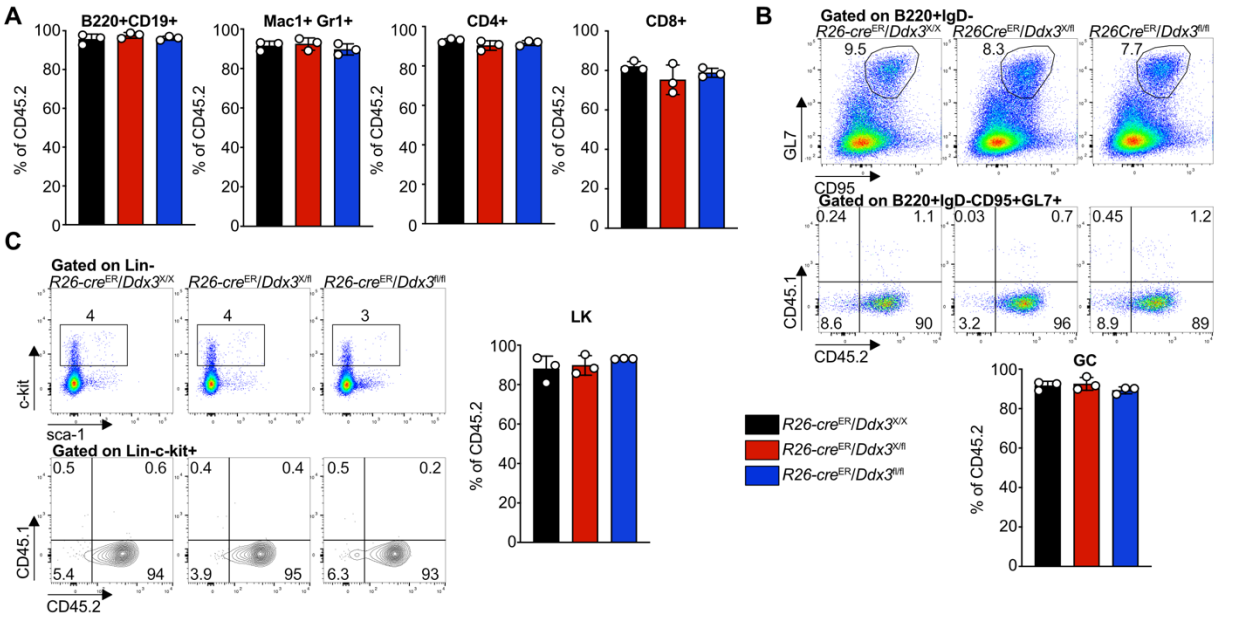


Figure 3-17: Validation of the BM reconstitution of CD45.1 transplanted mice

A, Percentage of CD45.2 cells measured by flow cytometry analysis of blood samples from CD45.1 recipient mice 8 weeks after BM transplantation (no tamoxifen injection). **B,** Percentage of CD45.2 cells measured from FACS analysis of spleen extracted from sacrificed CD45.1 recipient mice 8 weeks after BM transplant (no tamoxifen injection). **C,** Percentage of CD45.2 cells measured from FACS analysis of BM extracted from sacrificed CD45.1 recipient mice 8 weeks after BM transplant (no tamoxifen injection).

Percentages of CD45.2^{+} hematopoietic cells were very high and similar when recipients received *R26-cre*^{ER}/*Ddx3*$^{fl/fl}$, *R26-cre*^{ER}/*Ddx3*$^{X/fl}$ or *R26-cre*^{ER}/*Ddx3*$^{X/X}$ BM cells. Moreover, LK and GC cells, two populations lost in mice with the BM-*R26-cre*^{ER}/*Ddx3*$^{fl/fl}$ after tamoxifen injections, were for the majority CD45.2^{+} and were clearly reconstituted before tamoxifen injections, even in KO mice (**Figure 3-17B, C**). This demonstrated that the phenotypes observed in these populations

after tamoxifen injections were independent of the reconstitution of the hematopoietic system and instead caused by the loss of DDX3X. To summarize, *Ddx3x* acute deletion in adult female mice induced a loss of hematopoietic progenitors, DN2-DN3 T cells, and GC B cells as well as an increase of MZ B cells. Moreover, it caused a loss of RBCs provoking a severe and fatal anemia.

In this model, the deletion of one *Ddx3x* allele did not impact any hematopoietic cell population, as already observed in *Vav-cre*/*Ddx3*$^{X/fl}$ mice, again highlighting a compensation from one intact *Ddx3x* allele. Moreover, comparing the phenotypes caused by *Ddx3x*-deletion in CD45.1 mice that received *R26-cre*^{ER}/*Ddx3*$^{fl/fl}$ cells plus tamoxifen and *Vav-cre*/*Ddx3*$^{fl/Y}$ male mice informed us about the potential of *Ddx3y* compensation. Both models had a decrease of LKs, LSKs, DN2-DN3 T cells, GCs, RBCs and an increase of MZ B cells indicating an imperfect compensation from the *Ddx3y* gene. However, *Ddx3y* still had a strong compensatory effect in the context of a *Ddx3x*-deletion since the decrease of RBCs, LK and LSKs progenitors of *Vav-cre*/*Ddx3*$^{fl/Y}$ male did not affect their viability and life expectancy; by contrast to the effect seen in CD45.1 mice that received *R26-cre*^{ER}/*Ddx3*$^{fl/fl}$ BM cells. In addition, several other phenotypes found in *Vav-cre*/*Ddx3*$^{fl/Y}$ mice were not observed in CD45.1 mice that received *R26-cre*^{ER}/*Ddx3*$^{fl/fl}$ cells; for example, the decrease of several B cell progenitors from the BM (**Figures 3-11A-D and 3-16A, B**). This absence of a B cell phenotype in mice that received *R26-cre*^{ER}/*Ddx3*$^{fl/fl}$ BM cells may be because flow cytometry analysis was done quickly after tamoxifen injections, a consequence of the short viability of these mice. Moreover, the toxic effects of tamoxifen, which have been described previously (X. Tian & Zhou, 2021), were also clearly evident here most likely causing the variability of the results and the strong reduction of the DP pre-T cell population in the thymus for example (**Figure 3-15F, G**).

4. CHAPTER IV: DDX3X's role in lymphomagenesis

1. Generation of a B cell-specific KO mouse model

The main strategy to investigate the role of DDX3X in B cell lymphomagenesis was to use the conditional KO mouse model that deletes *Ddx3x* specifically in mature B cells and to cross these mice with models mimicking B cell lymphoma development. Not only is a specific B cell deletion better suited for these experiments compared to the *Vav-cre* deletion but it also allows circumventing the erythroid defect blocking embryogenesis in female full-KO mice. The effect of *Ddx3x*-deletion could then be assessed in models mimicking B lymphomagenesis in all KO mice: female full-KO as well as male hemizygous and female heterozygous.

The *CD21-cre* deleter which is active in transitional and peripheral B cells was first introduced into *Ddx3x*-floxed mice (Kraus et al., 2004). *CD21-cre/Ddx3*$^{X/fl}$ and *CD21-cre/Ddx3*$^{fl/Y}$ were viable, but *CD21-cre/Ddx3*$^{fl/fl}$ mice were never obtained which was probably the result of a "leaky" activity of this *cre* in early developmental stages causing a developmental arrest, as was observed by others (M. Tian et al., 2020). *Ddx3x*-floxed mice were then crossed with mice carrying the *Cd19-cre* deleter allele (Rickert et al., 1997). Although this *cre* deletes floxed allele starting at the pre-B cell step of differentiation, i.e. earlier compared to the *CD21-cre*; this *cre* allowed

obtaining all KO mice with *Cd19-cre/Ddx3*$^{X/fl}$, *Cd19-cre/Ddx3*$^{fl/Y}$ and *Cd19-cre/Ddx3*$^{fl/fl}$ genotypes.

The first step was to validate that a B cell deletion by the *Cd19-cre* allele caused similar phenotypes compared to what was observed with *Vav-cre* and *R26-cre*^{ER} KO models. In the following experiments, *Cd19-cre/Ddx3*$^{fl/Y}$ male KO mice were compared to *Cd19-cre/Ddx3*$^{X/Y}$ male controls while *Cd19-cre/Ddx3*$^{fl/fl}$ female KO mice were compared to *Cd19-cre/Ddx3*$^{X/X}$ female controls and *Cd19-cre/Ddx3*$^{X/fl}$ heterozygous mice. Early B cell populations from the BM of *Cd19-cre/Ddx3x*-floxed mice were analyzed by flow cytometry. B220^{+}CD19^{+} cells were decreased in male KO and almost absent in female KO, while other BM hematopoietic cells (B220^{-} CD19^{-}) remained unchanged (**Figure 4-1A, B**).

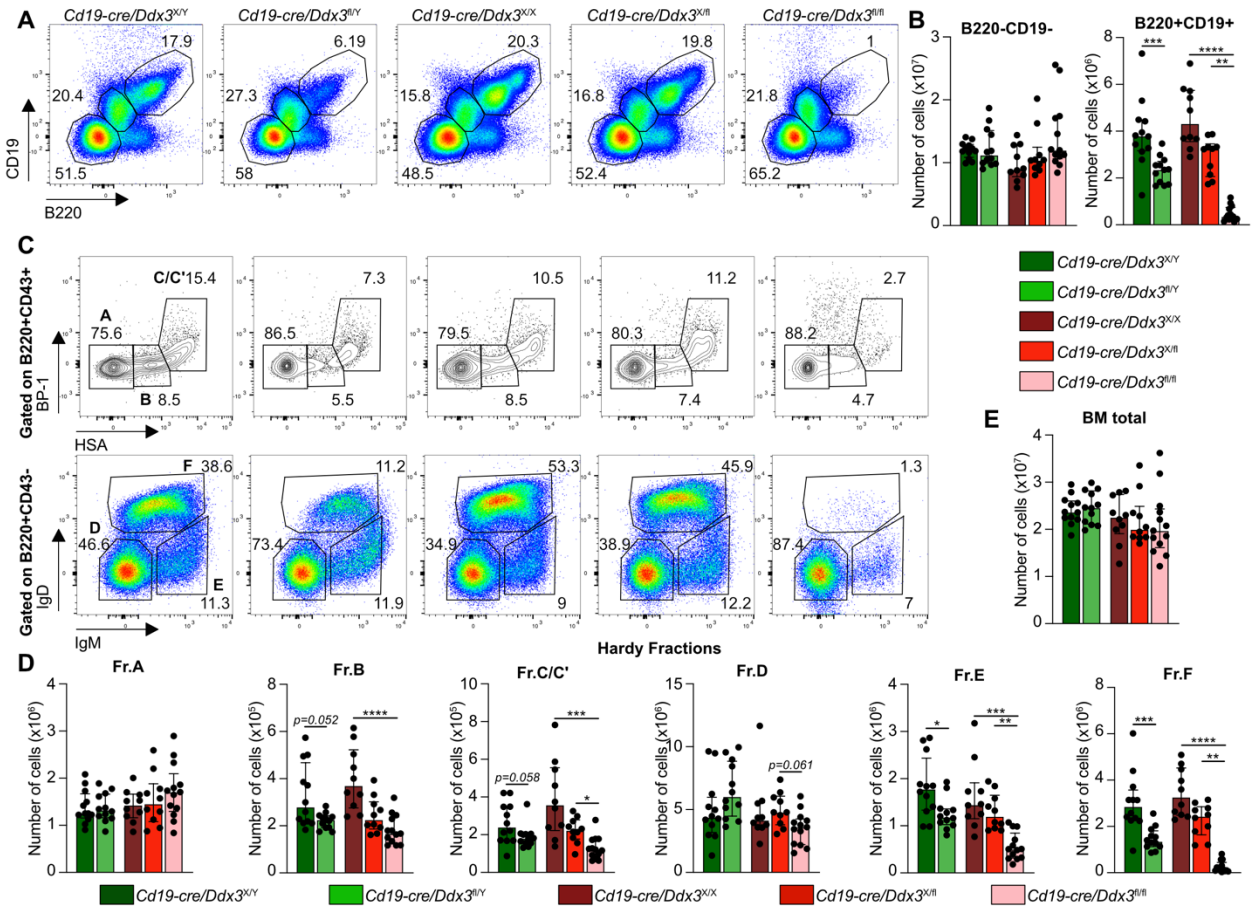


Figure 4-1: *Ddx3x*-deletion impairs several BM B cell populations in a sex-dependent way

A, Flow cytometry analysis of the B220/CD19 cells extracted from the BM of *Cd19-cre*/*Ddx3x*-floxed mice and quantification of absolute cell numbers in (**B**). **C,** Flow cytometry analysis of the B cell Hardy fractions from A to F. Populations were defined as follows: Fr.A or Pre-Pro-B cell ($B220^+CD43^+HSA^-BP1^-$), Fr.B or Pro-B cell ($B220^+CD43^+HSA^+BP1^-$), Fr.C-C' or large Pre-B cell ($B220^+CD43^+HSA^+BP1^+$), Fr.D or Pre-B cell ($B220^+CD43^-IgM^-IgD^-$), Fr.E or Immature B cell ($B220^+CD43^-IgM^+IgD^-$), Fr.F or mature or recirculating B cell ($B220^+CD43^-IgM^+IgD^+$). **D,** Quantification of Hardy fractions in absolute numbers. **E,** Total number of cells in the BM of *Cd19-cre*/*Ddx3x*-floxed mice. Kruskal-Wallis test was used to compare female KO with female controls and heterozygous and Mann-Whitney test was used to compare male KO with male controls. The p value is indicated as follows: *ns*, $p>0.05$, *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

Hardy fractions B, C, E and F were significantly decreased in female KO and diminished in male KO mice (**Figure 4-1C, D**). In addition, all of these quantitative changes in the Hardy fractions of KO mice were independent of the total number of cells in the BM similar whatever the genotype and sex of the mice (**Figure 4-1E**). By contrast, the total number of splenocytes was dramatically decreased in female KO mice (**Figure 4-2A**). Moreover, the splenic index was significantly decreased in female KO mice, indicating smaller spleens in those mice. $B220^+$ splenocytes were drastically reduced in female KO and slightly decreased in male KO mice (**Figure 4-2B, C**). Cellularity of the transitional, mature $B220^+IgD^+$ and FO B cell compartments was decreased in both male and female KO, whereas their MZ B cell subset was increased in percentage. In addition, female KO had a reduced absolute number of newly formed (NF), MZ as well as $B220^+Ig^-$ and $B220^+IgM^+$ B cells (**Figure 4-2B, C**).

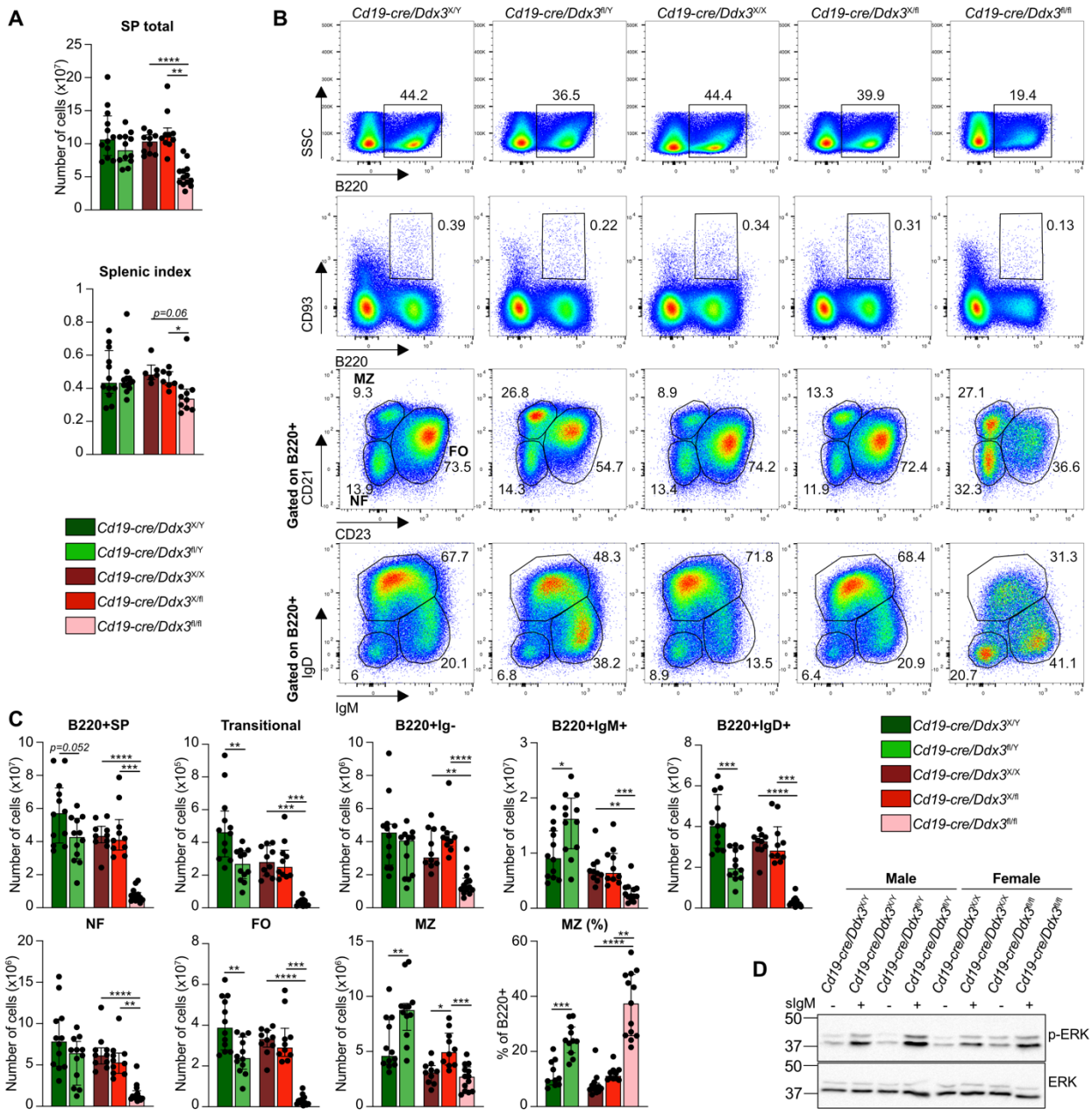


Figure 4-2: Mature B cells are significantly decreased in *Ddx3x*-KO mice

A, Quantification of the total number of splenic cells extracted from *Cd19-cre/Ddx3x*-KO mice and calculation of the splenic index defined as the weight of the spleen divided by the weight of the animal multiplied by 100. **B,** Flow cytometry analysis and (**C**) quantification in absolute number of splenic B cells in *Cd19-cre/Ddx3x*-KO mice compared to controls. The last panel represents the relative quantification of MZ B cells. Populations were defined as follows: transitional B cells ($B220^+CD93^+$), NF ($B220^+CD21^-CD23^-$), FO ($B220^+CD23^+$), MZ ($B220^+CD21^+CD23^-$). Kruskal-Wallis test was used to compare female KO with female controls and heterozygous and Mann-Whitney test was used to compare male KO with male controls. The p value is indicated as follows: *ns*, p>0.05, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. **D,** Western blot of isolated resting B cells from the spleen of *Cd19-cre/Ddx3x*-KO mice stimulated with secreted IgM. This experiment was done in triplicate. sIgM: secreted IgM.

Although significant changes were observed in many B cell subsets, splenic naïve B cells extracted from *Cd19-cre/Ddx3*$^{fl/Y}$, *Cd19-cre/Ddx3*$^{fl/fl}$ and sex-matched control mice were equally responsive to an anti-IgM stimulation *in vitro* suggesting the functionality of the BCR (**Figure 4-2D**). When put into the context of the B cell phenotypes discovered in *Cd19-cre/Ddx3*$^{fl/fl}$, this last result probably indicates that MZ B cells, constituting a vast proportion of the naïve B cells left in the spleens of *Cd19-cre/Ddx3*$^{fl/fl}$ animals are able to respond to an IgM stimulation. In addition, female KO exhibited a decrease of B220^{+}IgD^{-}CD38^{+} very likely linked to the decrease of B220^{+}IgD^{+} cells, whereas the PC subset was not affected by *Cd19-cre* deletion (**Figure 4-3A, B**).

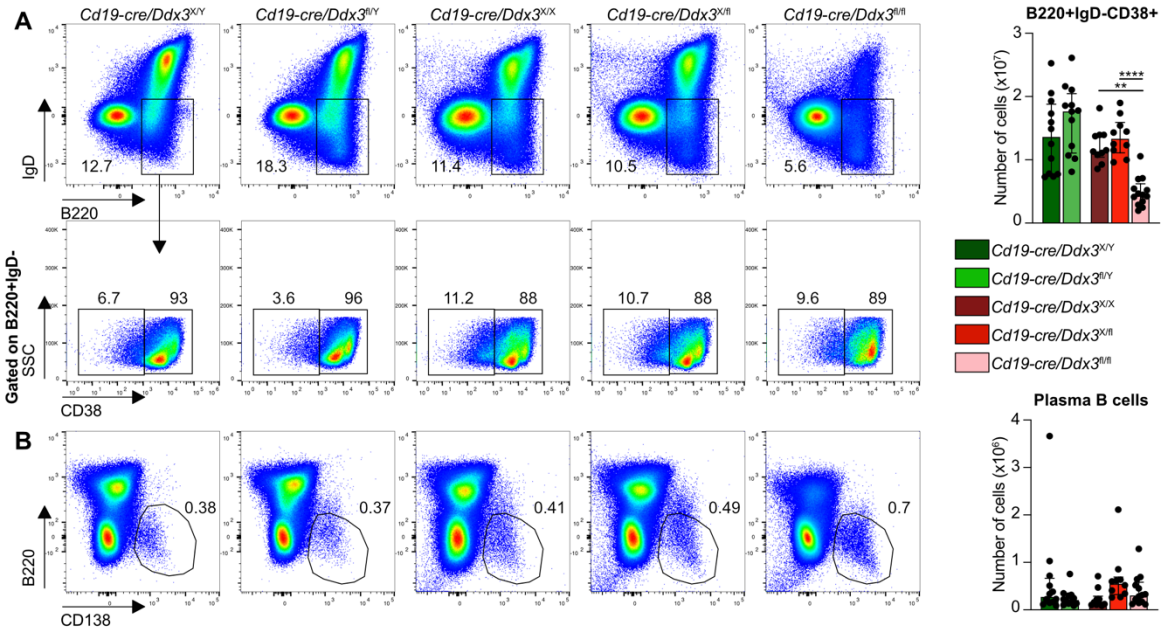


Figure 4-3: Last stages of B cell differentiation are poorly affected by *Ddx3x*-deletion

A, Flow cytometry analysis of splenic B220^{+}IgD^{-} B cells, B220^{+}IgD^{-}CD38^{+} B cells and PCs in (**B**) from the spleen of *Cd19-cre*/*Ddx3x*-floxed mice and quantification of absolute cell numbers. The population was defined as follows: Plasma B cells (B220^{-}CD138^{+}). Kruskal-Wallis test was used to compare female KO with female controls and heterozygous and Mann-Whitney test was used to compare male KO with male controls. The p-value is indicated as follows: *ns*, p>0.05, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

The majority of the phenotypes observed in the B cell differentiation were similar between male and female KO mice but were significantly more severe in females lacking both *Ddx3x* alleles, revealing again in this system a compensatory effect of the male-specific *Ddx3y* gene.

2. GC B cells are severely impaired in a sex-dependent way in *Ddx3x*-deleted mice

Given the critical role of GC B cells in initiating B cell malignancies, this specific B cell population was investigated in *Cd19-cre/Ddx3x*-floxed mice. GC B cells were significantly decreased in splenocytes from *Cd19-cre*/*Ddx3*$^{fl/Y}$ and *Cd19-cre*/*Ddx3*$^{fl/fl}$ mice (**Figure 4-4A**), a phenotype already observed in *Vav-cre*/*Ddx3*$^{fl/Y}$ and CD45.1 mice transplanted with *R26-cre*^{ER}/*Ddx3*$^{fl/fl}$ BM cells (**Figures 3-13A and 3-16G, H**).

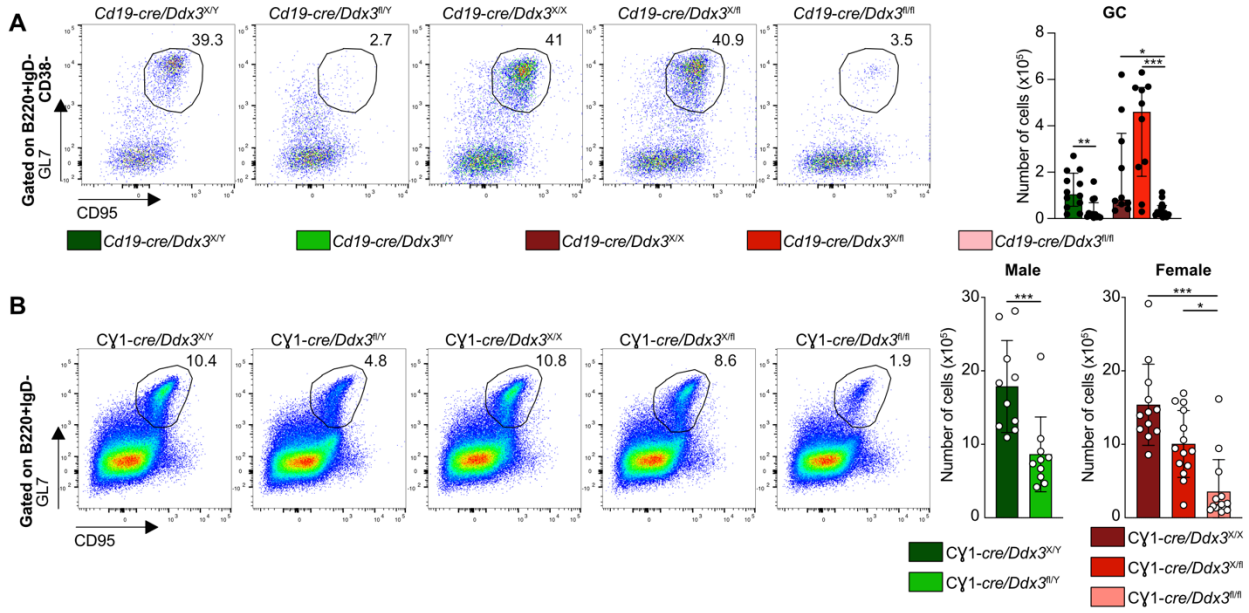


Figure 4-4: GC B cells are decreased in both physiological and sheep RBC immunization in *Ddx3x*-KO mice

A, Flow cytometry analysis of GC (B220$^+$IgD$^-$CD38$^-$CD95$^+$GL7$^+$) in the spleen of *Cd19-cre/Ddx3x*-floxed mice compared to controls and quantification in absolute number of cells. **B,** Flow cytometry analysis of GC (B220$^+$IgD$^-$CD95$^+$GL7$^+$) from spleens of Cγ1-*cre*/*Ddx3x*-floxed mice 10 days after immunization with sheep RBCs. For females, medians were compared using a Kruskal-Wallis test whereas for males, medians were compared using the Mann-Whitney *U* test. The p value is indicated as follows: *ns*, p>0.05, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

To determine whether this decrease of GC B cells resulted from defects observed in previous steps of B cell differentiation or an intrinsic effect caused by *Ddx3x*-deletion, mice with a GC-specific

deletion were generated using a Cγ1-*cre* allele which is inducible upon B cell activation, for instance by immunization with sheep RBCs (Casola et al., 2006). Under these conditions, GCs failed to expand in Cγ1-*cre/Ddx3*$^{fl/Y}$ males and Cγ1-*cre/Ddx3*$^{fl/fl}$ homozygous females 10 days after immunization compared to controls or heterozygous females (**Figure 4-4B**), demonstrating that the GC defect was intrinsic and not a consequence of an impaired earlier B cell differentiation in *Ddx3x*-KO mice. It was also noticed that GCs were more severely decreased in Cγ1-*cre/Ddx3*$^{fl/fl}$ animals compared to Cγ1-*cre/Ddx3*$^{fl/Y}$ mice.

Cγ1-*cre/Ddx3x*-floxed mice were bred to animals with a *R26*$^{mT/mG}$ reporter allele and GFP^{+} GCs were analyzed 10 days after sheep RBC immunization (**Figure 4-5A**).

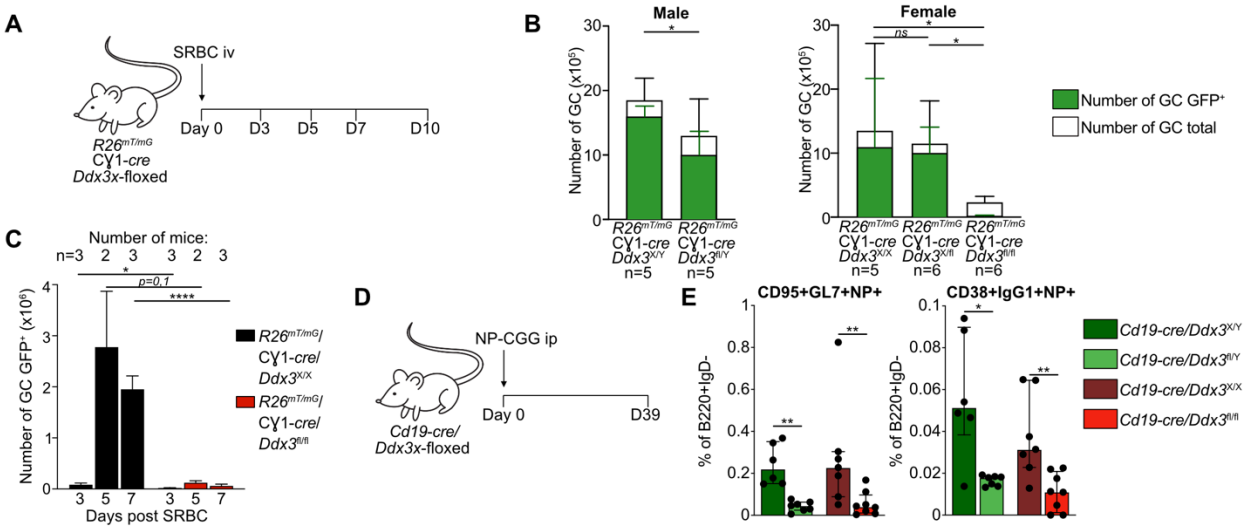


Figure 4-5: GC B cells are decreased in a sex-dependent way after immunization in *Ddx3x*-KO mice

A, Schematic representation of the sheep RBC immunization and the time points post-injection when GC GFP^{+} were analyzed. **B,** Absolute number of GFP^{+} GC (B220^{+}IgD^{-} CD95^{+}GL7^{+}) from spleens of Cγ1-*cre*/*Ddx3x*-floxed mice crossed with the *R26*$^{mT/mG}$ reporter mice 10 days after immunization. In females, GFP^{+} GCs were compared using a Dunnett's T3 test while in males, a Mann-Whitney *U* test was used to compare GFP^{+} GCs. **C,** Absolute number of GFP^{+} GCs (B220^{+}IgD^{-}CD95^{+}GL7^{+}) from spleens of Cγ1-*cre*/*Ddx3x*-floxed female mice crossed with the *R26*$^{mT/mG}$ reporter mice analyzed at day 3, day 5 and day 7 post-injection. Data from the same time point were compared with an unpaired *t*-test. **D,** Experimental design of the GC analysis of the *Cd19-cre/Ddx3x*-floxed mice 39 days post-NP-CGG immunization. **E,** Quantification of GC and Memory B cells after NP-CGG immunization. Populations were defined as follows: switched Memory B cells (B220^{+}IgD^{-}CD38^{+}IgG1^{+}NP^{+}), GC (B220^{+}IgD^{-}CD95^{+}GL7^{+}NP^{+}). Mann-Whitney *U* test to compared male KO with male controls and female KO with female controls. This experiment was done in collaboration with Dr. Julie Ross. The p value is indicated as follows: *ns*, p>0.05, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

GFP$^+$ GC B cells were significantly decreased in Cγ1-*cre/Ddx3*$^{fl/Y}$ males and extremely low in Cγ1-*cre/Ddx3*$^{fl/fl}$ females 10 days after sheep RBC immunization suggesting that GC B cells were eliminated in the absence of DDX3X (**Figure 4-5B**). Although GC cells were significantly decreased in KO males, GFP$^+$ GC B cells were clearly detectable suggesting that DDX3Y can partially compensate for *Ddx3x* loss in these cells. Similar to other cell populations, the GC compartment was intact in heterozygous Cγ1-*cre/Ddx3*$^{fl/X}$ females, excluding haploinsufficiency from *Ddx3x* locus in mice. To determine when the GC formation was impaired in Cγ1-*cre/Ddx3*$^{fl/fl}$ mice, earlier time points post sheep RBC injection were analyzed (**Figure 4-5C**). The number of GFP$^+$ GC B cells was still extremely low in spleens of Cγ1-*cre/Ddx3*$^{fl/fl}$ mice at earlier time points suggesting that GCs were never fully formed in female KO mice. Immunization of *Cd19-cre*/*Ddx3x*-floxed mice with NP-CGG was realized and the spleens were analyzed 39 days later to assess the quantity of GC cells and any impact of *Ddx3x*-deletion on switched MBC formation (**Figure 4-5D**). NP-CGG immunization demonstrated a similar phenotype: an impairment of female Cγ1-*cre/Ddx3*$^{fl/fl}$ and male Cγ1-*cre/Ddx3*$^{fl/Y}$ mice to generate antigen-specific, NP$^+$ GC cells, in addition to a lower quantity of switched memory B cells gated as B220$^+$IgD$^-$ CD38$^+$IgG1$^+$NP$^+$ (**Figure 4-5E**). While GCs were already decreased in non-immunized male KO (*Vav-cre*/*Ddx3*$^{fl/Y}$ and *Cd19-cre*/*Ddx3*$^{fl/Y}$) and female KO mice (CD45.1 mice transplanted with *R26-cre*^{ER}/*Ddx3*$^{fl/fl}$ cells and *Cd19-cre*/*Ddx3*$^{fl/fl}$); the decrease of B220$^+$IgD$^-$CD38$^+$ B cells was not observed in non-immunized male KO animals (**Figure 4-3A**); highlighting that the decrease of NP$^+$ switched memory B cells in this experiment may be due to a decrease of NP$^+$ GCs in male mice. In addition, no obvious difference was observed in the quantity of NP$^+$ GCs between male and female KO mice (**Figure 4-5E**), compared to what was observed in previous models (**Figures 4-4B and 4-5B**). This may be caused by the fact that only GCs that have responded to the NP-

CGG immunization were analyzed here while the total GC population was analyzed in previous experiments. Overall, these experiments demonstrated that GC maintenance and expansion critically require DDX3X or DDX3Y.

Several reports linked DDX3X to p53 and reported a role of this helicase in DNA damage response (Cargill et al., 2021; Chan et al., 2019; C. Y. Chen et al., 2016; W. J. Chen et al., 2017; M. Sun et al., 2013). To test whether the loss of DDX3X-deficient GC B cells is p53 dependent, *Trp53* KO mice (gene coding for murine p53) were used to generate apoptotic-resistant mice (Jacks et al., 1994). *Trp53*$^{+/-}$ and *Trp53*$^{-/-}$ mice were crossed with Cγ1-*cre/Ddx3x*-floxed animals and the GC population was analyzed 10 days after sheep RBC immunization to assess if GC loss could be rescued. However, the *Trp53* gene deletion did not rescue GC expansion neither in female Cγ1-*cre/Ddx3*$^{fl/fl}$ KO mice (**Figure 4-6A**) nor in male Cγ1-*cre/Ddx3*$^{fl/Y}$ KO mice (**Figure 4-6B**).

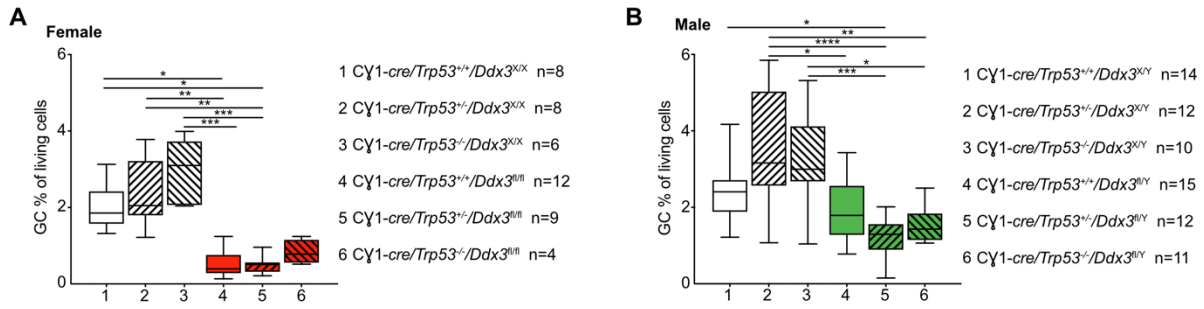


Figure 4-6: Preventing p53 apoptosis does not rescue the loss of GC in *Ddx3x*-deleted animals

Relative percentages of GC (B220^{+}IgD^{-}CD95^{+}GL7^{+}) from spleens of Cγ1-*cre*/*Ddx3x*-floxed mice females in (**A**) and males in (**B**) crossed with the *Trp53*-KO mice 10 days after sheep RBC immunization. A Kruskal-Wallis test was used to assess the statistical significance of this experiment. The p value is indicated as follows: *ns*, p>0.05, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

This result excluded a p53-dependent cell death as the underlying cause of the GC loss detected in *Ddx3x*-deleted mice.

In an attempt to better understand why GCs were lost when DDX3X is absent, an *in vitro* system allowing the expansion of "induced GC B cells", or iGB cells, was used (Haniuda & Kitamura, 2019). Naïve primary B cells were extracted from spleens of *R26*$^{mT/mG}$/Cγ1-*cre/Ddx3x*-

floxed mice and cultured with IL-4 on a 40LB feeder layer expressing B cell activating factor (BAFF) as a survival signaling; and CD40L required for B cell activation (**Figure 4-7A**).

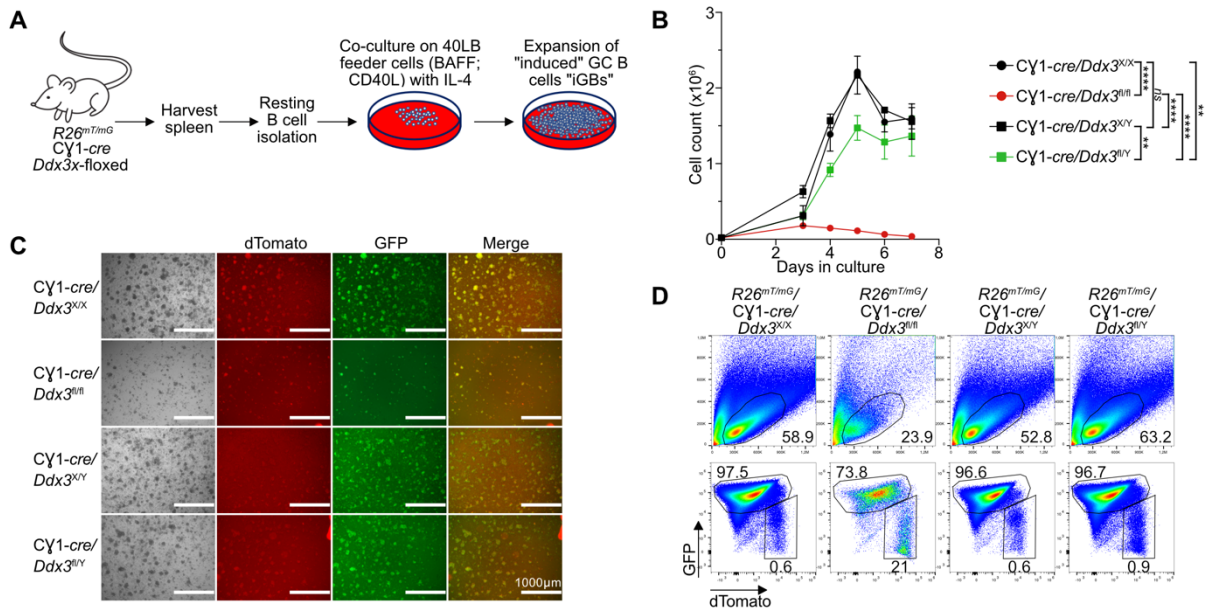


Figure 4-7: GC B cells survive *in vitro* but do not expand in the absence of DDX3X

A, Schematic representation of the *in vitro* co-culture system allowing iGB expansion. **B,** Trypan blue was used to count the iGBs and follow their proliferation. Day 0 marks when primary B cells isolated from *R26*$^{mT/mG}$/Cγ1-*cre*/*Ddx3x*-floxed mice were plated with 40LB feeder cells. Indicated statistics were calculated using a two-way ANOVA test on day 5: the peak of the iGB expansion. **C,** Microscopy pictures of iGBs expansion 5 days after co-culture on the 40LB feeder cells. **D,** iGBs isolated from were collected at day 5 and submitted to flow cytometry analysis to evaluate GFP and dTomato emissions. The p value is indicated as follows: *ns*, p>0.05, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

In vitro culture of murine primary B cells from *R26*$^{mT/mG}$/Cγ1-*cre/Ddx3x*-floxed mice demonstrated that a full *Ddx3x*-deletion almost entirely prevents GC expansion *in vitro* (**Figure 4-7B**). However, *Ddx3x*-deleted male B cells had a partial GC expansion, almost comparable to controls, which demonstrated again, the critical requirement of DDX3X or DDX3Y for GC expansion but also illustrated the potency of DDX3Y to compensate for the loss of DDX3X. The absence of an expansion of iGBs with a full *Ddx3x*-deletion was also observed by microscopy and flow cytometry (**Figure 4-7C, D**). GFP^{+} *R26*$^{mT/mG}$/Cγ1-*cre/Ddx3*$^{fl/fl}$ cells were still detectable by microscopy and flow cytometry (**Figure 4-7C, D**), suggesting that even if they were unable to expand, live female iGBs could be maintained and endure a *Ddx3x* full deletion when co-cultured

in vitro with this system. In contrast, male *R26*$^{mT/mG}$/Cγ1-*cre*/*Ddx3*$^{fl/Y}$ B cells were able to proliferate and expand almost to control levels suggesting that under these conditions, DDX3Y compensates and partially rescues DDX3X loss.

3. *Ddx3x* deletion delays MYC-driven lymphomagenesis

Since a high frequency of *DDX3X* LOF mutations was reported in B cell lymphoma where activation of the MYC oncogene plays a significant role, it was essential to test whether *Ddx3x*-deletion could influence MYC-driven lymphomagenesis. For this, E*μ*-*Myc* murine model was first used. These mice express a *Myc* transgene driven by the *Ig*H (E*μ*) enhancer and are prone to develop a spectrum of B-lymphoid tumors ranging from pre-B cells lymphoma to IgM^{+} B cell lymphoma (Adams et al., 1985; Harris et al., 1988; Langdon et al., 1986). These E*μ*-*Myc* mice were bred to *Vav*-*cre*/*Ddx3x*-floxed mice to assess the impact of *Ddx3x*-deletion on E*μ*-*Myc*-driven tumorigenesis. A first observation was that the majority of E*μ*-*Myc*/*Vav*-*cre*/*Ddx3*$^{fl/Y}$ males did not develop B lymphoma and had a significantly longer survival compared to E*μ*-*Myc*/*Vav*-*cre*/*Ddx3*$^{X/Y}$ control mice (**Figure 4-8A**).

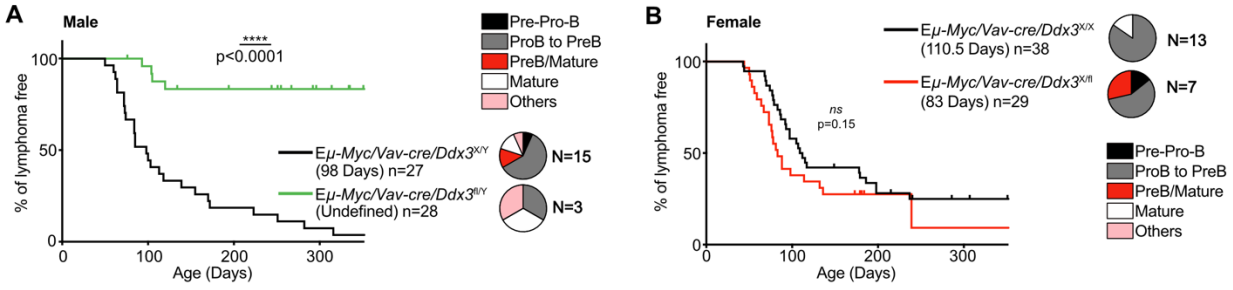


Figure 4-8: *Ddx3x*-deletion by *Vav-cre* almost exclusively prevents E*μ*-*Myc* tumorigenesis in male mice

A, Kaplan-Meyer curves representing the survival of E*μ*-*Myc*/*Vav*-*cre*/*Ddx3*-floxed male mice. **B,** Kaplan-Meyer curves representing the survival of E*μ*-*Myc*/*Vav*-*cre*/*Ddx3*-floxed female mice. Statistics on survival curves were assessed with the Mantel-Cox test. Median survival is indicated in brackets and is followed by the number of animals

in the cohort. The repartition of tumor subtypes was assessed by flow cytometry analysis of tumor samples. The p value is indicated as follows: *ns*, p>0.05, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

The tumor subtypes varied between E*μ-Myc*/*Vav-cre*/*Ddx3*$^{fl/Y}$ and E*μ-Myc*/*Vav-cre*/*Ddx3*$^{X/Y}$ tumors although no clear difference could be determined since the vast majority of E*μ-Myc*/*Vav-cre*/*Ddx3*$^{fl/Y}$ did not develop any tumors (85% of tumor-free E*μ-Myc*/*Vav-cre*/*Ddx3*$^{fl/Y}$ mice) and *Ddx3x*-KO tumor subtypes were only defined in three samples. In contrast, incidences and latency periods of B cell lymphoma were unchanged in female E*μ-Myc*/*Vav-cre*/*Ddx3*$^{X/fl}$ mice compared to E*μ-Myc*/*Vav-cre*/*Ddx3*$^{X/X}$ control mice (**Figure 4-8B**), correlating with the absence of B cell phenotype previously observed in *Vav-cre*/*Ddx3*$^{X/fl}$ mice.

These mice were then analyzed at the pre-tumor stage defined as 6-week-old mice not presenting any sign of disease. Although healthy, 6-week-old E*μ-Myc* mice already show a pre-B cell expansion and splenic enlargement, two main features of the pre-tumor stage (Vecchio et al., 2019). Pre-lymphomatous E*μ-Myc*/*Vav-cre*/*Ddx3*$^{fl/Y}$ mice lacked the pre-B cell expansion and splenic enlargement usually observed in young, lymphoma-free E*μ-Myc* mice (**Figure 4-9A, B**).

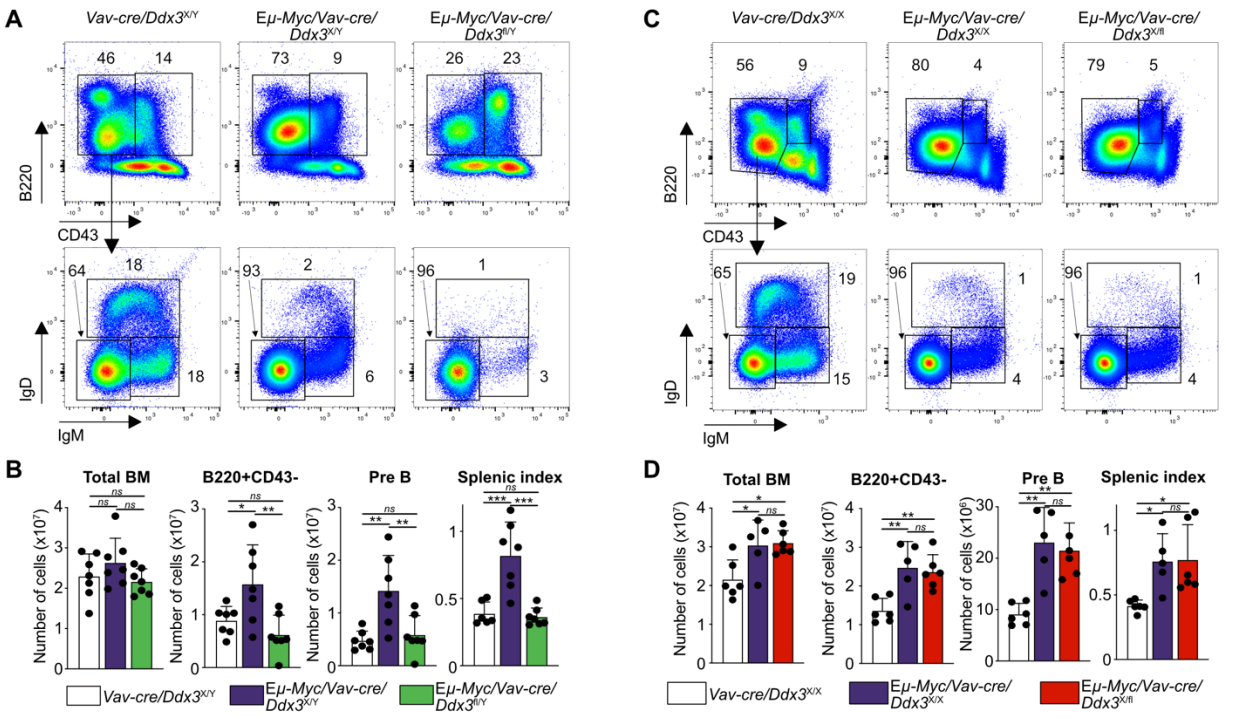


Figure 4-9: *Ddx3x*-deletion in E*μ-Myc* mice prevents pre-B cell expansion and splenic enlargement

A, Flow cytometry analysis of BM and spleens from 6-weeks-old E*μ-Myc/Vav-cre/Ddx3* male mice (pre-tumor phase) and quantification in absolute number of cells in (**B**). **C,** Flow cytometry analysis of BM and spleens from 6-weeks-old E*μ-Myc/Vav-cre/Ddx3* female mice (pre-tumor phase) and quantification in absolute number of cells in (**D**). The splenic index corresponds to the weight of the spleen divided by the weight of the animal multiplied by 100. Pre-B cell population (Fraction D) is defined as $B220^{+}CD43^{-}IgM^{-}IgD^{-}$. Statistical significance was assessed with a Kruskal-Wallis test. The p value is indicated as follows: *ns*, $p>0.05$, *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

Pre-lymphomatous E*μ-Myc*/*Vav-cre*/*Ddx3*$^{fl/Y}$ mice rather had a pre-B cell population and splenic index similar to those of *Vav-cre* mice not carrying an E*μ-Myc* transgene. The loss of pre-B expansion in pre-lymphomatous E*μ-Myc*/*Vav-cre*/*Ddx3*$^{fl/Y}$ mice was associated with a decrease of $B220^{+}CD43^{-}$ cells and an unchanged total number of BM cells. This suggested that *Ddx3x*-deletion in B cells with a high level of MYC was deleterious for these cells and may have caused their loss. By contrast, female E*μ-Myc*/*Vav-cre*/*Ddx3*$^{X/fl}$, E*μ-Myc*/*Vav-cre*/*Ddx3*$^{X/X}$ and E*μ-Myc*/*Vav-cre*/*Ddx3*$^{X/Y}$ controls have the characteristic features of E*μ-Myc* mice: a pre-B cell expansion and splenomegaly (**Figure 4-9C, D**). Further analysis of pre-malignant B cells from E*μ-Myc*/*Vav-cre*/*Ddx3*$^{fl/Y}$ mice revealed similar phenotypes to those found in *Vav-cre*/*Ddx3*$^{fl/Y}$(**Figures 3-11A-D and 3-12A-F**). They had a decreased number of lymphocytes in their BM, more particularly a decreased number of mature Hardy fractions E and F and showed an increase of fraction A and $B220^{+}CD43^{+}$ cells compared to E*μ-Myc*/*Vav-cre*/*Ddx3*$^{fl/Y}$ and *Vav-cre*/*Ddx3*$^{X/Y}$ mice (**Figure 4-10A**).

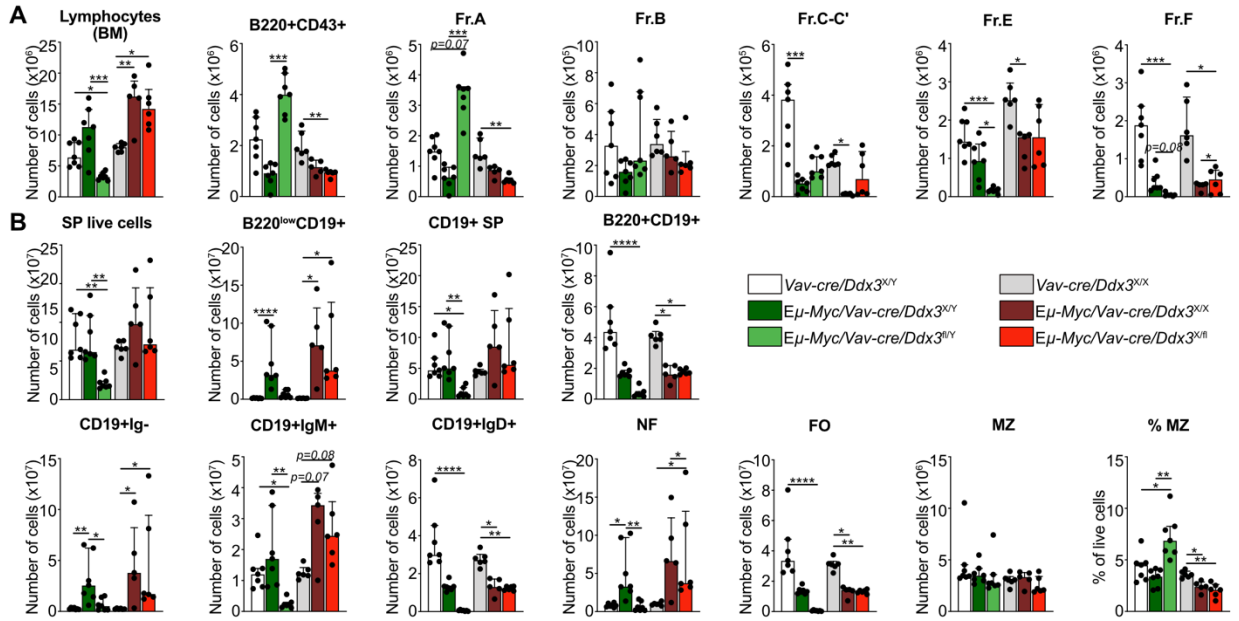


Figure 4-10: Pre-tumor analysis of E*μ-Myc* with and without *Ddx3x*-deletion

A, Quantification of BM B cell populations (Hardy fractions) from flow cytometric analysis from both male and female animals with indicated genotypes during the pre-lymphomatous phase. **B,** Quantification of splenic B cell populations from flow cytometric analysis from both male and female animals with indicated genotypes during the pre-tumor phase. Statistical significance was assessed with the Kruskal-Wallis test between mice of the same sex. The p value is indicated as follows: *ns*, p>0.05, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

E*μ-Myc*/*Vav-cre*/*Ddx3*$^{fl/Y}$ mice showed decreased numbers of splenocytes including B cells, CD19^{+}IgM^{+}, CD19^{+}IgD^{+}, NF and FO subsets, but a higher frequency of MZ B cells was detected in E*μ-Myc*/*Vav-cre*/*Ddx3*$^{fl/Y}$ mice (**Figure 4-10B**). This severe lymphopenia was not observed in control mice, and deletion of one *Ddx3x* allele in E*μ-Myc* mice did not impact B lymphocytes at the pre-tumor stage compared to E*μ-Myc*/*Vav-cre*/*Ddx3*$^{X/X}$ mice, again confirming that one intact *Ddx3x* allele compensates for the loss of the other (**Figure 4-10A, B**).

Since the pre-B cell expansion and splenomegaly typically observed in E*μ-Myc* mice were undetectable in young E*μ-Myc*/*Vav-cre*/*Ddx3*$^{fl/Y}$ males (**Figure 4-9A, B**), I suspected that the concomitant activation of *MYC* and *Ddx3x* deletion led to the elimination of those B cells that could potentially undergo malignant transformation and generate a lymphoma, which would explain why the majority of E*μ-Myc*/*Vav-cre*/*Ddx3*$^{fl/Y}$ mice were tumor-free although carrying the

Eμ-*Myc* transgene (**Figure 4-8A**). This notion was supported by the high level of Annexin V^{+} detected in splenic B cells from Eμ-*Myc*/*Vav-cre*/*Ddx3*$^{fl/Y}$ mice, even higher compared to Eμ-*Myc*/*Ddx3*$^{fl/Y}$ and *Vav-cre*/*Ddx3*$^{fl/Y}$ mice (**Figure 4-11A**).

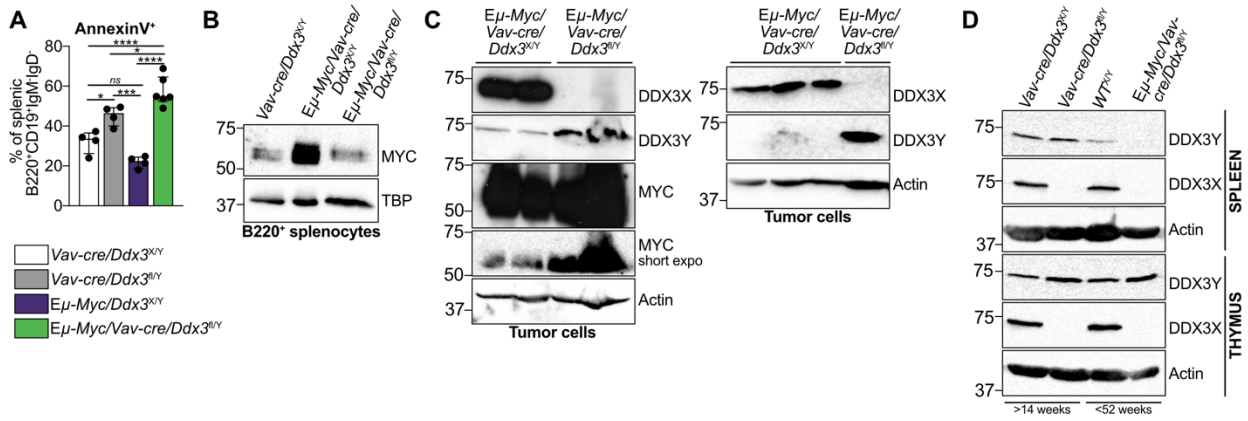


Figure 4-11: Concomitant activation of *c-Myc* and *Ddx3x*-deletion eliminates B cells

A, Flow cytometry analysis of splenocytes collected from adult tumor-free animals stained for Annexin V in addition to indicated surface markers. Significance was assessed by an ordinary one-way ANOVA test. **B,** Western blot analysis of nuclear extracts from splenic B220^{+} cells extracted from male animals with indicated genotypes during the pre-tumor phase. **C,** Western blot analysis of whole cell lysates from tumors developed in Eμ-*Myc/Vav-cre/Ddx3*$^{fl/Y}$ or Eμ-*Myc/Vav-cre/Ddx3*$^{X/Y}$ as controls. **D,** Western blot analysis of splenocytes and thymocytes extracted from animals with indicated genotypes and age; old Eμ-*Myc/Vav-cre/Ddx3*$^{fl/Y}$ mice (<52 weeks) are lymphoma-free animals.

In addition, MYC expression was lower in Eμ-*Myc*/*Vav-cre*/*Ddx3*$^{fl/Y}$ males than normally seen in Eμ-*Myc* transgenic mice and was comparable to the endogenous MYC levels of animals not carrying the Eμ-*Myc* transgene, which would be in agreement with the hypothesis that cells with high MYC expression and lacking DDX3X had been eliminated (**Figure 4-11B**). However, this did not explain why in rare cases (<15% of cases), tumors could arise from Eμ-*Myc*/*Vav-cre*/*Ddx3*$^{fl/Y}$ animals. Three out of the four *Ddx3x*-KO tumors obtained could be collected and were analyzed by Western blot. The absence of DDX3X protein was confirmed then excluding that these tumors were formed by a lack of *cre* efficiency or by escaping *Ddx3x*-deletion (**Figure 4-11C**). In addition, an upregulation of DDX3Y protein was detected in all three collected *Ddx3x*-KO tumors compared to *Ddx3x*-WT tumors expressing a low or undetectable level of DDX3Y

(**Figure 4-11C**). All tumors with a loss of DDX3X protein had a high level of DDX3Y protein, suggesting that DDX3X loss could be rescued in B cells by DDX3Y upregulation ensuring cell survival in the absence of DDX3X and allowing a B cell lymphoma development. *Ddx3y* compensation in the context of *Ddx3x*-KO Eμ-*Myc* tumors correlated with the compensatory effect previously observed while comparing B cell phenotypes in *Cd19-cre*/*Ddx3*$^{fl/Y}$ and *Cd19-cre*/*Ddx3*$^{fl/fl}$ mice (and other KO models previously described). Moreover, Western blot analysis of splenocytes from lymphoma-free Eμ-*Myc*/*Vav-cre*/*Ddx3*$^{fl/Y}$ animals (<52 weeks) revealed the absence of DDX3X as expected, but also the absence of DDX3Y whereas DDX3Y was clearly present in splenocytes from WT mice of similar age or *Vav-cre*/*Ddx3*$^{fl/Y}$ mice (**Figure 4-11D**). Hence the absence of lymphoma in Eμ-*Myc*/*Vav-cre*/*Ddx3*$^{fl/Y}$ animals correlated with the absence of DDX3Y, normally present in splenocytes, and suggested that B cells normally expressing DDX3Y had been lost in presence of an Eμ-*Myc* transgene. This was confirmed by DDX3Y detection in thymocytes, where the Eμ-*Myc* transgene is not expressed, regardless of age or whether *Ddx3x* was deleted or not (**Figure 4-11D**). This strongly suggested that a simultaneous transgenic expression of *c-Myc* and a deletion of *Ddx3x* is incompatible with cell survival and that high DDX3Y protein expression in the absence of DDX3X allows B cells to sustain *c-Myc* activation and lymphoma development.

To further investigate the role of DDX3X in B cell lymphomagenesis, Eμ-*Myc*/*Cd19-cre/Ddx3x*-floxed animals were generated. Not only was this model more accurate since the *Cd19-cre* deletes specifically in B cells compared to the *Vav-cre* deleter, but also it avoided the pan-hematopoietic effects of the *Vav-cre* deleter in full-KO female mice. This model allowed therefore to assess how Eμ-*Myc* lymphomagenesis is impacted by the deletion of both *Ddx3x* alleles specifically in B cells of female mice (**Figure 4-12A**).

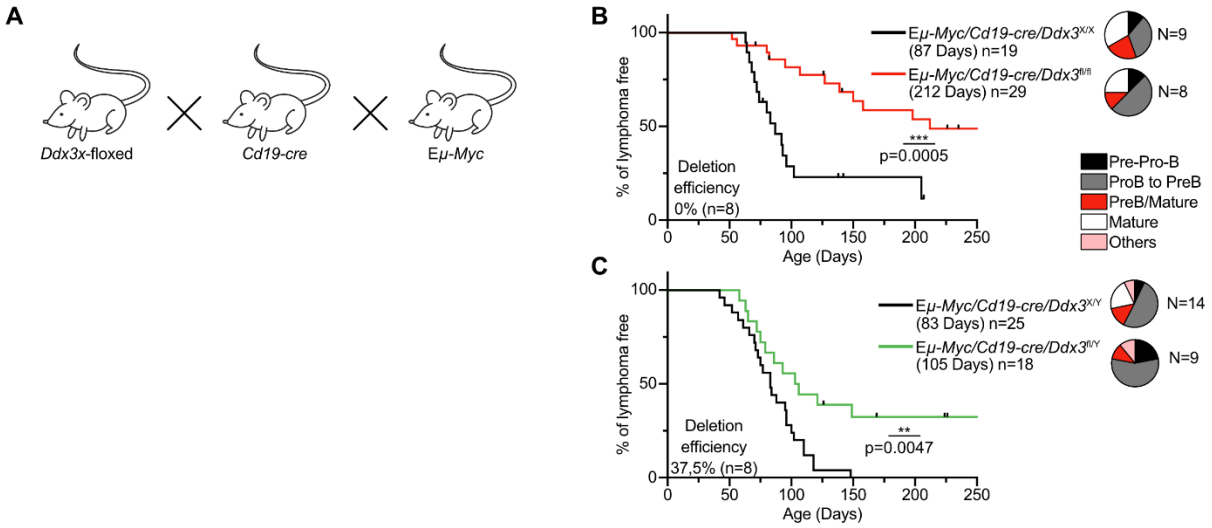


Figure 4-12: *Ddx3x*-deletion by *Cd19-cre* delays E*μ-Myc* tumorigenesis

A, Representation of the breeding strategy. **B,** Kaplan-Meyer curves representing the survival of females E*μ-Myc/Cd19-cre/Ddx3x*-floxed mice; male mice are represented in panel (**C**). Isolation and PCR genotyping of $CD19^+$ tumor cells from KO-mice allowed determining if a tumor achieved a complete deletion or not. At the bottom left of each survival curve, a deletion efficiency percentage was calculated based on PCR results. Statistics for survival curves were assessed with the Mantel-Cox test. The median survival is indicated in brackets and is followed by the number of animals in the cohort. The p value is indicated as follows: *ns*, $p>0.05$, *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

Ddx3x-deletion significantly delayed lymphomagenesis in E*μ-Myc*/*Cd19-cre*/*Ddx3*$^{fl/fl}$ animals compared to female mice carrying E*μ-Myc*/*Cd19-cre* transgenes and two intact *Ddx3x* alleles (**Figure 4-12B**). Similarly, *Ddx3x*-deletion in male mice significantly delayed lymphomagenesis in E*μ-Myc*/*Cd19-cre*/*Ddx3*$^{fl/Y}$ animals compared to E*μ-Myc*/*Cd19-cre*/*Ddx3*$^{X/Y}$ controls (**Figure 4-12C**). However, this delay was less significant in E*μ-Myc*/*Cd19-cre*/*Ddx3*$^{fl/Y}$ animals having a median survival of 105 days compared to E*μ-Myc*/*Cd19-cre*/*Ddx3*$^{fl/fl}$ mice having a median survival of 212 days. In addition, genotyping of $CD19^+$ tumor cells collected from E*μ-Myc*/*Cd19-cre*/*Ddx3*$^{fl/fl}$ mice revealed a 0% of deletion efficiency; i.e. all tumors that developed in female E*μ-Myc*/*Cd19-cre*/*Ddx3*$^{fl/fl}$ mice - without exception - were generated from cells that had escaped *Ddx3x*-deletion demonstrated by the presence of a "flox" band (**Figure 4-12B**). This indicated that cells lacking *Ddx3x* are counter-selected for this process and that DDX3X was critically required

for lymphomagenesis. Genotyping of $CD19^{+}$ tumor cells collected from E*μ-Myc*/*Cd19-cre/Ddx3*$^{fl/Y}$ mice revealed 37,5% of deletion efficiency (**Figure 4-12C**), indicating that most tumors (62.5%) escaped *Ddx3x*-deletion, which was not the case for tumors appearing in E*μ-Myc*/*Vav-cre/Ddx3*$^{fl/Y}$ mice (**Figure 4-8A**). It is therefore likely that cells more frequently escape a deletion induced by the *Cd19-cre* allele, which is less frequent or even impossible with the *Vav-cre* deleter, explaining the difference in survival curves between E*μ-Myc*/*Cd19-cre/Ddx3*$^{fl/Y}$ and E*μ-Myc*/*Vav-cre/Ddx3*$^{fl/Y}$ animals. However, 37,5% of tumors from E*μ-Myc*/*Cd19-cre/Ddx3*$^{fl/Y}$ mice achieved a complete *Ddx3x*-deletion, indicating that some tumors can emerge in the absence of DDX3X, suggesting that the loss of DDX3X can be compensated by DDX3Y in male mice to enable MYC-driven lymphomagenesis.

These findings and their conclusions were confirmed by using another murine model: λ-*Myc* transgenic mice that mimic B lymphomagenesis similarly to the E*μ-Myc* mice. λ-*Myc* mice develop spontaneously monoclonal mature B cell lymphoma with BL characteristics owing to deregulation of the *MYC* transgene by the λ light chain enhancer (Kovalchuk et al., 2000). λ-*Myc* tumors harbor some BL characteristics and are more mature compared to E*μ-Myc* tumors, and in this regard, represent a more suitable model for BL. λ-*Myc* were crossed to *Cd19-cre/Ddx3x*-floxed mice to assess the impact of *Ddx3x*-deletion in this model (**Figure 4-13A**).

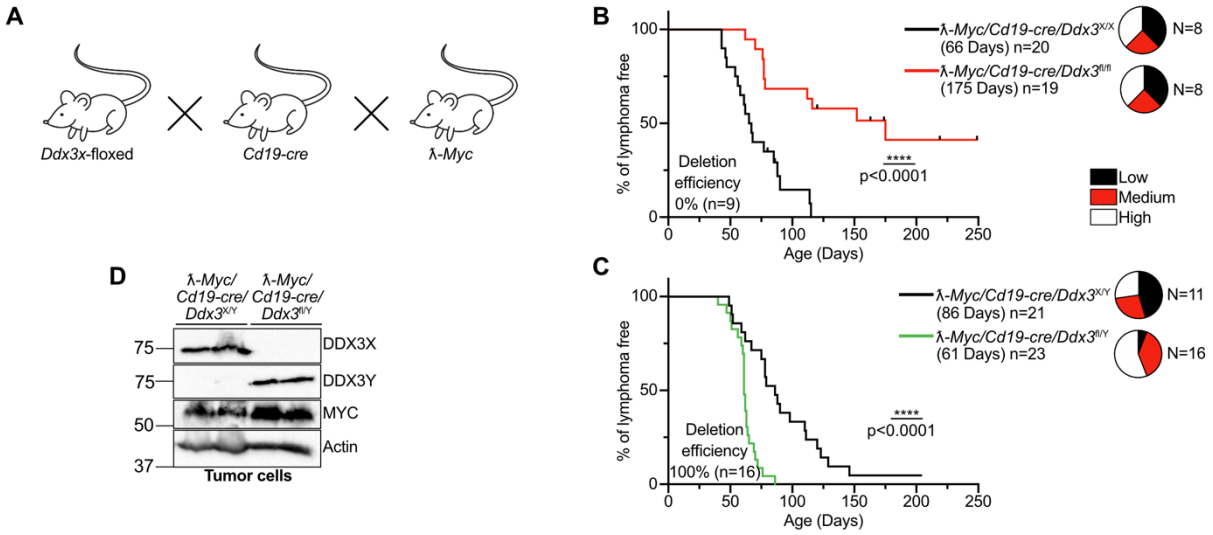


Figure 4-13: *Ddx3x*-deletion by *Cd19-cre* delays λ-Myc tumorigenesis

A, Representation of the breeding strategy. **B,** Kaplan-Meyer curves representing the survival of females λ-*Myc/Cd19-cre/Ddx3x*-floxed mice; male mice are represented in panel (**C**). Isolation and PCR genotyping of CD19^{+} tumor cells from KO-mice allowed determining if a tumor achieved a complete deletion or not. At the bottom left of each survival curve, a deletion efficiency percentage was calculated based on PCR results. Statistics for survival curves were assessed with the Mantel-Cox test. The median survival is indicated in brackets and is followed by the number of animals in the cohort. The p value is indicated as follows: *ns*, p>0.05, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. **D,** Western blot analysis of whole cell lysates from CD19^{+} tumor cells from λ-*Myc/Cd19-cre/Ddx3x*-floxed mice.

λ-*Myc*/*Cd19-cre/Ddx3*$^{fl/fl}$ female mice had a significant delay in lymphoma initiation compared to λ-*Myc*/*Cd19-cre/Ddx3*$^{X/X}$ mice (**Figure 4-13B**). Genotyping of CD19^{+} tumor cells revealed that all female tumors without exception, emerged from cells that had escaped *Ddx3x*-deletion, exactly as the female *Ddx3x*-deficient E*μ-Myc* mice, again confirming an absolute requirement of *Ddx3x* for B cell lymphomagenesis. In male mice, tumors developed in both λ-*Myc*/*Cd19-cre/Ddx3*$^{fl/Y}$ and λ-*Myc*/*Cd19-cre/Ddx3*$^{X/Y}$ mice (**Figure 4-13C**). Nevertheless, the deletion efficiency for λ-*Myc*/*Cd19-cre/Ddx3*$^{fl/Y}$ was 100%, meaning that all tested tumors had achieved a complete *Ddx3x*-deletion and none of them escaped it in this model. Western blot analysis confirmed the absence of DDX3X, but also showed upregulation of DDX3Y protein expression in *Ddx3x*-KO-tumors (**Figure 4-13D**), demonstrating the ability of DDX3Y to compensate for the loss of DDX3X also in mature MYC-driven B cell lymphoma. None of the λ-*Myc* tumors escaped the *Cd19-cre* deletion

(**Figure 4-13C**), suggesting that DDX3Y compensation was more efficient in these more mature, BL-like tumors compared to the malignancies emerging from earlier, less mature B cell stages in Eμ-*Myc*/*Cd19-cre/Ddx3*$^{fl/Y}$ mice (**Figure 4-12C**). Moreover, λ-*Myc*/*Cd19-cre/Ddx3*$^{fl/Y}$ tumors may appear more mature compared to control tumors (**Figure 4-13C**), again in favor of a more efficient compensatory effect of DDX3Y in mature B cell tumors. Finally, B cell lymphomagenesis appeared to be even accelerated in λ-*Myc*/*Cd19-cre/Ddx3*$^{fl/Y}$ animals compared to λ-*Myc*/*Cd19-cre/Ddx3*$^{X/Y}$ controls (**Figure 4-13C**). This could be due to a more efficient compensatory effect of DDX3Y in more mature B cell tumors compared to the less mature B cell lymphomas that emerge in Eμ-*Myc*/*Cd19-cre/Ddx3*$^{fl/Y}$ mice (**Figure 4-12C**). Alternatively, this acceleration may also indicate that in mature lymphomas, the loss of DDX3X and concomitant gain of DDX3Y represents an event that is favorable for MYC-induced malignant transformation and may reflect the situation found in male BL patients with *DDX3X* LOF mutations.

5. CHAPTER V: Conclusion

5.1. Discussion

5.1.1. Chapter III: DDX3X's role in hematopoiesis

At the time this project was initiated, one of the main questions was whether DDX3X is involved in B cell differentiation and hematopoiesis in general. Indeed, this project was started to better understand the role of DDX3X in lymphomagenesis, but basic knowledge about DDX3X's role in lymphocytes was lacking and an absolute pre-requisite for this study. Therefore, this was investigated by generating a conditional KO mouse model, as described in chapter III. New evidence from several mouse models is presented here and supports a sex-dependent, critical role of DDX3X for specific steps in erythropoiesis and lymphoid differentiation. Most significantly, it is shown that male and female mice lacking DDX3X have different phenotypes and compensation occurs in the absence of DDX3X by the male-specific *Ddx3y* gene.

During this investigation, two independent groups published reports about hematopoietic phenotypes in *Ddx3x*-KO mice, one being a non-peer reviewed report (K. Liu et al., 2018; Szappanos et al., 2018). In both studies, a conditional KO mouse model targeting *Ddx3x* exon 2 was generated and bred to the *Vav-cre* deleter, similar to the model used here. Both studies confirmed the majority of the results obtained in the work that forms the basis of the thesis: *Ddx3x*-deletion in male mice causes a decrease of B lymphocytes, more precisely of Hardy fractions B, D, E and F (K. Liu et al., 2018; Szappanos et al., 2018). Interestingly, fractions B and D (pro-B and small pre-B cells), where the V(D)J recombination of the *Ig* heavy and light chain genes occurs; as well as the DN2/DN3 stages of pre-T cell differentiation where the TCR β chain genes

are recombined, are affected in male mice in the absence of DDX3X. Since V(D)J recombination takes place at all these stages and is critical to produce mature lymphocytes, it supports the hypothesis of DDX3X's involvement in V(D)J recombination. Whether and how the DDX3X helicase affects these pathways requires further investigation. Whether this occurs through a direct regulation (for example, that DDX3X is directly involved in BCR or TCR signaling) or an indirect regulation (for example, translational or transcriptional regulation of factors involved in V(D)J recombination) remains to be shown too. A role for DDX3X in these critical steps of lymphoid development was previously suggested by a report indicating that *Ddx3x*-deleted small pre-B cells express lower levels of the bromodomain and WD repeat containing protein 1 *BRWD1* (K. Liu et al., 2018). This BRWD1 protein restricts V(D)J recombination at the *Igk* locus and *Brwd1* mutant mice harbor similar defects to *Ddx3x*-deficient animals: a decrease of Hardy fractions D, E and F and peripheral B cells (Mandal et al., 2015).

In agreement with the data presented in this thesis, Liu and colleagues also demonstrated a decrease in transitional B cells, FO, and GC in addition to the MZ expansion (K. Liu et al., 2018). In this same report, the MZ expansion is absent from CD45.1 transplanted mice, although the percentages of MZ cells seemed to increase, and the authors suggested that this may be the consequence of an indirect effect of *Ddx3x*-deletion caused by extrinsic factors (K. Liu et al., 2018). In contrast, the transplantation of *R26-cre*^{ER}/*Ddx3*$^{fl/fl}$ BM cells into CD45.1 recipients described here resulted in a MZ cell expansion, rather suggesting an intrinsic effect of *Ddx3x*-deletion (**Figure 3-16G, H**). While I analyzed the percentages of living cells, Liu and colleagues analyzed the total number of MZ cells that is likely affected by the low amount of live splenocytes (Figure 4 from (K. Liu et al., 2018)), which would support the notion that the observed MZ expansion is an intrinsic defect of *Ddx3x*-deletion. However, this expansion never led to any tumor

formation in *Vav-cre/Ddx3*$^{fl/Y}$ animals even when they were maintained until one year of age. It is therefore unlikely that the MZ B cell expansion is associated with malignant transformation. The observation of a concomitant decrease of FO and increase of MZ B cells could indicate a defect at this specific step of differentiation where transitional B cells mature into MZ or FO B cells (**Figure 1-2**). This cell fate decision depends on Notch2 and BAFF/NF-κB signals (Cariappa et al., 2000; Hwang et al., 2018; T. Saito et al., 2003), that are known to direct the differentiation towards MZ B cells when over-activated and towards FO when inactivated (Gibb et al., 2010; Lechner et al., 2021; Mackay et al., 1999; Moran et al., 2007; J. J. Wang et al., 2013). In addition, the strength of BCR signaling also influences this cell fate decision (Carey et al., 2008; Cariappa et al., 2001; Hampel et al., 2011; Martin & Kearney, 2000b; Pillai et al., 2005; Wen et al., 2005), and several studies demonstrated for instance, that a weak BCR signal strength directs differentiation to MZ over FO. Whether a MZ/FO unbalance in *Ddx3x*-KO mice results from a poor quality of the BCR signal in the absence of DDX3X or is the result of FO cells trans-differentiating into MZ B cells remains to be shown (Lechner et al., 2021; Srivastava et al., 2005; Vinuesa et al., 2003). Also, whether MZ/FO unbalanced phenotype could also be a consequence of the defective V(D)J recombination process(es) remains to be investigated.

While DDX3X has been shown to be crucial for the early stages of embryonic development (C. Y. Chen et al., 2016; Q. Li et al., 2014), I demonstrated a specific requirement of DDX3X for fetal erythropoiesis that was never described before. While Chen and colleagues demonstrated a severe impact of *Ddx3x* hemi- and heterozygous deletion in early embryogenesis (C. Y. Chen et al., 2016), a phenotype in fetal hematopoiesis was only observed when both *Ddx3x* alleles were deleted, pointing to a cell-type specific compensation. Interestingly, the *Vav* transcripts are first detected around developmental stage E11,5 suggesting that the *Vav-cre* deleter is active from this

stage of development (Bustelo et al., 1993). However, data obtained with the *R26*$^{mT/mG}$ reporter allele demonstrated that the *Vav-cre* deleter is more active in definitive erythrocytes compared to primitive erythrocytes in control embryos (**Figure 3-2C**, top panels), correlating with the block of definitive erythropoiesis observed in *Ddx3x*-deleted embryos.

Not only did this first part of the study reveal the importance of DDX3X in hematopoietic progenitors, lymphocytes, and erythrocytes, but it also provided first clues about a sexual dimorphism caused by the localization of the *Ddx3x* gene at the X chromosome. While most studies focused on male *Vav-cre*/*Ddx3*$^{fl/Y}$ mice (K. Liu et al., 2018; Szappanos et al., 2018), the analysis of female KO and heterozygous animals in various *cre* systems presented here provided a more complete picture of sex-dependent hematopoietic phenotypes. A common observation in all models was that one *Ddx3x* allele is sufficient to compensate well for all the hematopoietic phenotypes detected in full KO female mice. Even if both *Ddx3x* alleles are active, B220^{+} cells with one allele deleted express a similar level of DDX3X protein compared to B220^{+} cells with two intact alleles (**Figure 3-3E**), excluding a haploinsufficiency or a dosage effect from the *Ddx3x* gene, at least in B cells. It is possible that this is due to the so far unknown mechanisms adjusting DDX3X protein levels after the loss of one allele, at least in murine B lymphocytes. These findings are in agreement with observations made in hepatocytes where a full *Ddx3x* deletion sensitizes mice to liver tumorigenesis, while deletion of one allele does not confer any phenotype, revealing a similar compensation by one intact *Ddx3x* allele in hepatocytes (Chan et al., 2019). However, this compensation may not occur in all cell types and may not be perfect, as observed in the murine brain. A *Ddx3x*-KO in neural progenitors induced a limited phenotype in heterozygous mice, much less severe compared to the full DDX3X-KO which caused microcephaly (Hoye et al., 2022). This indicates that in contrast to B cells, protein and RNA levels are regulated differently in neural

progenitors where haploinsufficiency exists (Boitnott et al., 2021; Hoye et al., 2022). Another example is provided by a study on the role of DDX3X in medulloblastoma where deletion of one *Ddx3x* allele does not confer any phenotype in a model of Wnt-activated medulloblastoma; in contrast to a model of Sonic-hedgehog-activated medulloblastoma (Patmore et al., 2020). Since those tumor types emerge from different cells of origin (Gibson et al., 2010), this demonstrates again that the *Ddx3x* haploinsufficiency model can exist, but varies even within the same organ and is therefore cell-type and context-dependent. In humans, an example of *DDX3X* haploinsufficiency may be illustrated in ID patients. These patients are almost exclusively females with a heterozygous germline *Ddx3x* mutation whereas male ID patients with such a germline *DDX3X* mutation are very rare. This indicates that one *DDX3X* intact allele in female patients is beneficial and allows embryonic development by contrast to males, but there is still an imperfect compensation from this intact *DDX3X* allele causing severe phenotypes during development. However, a skewed or incomplete X-chromosome inactivation may further complicate the situation in these patients (Snijders Blok et al., 2015).

The data presented here study also provide the first answers to the question of whether the male homolog of DDX3, DDX3Y, is expressed during blood cell formation and whether it can exert the same role as DDX3X. In the context of this project, the first DDX3Y-specific murine antibody was generated and allowed to demonstrate the expression of this Y-linked DDX3 paralog in murine thymocytes and splenocytes (**Figure 3-3A-D**). The observation that *Vav-cre*/*Ddx3*$^{fl/Y}$ pups were produced at a mendelian ratio but live-born *Vav-cre*/*Ddx3*$^{fl/fl}$ female mice were never obtained would be consistent with the view that DDX3Y can indeed compensate for DDX3X loss, at least in fetal erythropoiesis. Moreover, *in vitro* expansion of induced GC B cells and *in vivo* stimulation of GC B cells are other examples where DDX3Y compensates for DDX3X loss. A

DDX3Y compensatory effect was also evident in adult erythropoiesis since *Vav-cre*/*Ddx3*$^{fl/Y}$ mice, although having a decreased hematocrit (**Figure 3-4E**), do not show the severe anemia that affects the survival of female DDX3X-KO mice that received *R26-cre*^{ER}/ *Ddx3*$^{fl/fl}$ BM cells (**Figure 3-15B, C**). In addition to the fact that the DDX3Y protein was presumably expressed in several tissues in mice, several groups recently claimed a DDX3Y compensation in the case of DDX3X depletion even though DDX3Y protein expression had never been properly demonstrated in these models because of the lack of suitable specific antibodies. These claims were based on the observation that male *Ddx3x*-KO mice had a mild phenotype compared to female full-KO mice that were more severely impacted by DDX3X homozygous loss in hematopoietic cells (K. Liu et al., 2018; Szappanos et al., 2018), hepatocytes (Chan et al., 2019), as well as in murine brain cells (Hoye et al., 2022; Patmore et al., 2020). Furthermore, one study even raised the question of whether DDX3Y ectopic expression may explain why in rare cases, male ID patients with a *DDX3X* germline mutation are viable (Hoye et al., 2022). However, the data presented here and published findings clearly indicate that any compensatory effect of DDX3Y is imperfect and very likely context- and cell type-dependent, and in this regard, comparable to the compensation of one *Ddx3x* allele in females discussed above. The context dependency and the degree of compensation that can be provided by DDX3Y are most likely due to its variable expression level among different cell types, differences in its subcellular localization (compared to DDX3X), differences in its enzymatic activity (also compared to DDX3X), a combination of these reasons, and/or any other unknown function that may differ from DDX3X and remain to be clarified. A recent report showed that human DDX3X and DDX3Y enzymes are functionally redundant in mRNA translation catalyzing protein synthesis (Hoye et al., 2022; Venkataramanan et al., 2021), but their functions may differ in other contexts such as stress response for instance (Shen et al., 2022). In this thesis

work, I could assess the expression of the DDX3Y protein in bulk populations (splenocytes and thymocytes), but the many attempts to assess its expression in smaller, more specific populations were ineffective. A Western blot of 293T cells (a female human cell line) transfected with a murine Flag-tagged DDX3Y revealed that the anti-DDX3Y antibody has a good capacity of detection compared to the anti-Flag antibody (**Figure 5-1**).

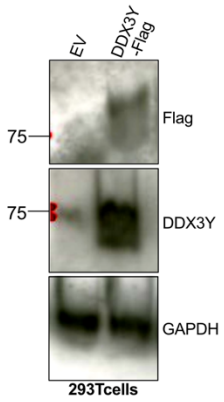


Figure 5-1: Validation of the anti-DDX3Y antibody sensitivity
HEK293T cells were transiently transfected with a Flag-tagged DDX3Y vector expressing the murine DDX3Y protein or empty vector as control. This experiment was done by Dr. Hugues Beauchemin.

This suggests that the difficulties encountered with the detection of DDX3Y protein detection are most likely linked to its low level of expression and not to a low sensitivity of the anti-DDX3Y antibody. However, it cannot be excluded that cell type-specific post-translational modifications of DDX3Y interfere with the recognition of the protein by this antibody. Based on the available data, I hypothesize that a low level of DDX3Y in splenocytes and thymocytes is at least one of the reasons why DDX3Y imperfectly compensates for *Ddx3x*-deletion.

5.1.2. Chapter IV: DDX3X's role in lymphomagenesis

Chapter IV of this thesis describes experiments that were performed to better understand the role of DDX3X in B lymphomagenesis using the conditional KO mice described in chapter III. *DDX3X* LOF mutations have frequently been reported in GC-derived B cell lymphoma: estimates

indicate that approximately 30% of BL and 14-28% of MYC dysregulated DLBCL harbor *DDX3X* mutation, and their consequence for tumorigenesis was unknown (Burkhardt et al., 2022; Cucco et al., 2020; Gong et al., 2021; Miyaoka et al., 2022; N. Thomas et al., 2023). This was investigated by breeding a *Ddx3x*-conditional KO mouse model with models that mimic MYC hyper-activation and spontaneously develop B cell lymphoma. These experiments provided new evidence for a requirement of DDX3X for GC physiology and MYC-driven lymphomagenesis and support the incompatibility of *Ddx3x*-deletion in B cells highly expressing MYC and the ability of DDX3Y to compensate for the loss of DDX3X and initiate lymphomagenesis. My results present a sexual dimorphism of murine DDX3X and DDX3Y RNA helicases in B cell physiological and malignant development.

Using the *Cd19-cre* deleter allele allowed a clear comparison between female KO, heterozygous and male KO mice within the same experimental system. This confirmed the compensatory effects by one *Ddx3x* allele and *Ddx3y* allele in B cells described in chapter III. I noticed that *Ddx3x*-deletion negatively affects the maintenance of GC B cells in physiological conditions but also their expansion in an immunization context. CSR and SHM occurring in GC B cells require DNA strand breaks that must be repaired without generating an abortive DNA damage response for instance through TP53 activation (Bahjat & Guikema, 2017). Experimental results obtained with *Trp53/Ddx3x* double KO mice demonstrated however that TP53 activation is still intact in *Ddx3x*-deficient GC B cells, and excluded p53-dependent apoptosis as the cause of a loss of GC B cells in these mice. This suggests that DDX3X probably regulates the proliferative expansion of GC B cells, but this still needs to be clarified in detail. Interestingly, *in vitro* studies with the iGB co-culture system resulted in the survival of DDX3X-depleted female GCs, but not in their expansion. It is therefore likely that DDX3X exerts an intrinsic role in GC

expansion, but the absence of DDX3X-deficient GC B cells *in vivo* may be caused by extrinsic factors. Robinson and colleagues have found a similar phenotype using the iGB *in vitro* system when B cells are co-cultured with the BAFF and CD40L but in the absence of IL-4 (Robinson et al., 2019). BAFF and CD40L signals are indeed sufficient for B cell survival and to induce a GC-like phenotype, but are insufficient for their expansion. This could point to the possibility that *Ddx3x*-KO cells harbor defects in their IL-4 receptor and/or IL-4 signaling cascade which block their proliferation expansion even if they survive the Cγ1-*cre* deletion *in vitro*.

The high frequency of somatic LOF mutations in *DDX3X* in human BL has raised interest in the role of DDX3X in B cell lymphomagenesis (Arthur et al., 2018; Burkhardt et al., 2022; Cucco et al., 2020; Grande et al., 2019; M. Kim et al., 2022; Miyaoka et al., 2022; Richter et al., 2012; Schmitz et al., 2012; N. Thomas et al., 2023; Zhang et al., 2020). In chapter IV, the observation that male E*μ-Myc/Vav-cre* mice lacking DDX3X are almost free of lymphoma indicated a strict requirement of DDX3X for MYC-driven lymphomagenesis. Although this was recently suggested for human B lymphoma (Gong et al., 2021), it was not yet shown in a murine lymphoma model. The experiments described in chapter IV provide new evidence for a requirement of DDX3X for effective B cell lymphomagenesis from two mouse models, the E*μ-Myc* and λ-*Myc* transgenic mice, the latter being a model for human BL (Kovalchuk et al., 2000). While both models showed that the loss of DDX3X impedes MYC-driven tumorigenesis, another study showed that human GC B cells co-transduced with MYC in addition to a DDX3X LOF mutant acquire a competitive growth advantage (Gong et al., 2021). Although these are very different experimental systems and cellular contexts, the differences between these experiments and the data presented here were intriguing and could also reveal a dominant negative effect of *DDX3X* mutants used in the human system that cannot be observed when DDX3X is entirely

depleted, as it is the case in the conditional KO mice. The combination of the E*μ-Myc* transgene and the pan-hematopoietic *Vav-cre* deleter showed that the pre-B cell expansion that usually takes place during the pre-lymphomatous phase in E*μ-Myc* mice is not detected in male mice when DDX3X is absent, suggesting that the combination of *Ddx3x*-deletion and MYC activation eliminates these cells, possibly through cell death triggered by increased cell stress as previously suggested (Gong et al., 2021). It is thus conceivable that a simultaneous *Ddx3x*-deletion and MYC activation eliminates the cells available for MYC-driven tumorigenesis thereby protecting mice against lymphoma. This is also supported by my finding that only B cells that upregulate DDX3Y expression can tolerate *Ddx3x*-deletion and MYC activation and therefore can develop a lymphoma, whereas DDX3Y is undetectable in the spleen of E*μ-Myc*/*Vav-cre*/*Ddx3*$^{fl/Y}$ lymphoma-free mice. Lymphomagenesis in male mice lacking DDX3X occurs therefore only when DDX3Y is upregulated, a conclusion supported by two studies which demonstrated that DDX3Y, although not present in normal human B lymphocytes, is expressed in a malignant context, especially in BL (Gong et al., 2021; Rosinski et al., 2008).

The use of a B cell-specific *Cd19-cre* deleter in the E*μ-Myc* and λ-*Myc* models showed a clearer picture since it avoided the effects of a pan-hematopoietic deletion caused by the *Vav-cre* deletion in females. Data from these two models provided strong support for the notion that B cells do not tolerate *Ddx3x* loss in the context of an activated *Myc* since lymphomas with full deletion of both *Ddx3x* alleles were never observed in E*μ-Myc* or λ-*Myc* female mice. Moreover, the critical requirement of DDX3 activity in general for B cell lymphomagenesis was also evident in male mice. Indeed, 37,5% of E*μ-Myc/Cd19-cre/Ddx3*$^{fl/Y}$ and all λ-*Myc*/*Cd19-cre/Ddx3*$^{fl/Y}$ mice developed tumors that had a full *Ddx3x*-deletion indicating that DDX3Y can compensate for DDX3X loss, sustain *c-Myc* activation and enable the development of a B cell lymphoma. This

result is in agreement with the report indicating that DDX3X loss in male BL patients is compensated by an ectopic expression of DDX3Y (Gong et al., 2021). These data provide at least one explanation as to why BL patients with a *DDX3X* mutation are almost exclusively male patients (Burkhardt et al., 2022; Gong et al., 2021; N. Thomas et al., 2023).

Another recent study demonstrated that DDX3X and DDX3Y have redundant functions in translational regulation confirming that one can compensate for the loss of the other (Venkataramanan et al., 2021). It is therefore plausible that DDX3Y compensates for the deleterious effects caused by DDX3X LOF mutations in BL cells allowing MYC-driven lymphomagenesis to occur in BL patients. While the results presented in this thesis suggest that DDX3Y is expressed in normal murine lymphocytes, which is a fundamental difference compared to humans, as shown by Gong and colleagues (Gong et al., 2021), the murine lymphoma models still led to a very similar conclusion compared to the Gong study: DDX3Y enables lymphoma development in case of DDX3X absence. It is thus well possible that the co-occurrence of DDX3X loss and a gain of MYC represents a synthetic lethal combination that could be exploited for the development of new therapeutical options for human MYC-driven B cell lymphoma, but it is also conceivable that DDX3Y represents the target of interest in male patients harboring *DDX3X* mutations.

5.2. Limitations

The immuno-phenotyping analysis of hematopoietic populations in *Ddx3x*-KO mice was realized in a physiological context, i.e. without any hematopoietic stimulation. Therefore, it is not excluded that DDX3X plays a role in populations that were found unaffected in percentage and/or in number under these conditions and that this role only reveals itself under specific conditions,

for example in the presence of specific cytokines or other immune modulators. One example of this is myeloid cells that were not affected in numbers in *Ddx3x*-KO mice (**Figure 3-6D**), as confirmed by Samir and colleagues that used *Ddx3x*-KO mice bred with a myeloid-specific *cre* deleter (Samir et al., 2019). However, the authors also demonstrated that when the NLRP3 inflammasome is activated, *Ddx3x*-deletion protects against inflammation and cell death (Samir et al., 2019). Another example is found in the Szappanos study: even if the percentages and total numbers of myeloid cells are normal in *Ddx3x*-KO mice, *Ddx3x*-KO BM-derived macrophages respond less efficiently *in vitro* to *Listeria monocytogenes* infection or Toll-like receptor activation (Szappanos et al., 2018). Such phenotypes could not have been found in my model since the immuno-phenotyping analysis was done in a physiological context without any NLRP3 activation or bacterial stimulation for example. Consequently, it is not excluded that other hematopoietic populations found unchanged in my models may be affected by *Ddx3x*-KO when stimulated; for example, the granulocytes although unaffected in quantity (**Figure 3-6D**) may still be affected by *Ddx3x*-KO when stimulated with an allergen.

Since deletion of both *Ddx3x* alleles in females is incompatible with life past mid-gestation, it was necessary to model a full *Ddx3x*-KO by transplantation of cells in which the *Ddx3x* gene could be acutely inactivated in adult animals. However, even if the *R26-cre*^{ER} murine model has allowed generating an inducible *Ddx3x*-KO in adult female mice and has demonstrated DDX3X's role for the maintenance of hematopoietic cells, more particularly BM progenitors and erythrocytes, the flow cytometry results are clearly affected by tamoxifen. This drug is known to be toxic (X. Tian & Zhou, 2021), and its injection into *R26-cre*^{ER} mice results in a considerable variation in cell population percentages as well as some reduced populations, the DP T cells for example (**Figure 3-15F, G** compared to **Figure 3-8C**). Consequently, this model has severe

limitations which can at least be reduced or avoided by using more sensitive *cre* systems that can be activated with lower tamoxifen doses to decrease these undesirable side effects (Indra et al., 1999; X. Tian & Zhou, 2021).

One of the main limitations of the study described here resides in the murine models themselves. First, I clearly demonstrated that the DDX3Y protein is expressed in murine B cells under physiological conditions, while this is not the case for human B lymphocytes according to the study of Gong and colleagues (Gong et al., 2021). Consequently, the absence of DDX3X in murine malignant B cells is compensated by an upregulation of the murine DDX3Y protein instead of the ectopic induction of DDX3Y protein expression observed in human tumors (Gong et al., 2021). Furthermore, it has been suggested that the differences in phenotypes between male and female *Ddx3x*-KO mice result from compensation by the male-specific *Ddx3y* gene, although this has never been formally demonstrated. Even if *Ddx3y* is very likely the factor compensating for the loss of DDX3X, it cannot be ruled out that compensation from other male-specific factors may occur. Such a demonstration would require the generation of a double *Ddx3x*/*Ddx3y* KO mice allowing testing whether the absence of both *DDX3* genes causes the same defects as those detected in female KO mice.

Second, mice carry another *DDX3* homolog on their chromosome 1 named *D1Pas1* (or *Pl10*) which is believed to not exist in humans. This gene is highly similar to murine and human DDX3 proteins (**Figure 1-5**), and is known to be important for male fertility in the mouse (Inoue et al., 2016; Matsumura et al., 2019; Session et al., 2001; Vong et al., 2006). *D1Pas1* has been poorly characterized outside of murine male germ cells and its pattern of expression or function remains totally unknown. Therefore, this could potentially contribute to the biological difference(s) observed between humans and mice although the analysis of single cell RNA-seq

data from splenocytes after immunization shows that transcripts of *D1Pas1* are not readily detected in murine B cells (T. Möröy, personal communication).

Third, the E*μ*-*Myc* or λ-*Myc* mice do not represent the best models *per se* to recapitulate GC-derived B cell lymphomagenesis. Indeed, E*μ*-*Myc* tumors emerge from pre-B to naïve B cells and lack surface *Ig*, hence their "immature" phenotype (Adams et al., 1985; Harris et al., 1988; S. N. Meyer et al., 2021; Pasqualucci & Klein, 2021). In this regard, they resemble more human lymphoblastic leukemia instead of GC-derived B cell lymphoma. λ-*Myc* tumors are more mature compared to E*μ*-*Myc* tumors (IgM^+) and mimic much better a mature human B cell lymphoma. However, λ-*Myc* tumors still express some immature markers (e.g. CD43) and lack evidence of SHM suggesting these tumors emerge from transitional or pre-GC B cells (Kovalchuk et al., 2000; S. N. Meyer et al., 2021; Pasqualucci & Klein, 2021). Accordingly, none of these models faithfully recapitulate the GC-derived B cell lymphomagenesis and alternative BL models have to be used in the future (Caeser et al., 2019; S. N. Meyer et al., 2021; Mossadegh-Keller et al., 2021; Pasqualucci & Klein, 2021; Sander et al., 2012). One of them, which is the best mouse model so far to mimic BL, is the model developed by Sander and colleagues (Sander et al., 2012). In this model, transgenic mice express high levels of MYC and a constitutively active form of PI3K (Srinivasan et al., 2009) specifically in B cells undergoing a GC reaction using the Cγ1-*cre* deleter. These mice generate tumors very closely resembling human BL since they express GC markers ($CD95^+$ $GL7^+$ $BCL6^+$), carry evidence of AID-dependent SHM validating their GC origin and are histologically similar to BL (Sander et al., 2012).

Fourth, *DDX3X* mutations reported in patients are mostly missense, nonsense mutations or truncations. Accordingly, the conditional KO mice may, to some extent, imitate the consequence of some mutations found in patients, for example truncations or nonsense mutations occurring

early in the DDX3X amino acid sequence. However, deleting the entire gene very likely does not provoke the same effect as a missense mutation, a nonsense mutation, or a truncation, occurring in the middle or the end of the amino acid sequence. Therefore, a potential dominant negative effect exerted by some mutants is impossible to assess in this system. An attempt to generate two humanized murine models was done (collaboration with Ingenious Targeting Laboratory and Dr. Peiman Shooshtarizadeh). These two additional murine models both contained a lox-stop-lox in the *Rosa26* locus upstream of the human Flag-*DDX3X* sequence, either WT or carrying a mutation encoding the DDX3X-E348K LOF protein (Jiang et al., 2015). However, these transgenes were insufficiently transcribed, and the human DDX3X-WT failed to rescue, even partially, the phenotypes observed in DDX3X KO mice (data not shown). Alternative strategies should be used to generate such models and ensure to include transcriptional regulator elements that allow a proper expression from the ROSA locus.

5.3. Futures directions

Even if this work brought a considerable amount of information about the DDX3 helicases, many questions are still open and remain to be investigated. Results from chapter III revealed that many, but not all hematopoietic cell types require DDX3X. It would then be important to understand the underlying causes of these population defects and to assess whether the mechanisms impaired in the absence of DDX3X are cell type-specific or not. Since the *Vav-cre* allele deletes in a wide spectrum of cells, defects in erythropoiesis or T lymphopoiesis for example should be assessed with a more specific *cre* system (e.g. Lck-*cre* (Hennet et al., 1995); CD4-cre (P. P. Lee et al., 2001)); similarly, to the strategies used to analyze the B cell lineage. This would

also be informative to determine whether phenotypes associated with loss of DDX3X are intrinsic or the results of earlier defects of differentiation or altered cellular environments.

The intestinal phenotype discovered when the acute deletion of *Ddx3x* in adult female mice was assessed, is of interest (**Figure 3-14**). Transplantation experiments indicated that this phenotype is independent of immune cells; it is therefore likely that an acute deletion of *Ddx3x* in adult female mice disrupts intestinal epithelial cell homeostasis provoking perforations of the intestine, bacterial infections and subsequent fatal inflammatory reactions. It would be relevant to assess which cell type/s is/are affected by *Ddx3x*-deletion causing such a fatal and rapid outcome. A similar premature death was found after deleting the SREBP cleavage-activating protein (*Scap*) gene in intestinal epithelial cells of adult mice (McFarlane et al., 2015). The *Scap* gene encodes an endoplasmic reticulum membrane protein and its deletion in the intestinal mucosa was associated with a defect in the proliferation of intestinal crypts (McFarlane et al., 2015). Given the similarities between the phenotype of *Scap* null mice and the consequences of *Ddx3x* deletion, it is possible that DDX3X is involved in maintaining the intestinal epithelium structure. Understanding DDX3X in the intestine would be informative for fundamental knowledge, as well as for translational research, given that several intestinal dysregulations in humans have been linked to DDX3X (Cording et al., 2022; Okano et al., 2020; Tang et al., 2021; Tantravedi et al., 2017). In addition, knowing whether this phenotype is associated with DDX3X enzymatic activity or not would be of great interest regarding the potential toxicities of drugs inhibiting DDX3X helicase activity as anti-cancer or anti-viral therapeutics.

Although the critical importance of DDX3X in B lymphopoiesis has been demonstrated in this thesis, it is still unknown why and how B cells are eliminated in the absence of DDX3X. A DDX3X knockdown in human B cells has recently been shown to reduce the translation efficiency

of mRNAs encoding proteins of the translational and protein synthesis machinery (Gong et al., 2021). Whether this is also the case in murine B cells needs to be investigated using technics such as CLIP-seq, RNAseq or Riboseq. Moreover, specific experiments to reveal how DDX3X is mechanistically involved in B cells regarding its RNA binding ability or RNA helicase activity or independently of these characteristics have to be done and may point to the translational regulation of proteins necessary for V(D)J recombination, BCR signaling or CSR recombination for example. It was initially demonstrated that RNA helicases unwind dsRNA but it is now known they can also unwind structures such as G-quadruplexes or R-loops known to be important for B cell physiology, particularly for CSR (Deze et al., 2023; Pavri, 2017). Recent findings that identified DDX3X as an interactor of RNA G-quadruplex support this view (Herdy et al., 2018; Varshney et al., 2021). Many DEAD box RNA helicases have recently emerged as essential factors in unwinding R-loops such as DDX19 (Hodroj et al., 2017), DDX39B (Perez-Calero et al., 2020), DDX21 (Song et al., 2017), DDX23 (Sridhara et al., 2017), DDX41 (Mosler et al., 2021), DDX5 (Mersaoui et al., 2019; Z. Yu et al., 2020) or the DEAH-RNA helicase DHX9 (Yuan et al., 2021). More specific to the B cell context, it was shown that the DDX1 helicase, another member of the DEAD box family, converts G-quadruplex structures of the Sμ switch region into R-loops, thus promoting CSR in B cells (Ribeiro de Almeida et al., 2018). Future investigations could aim to determine whether DDX3X is involved in R-loop, G-quadruplex regulation and CSR.

Not only the mechanistic role of DDX3X in B cells is yet to be discovered but the impact of DDX3X mutations detected in GC-derived B cell lymphoma needs to be determined more clearly. So far, only one study demonstrated that a *DDX3X* mutation is an advantageous acquisition for human GC B cells with high MYC levels since its counterbalances the proteotoxic stress associated with high MYC levels (Gong et al., 2021). Another open question is whether these

mutations only affect the enzymatic activity of DDX3X or other functions independent of its helicase activity, for instance its role as a general regulator of mRNA translation and/or other yet unknown function(s). Since unresolved R-loops cause genomic instability and increase the probability of translocations, particularly at the *Ig*H and c-*Myc* loci (Y. Yang et al., 2014), it will be of great interest to investigate the possibility that *DDX3X* mutation impairing its helicase activity impacts R-loop resolution at the *Ig*H Sμ region and provokes *Myc* translocations.

Although focused on the role of DDX3X in hematopoiesis, the present thesis work revealed an unexpected primordial role for the less studied DDX3 Y-linked paralog. This protein is less known, likely due to its restrained expression pattern in humans. However, it is clear that data presented in this thesis highlight its relevance in the pathological context demonstrating the need for future research to describe more precisely DDX3Y properties and functions. Understanding DDX3Y's mechanistic role, particularly alongside its X-linked paralog DDX3X, would be fundamental in order to determine at which degree these proteins play redundant functions, as already been suggested for the translational regulation (Venkataramanan et al., 2021). It would be important to assess whether and how they can replace each other in a pathological context. In this regard and as stipulated in the limitation section, assessing experimentally whether *Ddx3y* is the male-specific factor compensating for *Ddx3x* deletion could be a priority, particularly considering the lymphoma context. Also, because both paralogs are highly similar but not entirely identical, understanding their differences, like in the report from Shen and colleagues (Shen et al., 2022); could be of great interest too. This could provide explanations regarding the sexual dimorphism observed in the physiological as well as in the pathological context. It could lead to a better understanding of the sex bias observed in many diseases; for instance in BL (Burkhardt et al., 2022; Gong et al., 2021; N. Thomas et al., 2023), but also in melanoma (Alkallas et al., 2020;

Phung et al., 2019), hepatocellular carcinoma (Chan et al., 2019), ID (Johnson-Kerner et al., 2020), or Hirschsprung disease (Cardinal et al., 2020).

Finally, future work may focus on the therapeutic opportunities suggested by the results presented here. Several concepts need to be validated, particularly in human cells; one of them being the demonstration that DDX3X depletion in B cells with high MYC levels causes their death. In this thesis, the incompatibility of high MYC levels and DDX3X deficiency was clearly observed in murine B cells; and is a concept that could be exploited for therapeutic strategies. Whether this effect depends on the elimination of the DDX3X protein or its helicase activity only needs to be elucidated as well. Whether and to what extent targeting DDX3X affects normal B cells compared to B cells with high MYC levels also needs to be assessed since it could support a synthetic lethality concept and provide evidence for a specific therapeutic window of DDX3X inhibition in MYC-deregulated lymphoma.

Other potential therapeutic opportunities reside in the concept of interdependency of both DDX3X and DDX3Y paralogs. It has been proposed that the paralogs encoded by the sex chromosomes play redundant functions and the concept of paralog dependency in tumor cells was experimentally validated (Koferle et al., 2022). It means that cancer cells (but not healthy cells) with an impairment in one paralog; for example, a *DDX3X* mutation or a loss of Y chromosome, rely on the other paralog; respectively, DDX3Y or DDX3X. Accordingly, targeting the intact paralog represents an attractive therapeutic strategy based on synthetic lethality (Koferle et al., 2022). Targeting DDX3Y may be highly efficient to eliminate human malignant B cells carrying a *DDX3X* mutation. In addition, targeting DDX3Y may be highly specific to malignant B cells since the DDX3Y protein is not expressed in humans, except in male germ cells in addition to the

malignant B cells harboring a *DDX3X* mutation (Gong et al., 2021). This interdependency concept of DDX3X/Y was partially demonstrated for B lymphoma *in vitro* in one study (T. Wang et al., 2015). The authors used the Raji cell line, a B lymphoma cell line with a *DDX3X* LOF mutation generating a splicing variant, and demonstrated that the additional loss of DDX3Y results in cell death of this line (Figure 4F from (T. Wang et al., 2015)). Consequently, results from the Gong study in addition to the data presented in this thesis are relevant in this context since we both demonstrated in humans and mice that DDX3Y compensates for a DDX3X LOF. Future work should aim to confirm experimentally that the DDX3Y protein indeed has such a compensatory role in the case of DDX3X LOF and that both have redundant functions in B cells. It would also be important to demonstrate experimentally that the targeting of DDX3Y represents a synthetic lethal strategy in B cells MYC-activated harboring a *DDX3X* alteration similar to the LOF found in BL and DLBCL patients. This could even be extrapolated to demonstrate that targeting DDX3X represents a synthetic lethal strategy in malignant B cells which have lost their Y chromosome, a common feature in many cancers. Validation of the interdependency theory of DDX3X/Y for B lymphoma could lead to a stratification of patients based on their sex and DDX3 status (Lacroix et al., 2022). Patients with a *DDX3X* mutation (regardless of the sex but BL patients with a *DDX3X* mutation are almost exclusively males), or male patients with malignant B cells that have lost their Y chromosome would represent suitable candidates for whom targeting the intact paralog may be beneficial. This could ultimately result in personalized therapeutic options for B lymphoma patients.

6. Final conclusion and summary

The high frequency of mutations in the X-linked gene *DDX3X* in GC-derived B cell lymphoma has raised interest in understanding its role in hematopoietic cells and their malignant transformation. Since a large proportion of *DDX3X* mutations found in malignancies generate LOF variants, a conditionally deficient mouse model was generated to investigate the role of DDX3X in hematopoiesis and lymphomagenesis. I present new evidence from these mouse models supporting a sex-dependent, critical role of DDX3X for specific steps of erythropoiesis as well as lymphoid differentiation. Most significantly, it is shown that male and female mice lacking *Ddx3x* behave differently, notably in MYC-driven lymphomagenesis. I also demonstrate that a DDX3X depletion is compensated by the male-specific DDX3Y to permit B cell lymphomagenesis. These discoveries present insight toward new and personalized therapeutic strategies for B cell lymphoma.

7. References

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