The role of Sel1L-Hrd1 ER Associated Degradation in B cell development and function.

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Hana Kim

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The Role of Sel1L-ER Associated Degradation in B cell Development and Function

Hana Kim, Ph. D.
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The Endoplasmic Reticulum (ER) serves as the site for protein synthesis, folding, maturation and modification in the cell. In mammalian cells, approximately one-third of the newly synthesized proteins enter the ER in an unfolded state. Despite various folding machineries in the ER, protein folding is inherently error prone and often results in the generation of irreparable misfolded proteins which disrupts ER homeostasis. ER-associated degradation (ERAD) is a principle quality-control mechanism responsible for restoring ER homeostasis by targeting misfolded proteins in the ER for cytosolic proteasomal degradation. The best characterized ERAD machinery in mammals is the highly conserved Sel1L-Hrd1 complex, consisting of E3 ubiquitin ligase Hrd1 and its adaptor protein Sel1L. Although studies using tissue specific knockout mice, have provided insights into the regulatory role of ERAD in highly secretory cell types such as adipocytes and intestinal epithelium, the physiological importance of Sel1L-Hrd1 ERAD in cell differentiation and development has not been explored to date.

To this end, we chose to investigate B cell development as a model system, as it requires tightly orchestrated expression of many growth factors and cell surface-associated proteins for different stages of development. We generated B cell specific Sel1L knockout mice driven by CD19-Cre (*Sel1L*^{CD19}). Here, we report that B cell development, which requires a sequential progression of differentiation in the bone marrow, was blocked at the large pre-B cell stage in *Sel1L*^{CD19} mice. We show that ERAD manages an early checkpoint in B cell development by selectively targeting the pre-BCR complex for proteasomal degradation, hence terminating pre-BCR signaling. In the absence of Sel1L-Hrd1 ERAD, pre-BCR accumulates at the cell surface,

leading to persistent pre-BCR signaling and cell cycling. Strikingly, our study shows that pre-BCR complex, but not BCR complex, as an endogenous protein substrate of ERAD in B cells. To further understand the physiological importance of ERAD in B cell function, we examined the mucosal immunity of *Sel1L*^{CD19} mice. While IgG/IgM secretion in *Sel1L*^{CD19} mice is comparable to the wild type mice, the IgA secretion level was significantly reduced. Surprisingly, *Sel1L*^{CD19} mice exhibited exacerbated inflammatory response to dextran sodium sulfate (DSS)-induced colitis. Interestingly, this colitogenic phenotype was transmissible only between littermates bred from *Sel1L*^{CD19} dam. Collectively, the works described in this thesis provide novel insight into the physiological importance of ERAD in B cell development. Further studies based on this dissertation may provide important mechanistic insight into protein complex substrate selection by ERAD.

BIOGRAPHICAL SKETCH

Hana Kim was born in Seoul, South Korea in 1984 and spent her early years in Seoul, South Korea and Vancouver, Canada. The year she moved to Los Angeles with her family, she started her studies in Microbiology at California State Polytechnic University-Pomona. After receiving her bachelor's degree, she continued her pursuit of knowledge by entering master's program in Biological Sciences at California State Polytechnic University-Pomona, where she worked as a laboratory manager, while writing her thesis, Characterization of Efficacy and Immune Response of VesiVax® Universal Liposomal M2e Vaccine (L-M2e) for Scale-Up Production. Her research experience during her undergraduate and graduate years led to her interest in pursuing her Ph.D. in Immunology and Infectious Diseases at Cornell University in the Fall of 2011. In June of 2012, she joined Dr. Ling Qi's laboratory where she enjoyed the collaborative work with the members of the Qi lab. Her research focused on the ER quality control machinery and its physiological significance in B cell development and functions.

Dedicated to my parents, WonKyu and MoonSun Kim

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CHAPTER ONE: INTRODUCTION AND LITERATURE REVIEW

1.1 ENDOPLASMIC RETICULUM

Endoplasmic reticulum (ER) is the largest eukaryotic organelle and is a major site for: (a) protein synthesis and transport, protein folding; (b) lipid metabolism; (c) calcium (Ca^{2+}) storage and release into the cytoplasm (Ma et al., 2001; Matlack et al., 1998; McMaster, 2001; Meldolesi et al., 1998). This multifunctional organelle is defined as an interconnected network with a continuous membrane, which forms abundant membrane contact sites to interact with other cellular organelles such as nucleus, Golgi, mitochondria and endosomes.

The largest domain of the ER flattens around the cell nucleus to form the nuclear envelope. The peripheral ER, which branches out from the outer nuclear envelope and spread throughout the cytoplasm, consists of two structural domains: smooth tubules and rough sheets. There are distinctive morphological differences in these two domains: the outer surfaces of the rough sheets are covered with ribosomal particles (known as rough ER or rER) for the synthesis of nascent peptides into its lumen, whereas smooth ER tubules (known as smooth ER or sER) associate significantly less with ribosome particles (Shibata et al., 2006). While rER and sER are found in all eukaryotic cells (Staehelin, 1997), the ratio of rER and sER depends on the type of cells and their functions. For example, cells that secrete large amounts of protein, such as plasma cells or pancreatic β-cells, are rich in rER, while cells that are highly involved in lipid synthesis or calcium signaling have abundant sER. In addition to their morphological differences, rER and sER perform distinct biological functions.

1.1.1. PROTEIN FOLDING AND POST-TRANSLATIONAL MODIFICTIONS

One of the major function of the rER is to serve as a site for protein synthesis for secreted, luminal and membrane proteins (Jan et al., 2014; Reid et al., 2015). After translation, proteins can be translocated into the ER either by free ribosomes in the cytosol (post-translationally), or

during translation by ER membrane bound ribosomes (co-translationally) (Wickner et al., 2005). Co-translational translocation initiates as a signal recognition particle (SRP) recognizes and binds to the signal peptide on the emerging polypeptide. The binding of SRP stalls translation until the ribosome docks onto the translocon complex, Sec61 (SecY in yeast) complex (Robson et al., 2006). Sec61, a heterotrimeric protein channel, facilitates the translocation of polypeptides across the hydrophobic ER membrane. The active movement of the elongating polypeptide drives the translocation of growing polypeptide into the ER lumen (Park et al., 2012). On the other hand, post-translational translocation does not require SRP. The hydrophobic signal sequence in the nascent polypeptide chain is recognized by receptor proteins of the Sec62/63 complex, which is associated with Sec61 in the ER membrane (Ng et al., 1996). In the luminal side of the ER membrane, the chaperone binding immunoglobulin protein (BiP) binds the polypeptide and pulls it into the ER lumen.

The polypeptide chains fold into their correct three-dimensional conformation and polypeptides are assembled into multi-subunit proteins as soon as it enters the ER lumen (Ellgaard et al., 2003). During folding, proteins undergo transient conformational changes until they achieve the most stable and functional state, known as native state of protein. In order for nascent polypeptides to reach the final native state of protein, an array of ER resident molecular chaperones and enzymes assist folding and modification (Anfinsen et al., 1975). Folding of the polypeptide occurs while it is modified by processes such as N-linked glycosylation or disulfide bond formation.

Nascent polypeptides are glycosylated (N-linked glycosylation) while they are translocated into the ER. An oligosaccharide with 14 sugars is transferred to the side chain of an asparagine residue in the consensus sequence Asn-x-Ser/Thr by a membrane bound

oligosaccharyl transferase (Hubbard et al., 1981; Kornfeld et al., 1985). Some proteins require N-linked glycosylation for proper folding in the ER. After addition of the core oligosaccharide, two glucoses are sequentially removed by glucosidase I and glucosidase II. The calnexin/calreticulin machinery, which specifically recognizes the monoglucosylated glycan tag, interacts with sugar residues in folding intermediates and retains them in the ER (Ellgaard et al., 2003; Hebert et al., 1995). When a third glucose is removed from the glycan, the protein dissociates from calnexin/calreticulin and can leave the ER. If the protein is still unfolded, calnexin/calreticulin promotes their entry/ re-entry into the folding cycles as UDP glucose –glycoprotein glucosyl transferase (UGT) adds another glucose residue to the unfolded protein and goes through another cycle of interaction with calnexin/calreticulin. An unfolded protein undergoes continuous cycles of de- and re-glucosylation, and maintains an affinity for calnexin and calreticulin until it achieves its fully folded state.

As nascent chain emerges from the translocon, it associates with BiP (also known as Grp78), one of the most abundant ER chaperones (Hendershot, L. M., 2004). Interaction with chaperones consumes ATP and the folding of many secretory proteins is inhibited by depletion of ATP (Dorner et al., 1990); hence, the ATP cycle of BiP is tightly linked to its substrate binding (Behnke et al., 2015). BiP consists of two domains; a C-terminal substrate-binding-domain, which allows interaction with hydrophobic regions on nascent chains, and a N-terminal nucleotide-binding-domain with ATPase function (Behnke et al., 2015; Gething, 1999; Otero et al., 2010). In its ATP bound form, the substrate-binding-domain is in its open conformation, allowing binding and release of substrate. However, upon ATP hydrolysis, the lid of the substrate-binding domain closes, thus binding tightly to its substrate (Gething, 1999; Otero et al., 2010). Therefore, BiP has the ability to switch between substrate-bound and substrate free form,

which prevents misfolding and aggregation of the polypeptide and keeps it on the folding path. In addition to translocating the newly synthesized polypeptides into ER lumen and facilitating the folding and assembly of proteins, BiP also plays a critical role in many cellular processes: 1) targeting misfolded proteins for ER-associated degradation (ERAD), 2) regulating calcium homeostasis, and 3) serving as a master regulator of unfolded protein response upon ER stress (Hendershot, L. M., 2004; Lee, A. S., 2005; Li, J. et al., 2006).

In addition, disulfide formation between the side chains of cysteine residues (S-S) further assists protein folding. In the ER, formation, reduction, and isomerization of disulfides are catalyzed protein disulfide-isomerase (PDI) family. Most PDIs have several thioredoxin-like domains, and catalytic domains with the amino acid motif CXXC (C, Cys; X, any amino acid) which contains two redox-active (Benham, 2012; Kulp et al., 2006). Most redox-active PDIs have oxidase activity and disulfide isomerase activity. When oxidized PDI introduces disulfides to unfolded/reduced substrates, the PDI has open formation (Wang, C. et al., 2013). As the disulfide of the active site transfers to the substrate via a thiol/disulfide reaction, PDI switches to a closed reduced conformation and subsequently releases of oxidized substrate. If an introduced disulfide is non-native, the substrate can bind to a molecule of reduced PDI for isomerization (Wang, C. et al., 2013). This process stabilizes the structure of secreted or membrane surface proteins. For example, the disulfide bond between subunits of immunoglobulins, in its membrane form or secreted form, stabilizes the multimer complex (Elkabetz et al., 2008; Vinci et al., 2004).

Correctly folded proteins are transported to the Golgi and proceed in the secretory pathway, *en route* to the plasma membrane or the extracellular space. However, despite various folding machineries, protein folding is innately prone to error, due to its slow and inefficient process. As a result, a subpopulation of newly synthesized polypeptides fails to reach their native

conformation and become permanently entrapped in the ER (Guerriero et al., 2012), subsequently inducing ER stress. ER stress has been observed during physiological conditions, such as nutrient deprivation and the differentiation of B lymphocytes into plasma cells, as well as in pathological conditions such as diabetes, neurodegeneration, and cancer. In the case of prolonged or overwhelming ER stress, the cell dies by apoptosis. In order to alleviate ER stress, the terminally misfolded or unfolded proteins are dislocated into the cytoplasm and degraded by the ubiquitin/proteasome system, a process known as ERAD. In addition, cells can develop an adaptive mechanism to ER stress, known as unfolded protein response (UPR).

1.2 UNFOLDED PROTEIN RESPONSE

In eukaryotic cells, approximately one third of the newly synthesized membrane and secreted proteins enter the ER in an unfolded state for further modification and folding with the help of a myriad of chaperones and enzymes. Once the proteins reach their native conformation, they are transported to the Golgi complex *en route* to their final destinations. However, protein folding is a highly error-prone process, even with the assistance of many factors. In some cases, proteins which contain disease-associated mutations, such as cystic fibrosis transmembrane conductance regulator (CFTR), are structurally incapable of reaching a native conformation and become trapped in the ER (Guerriero et al., 2012). The accumulation of misfolded proteins in the ER disrupts ER homeostasis and leads to ER stress, which has been strongly associated with human disease pathogenesis (Lin, J. H. et al., 2008). In response to ER stress, eukaryotic cells have developed three pathways of protein quality control to maintain ER homeostasis and cope with the stress for survival: unfolded protein response (UPR), ER-associated degradation (ERAD), and autophagy.

Unlike in yeast cells, where UPR is initiated by single signaling cascade, Ire1p (a type I transmembrane ER protein) (Ron et al., 2007), UPR has evolved in mammalian cells to be governed by three major signaling cascades, Inositol-requiring enzyme 1α (IRE1α), PKR-like ER Kinase (PERK), and activating transcription factor 6 (ATF6) (Figure 1.1). The activated UPR promotes: 1) increased ER folding capacity, 2) enhanced ERAD, and 3) attenuation of global translation to restore ER homeostasis. However, failure to reverse ER stress can induce cellular apoptosis (Walter et al., 2011).

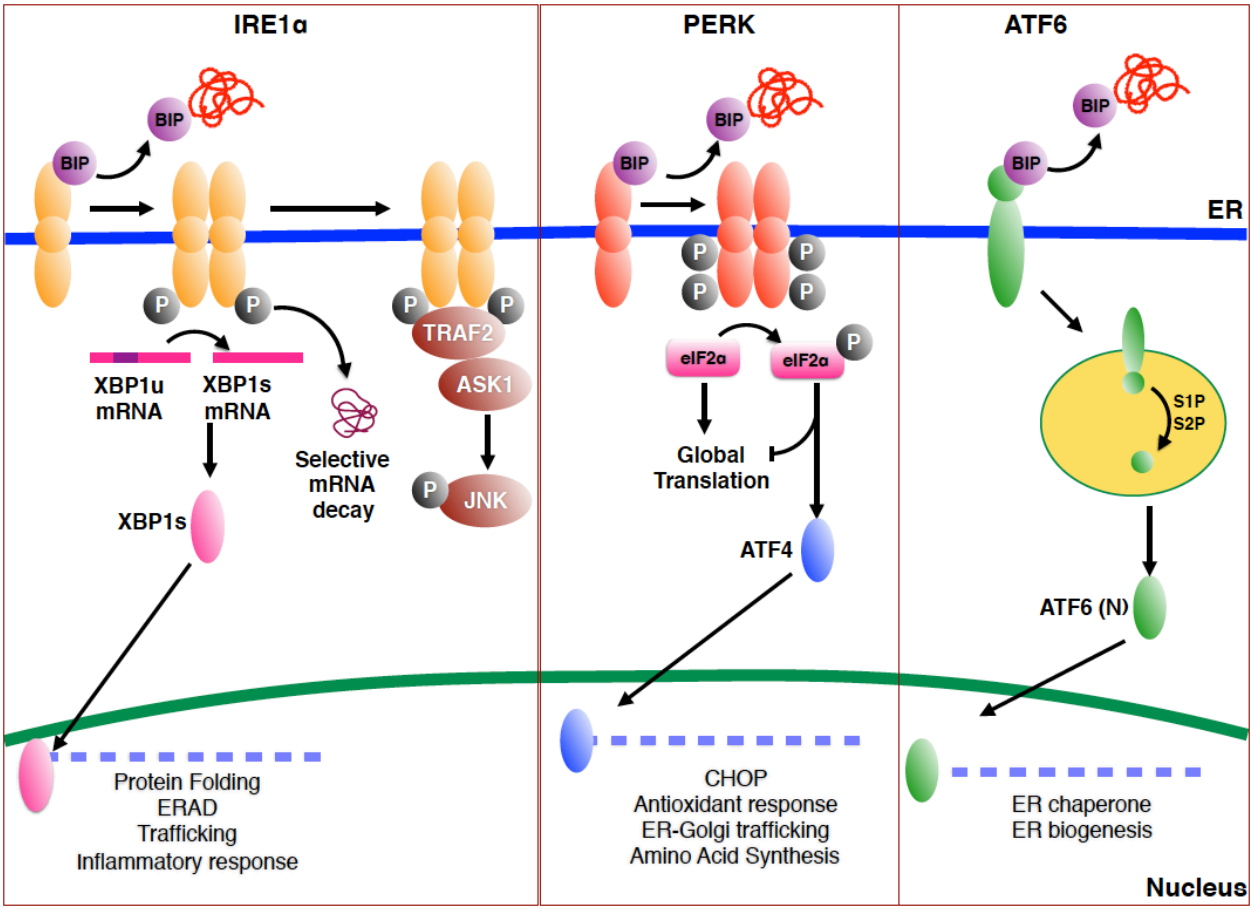

Figure 1. 1 The unfolded protein response (UPR) in mammals.

Accumulation of misfolded of unfolded proteins in the ER activates three UPR pathways: IRE1α, PERK and ATF6. (a) Activated IRE1α alternatively splices *Xbp1* mRNA to generate Xbp1s which translocates into the nucleus and regulates different genes. Binding of TRAF2 to active IRE1 cytosolic domain activates the JNK-pathway. Furthermore, activated IRE1α can selectively degrade particular mRNAs by a process called regulated IRE1-dependent decay (RIDD). (b) Active PERK phoshporylates eIF2α which leads to a global reduction in protein translation and translation of *Atf4* mRNA. (c) Activated ATF6 translocates to the Golgi where it is proteolytically cleaved into an active fragment, which results in the transcription of *Xbp1* mRNA and other genes related to ER chaperones and ER folding.

1.2.1. IRE1 PATHWAY

Inositol-requiring enzyme 1(IRE1) is the most evolutionarily conserved UPR branch among three groups, as it is the only branch identified in lower eukaryotes such as yeast, plants, worms, flies and vertebrates (Cox et al., 1993; Koizumi et al., 2001; Mori, 2009; Shen et al., 2001; Wang, X. Z. et al., 1998). Ire1 is a type I ER-resident transmembrane protein with: 1) an N-terminal luminal dimerization domain, 2) a 20 amino acid transmembrane domain, and 3) a C-terminal cytosolic domain with Ser/Thr kinase and endoribonuclease activity (Liu, C. Y. et al., 2002). It was first identified as an indispensable gene for yeast, *Sacchromyces cerevisiae*, growth on inositol-deficient medium (Nikawa et al., 1992), and later was also found to contain site-specific endonuclease activity to splice bZIP transcription factor, *HAC1* mRNA (Nikawa et al., 1992; Sidrauski et al., 1997). In the budding yeast, *HAC1* is the only known substrate of IRE1 (Nikawa et al., 1992) and the functional homolog *HAC1* in mammals, worms, and flies is the transcription factor X-box binding protein 1(XBP1) (Calfon et al., 2002; Ryoo et al., 2007; Souid et al., 2007; Yoshida et al., 2001).

In mammalian cells, there are two isoforms of IRE1, IRE1α and IRE1β, both of which display kinase and RNase domain that is highly conserved to yeast Ire1p (Tirasophon et al., 1998; Wang, X. Z. et al., 1998). IRE1α is ubiquitously expressed in most tissues with relatively high expression in pancreas and placenta, while IRE1β is selectively expressed in gastrointestinal epithelial cells and lung (Bertolotti et al., 2001; Martino et al., 2013). Hence, global deletion of *IRE1α* can result in early embryonic lethality (Iwawaki et al., 2009; Zhang, K. et al., 2005), while *IRE1β* deletion disrupts the regulation of airway mucin production (Iwawaki et al., 2009) and increased ER stress and exacerbated colitis induced by dextran sodium sulfate (DSS) (Bertolotti et al., 2001; Kaser et al., 2008).

The mechanism by which IRE1 is activated is still ambiguous. It was first proposed that under homeostatic conditions, BiP binds to the luminal domain of IRE1, which renders IRE1 monomeric and inactive (Bertolotti et al., 2000; Kimata et al., 2003). During stress conditions, BiP dissociates from IRE1, to assist in protein folding. This subsequently results in dimerization, oligomerization, and trans-autophosphorylation of IRE1 which leads to a conformational change that activates the RNase domain. Upon activation, the IRE1α kinase and RNase domain cleave *Xbp1* mRNA, mediate mRNA decay, or regulate inflammatory/apoptotic pathways. However, studies demonstrated that a mutant IRE1p, lacking the BiP binding region, can still form oligomers and splice *HAC1* mRNA (Kimata et al., 2003; Pincus et al., 2010). Moreover, crystal structure analysis of the IRE1p reveals that the ER luminal domain of IRE1p displays a major histocompatibility complex (MHC)-like groove and mutations in amino acids of the groove or in the dimerization surface abrogate the ability of IRE1p to engage the UPR (Credle et al., 2005). Hence it is more likely that the physical interaction of BiP is a fine-tuning mechanism to ensure Ire1p activation (Pincus et al., 2010).

In contrast to yeast IRE1p, the crystal structure of human IRE1α revealed that the MHC-like groove might be too narrow to accommodate an unfolded protein peptide (Zhou, J. et al., 2006). Interestingly, mammalian IRE1α with luminal domain mutants exhibit low affinity for BiP. Moreover, the luminal fragments of mammalian IRE1α does not interact with unfolded proteins in an in vitro assay (Oikawa et al., 2009). These studies suggest that the regulation of mammalian IRE1α may actually rely more on the dissociation of BiP and maybe independent of misfolded protein binding. It is likely that the IRE1 sensing mechanisms is not conserved between yeast and mammalian, due to the differences in protein structure within the sensor

domain (Zhou, J. et al., 2006). Unlike IRE1α, a recent study revealed that mammalian IRE1β is not able to associate with BiP, while it still binds to unfolded proteins (Oikawa et al., 2012).

IRE1-XBP1(HAC1) pathway

XBP1 is a member of the cAMP response element-binding/activating transcription factor (CREB/ATF) basic region-leucin zipper (bZIP) family (Clauss et al., 1996). It was initially identified during screening for proteins that were bound to X-box, a sequence which regulates transcription of the major human histocompatibility complex (MHC) (Liou et al., 1990). In yeast, activated Ire1p cleaves the 5' and 3' exon-intron splice junction in *HAC1u*, to remove the 252-nucleotide intron (Sidrauski et al., 1997). Then two exon fragments are ligated by yeast tRNA ligase RLG1/TRL1 (Cox et al., 1996; Sidrauski et al., 1996), which yields in a mature HAC1i mRNA. The resulting *HAC1i* mRNA can be translated into a functional transcription factor that translocates to the nucleus and binds to the UPR element (UPRE) (consensus motif: CAGCGTG) and upregulates the entire yeast transcriptome (Mori et al., 1996; Sidrauski et al., 1996).

In metazoans, activated IRE1 excises 26 nucleotides (23 nucleotides in *C. elegans*) intron from the *XBP1u* mRNA. This causes a frame shift to avoid the premature stop codon and leads to the expression of more stable and active form – X*bp1s* (Calfon et al., 2002; Lee, K. et al., 2002; Yoshida et al., 2001), which translocate into the nucleus and subsequently leads to translation of a 370 amino acid protein, as opposed to the 266 amino acid chain that is translated from *Xbp1u*. While the translation of the *HAC1u* is blocked by a mechanism that is poorly understood (Hetz et al., 2009), it has been demonstrated that *XBP1u* in mammalian can be translated into XBP1u, a protein with a rapid rate of degradation (Chen, X. et al., 2014). Two known substrates of XBP1u are XBP1s (Yoshida et al., 2006) and the active form of ATF6 (Yoshida et al., 2009). By

targeting these two proteins for degradation, XBP1u functions as a negative regulator of the UPR (Chen, X. et al., 2014).

The XBP1s activates an array of genes associated with protein folding, ERAD, lipid synthesis, protein translocation to the ER, and protein secretion (Acosta-Alvear et al., 2007; Lee, A. H., Iwakoshi, & Glimcher, 2003). For example, under ER stress condition, XBP1s induces the transcription of genes encoding ER chaperones, such as BiP, and proteins involved in ERAD, such as Derlin-2 and ER degradation enhancing a-mannosidase-like protein (EDEM), to restore ER homeostasis and promote cell survival (Lee, A. H., Iwakoshi, & Glimcher, 2003; Oda et al., 2006; Walter et al., 2011; Yoshida et al., 1998). Furthermore, given its role in upregulating genes involved in protein secretion, XBP1 or IRE1-XBP1 pathway has also been implicated in development and function of many secretory cell types. Studies with either a tissue specific, or whole body knockout of IRE1 or XBP1 have shown that this pathway is necessary for cardiac myogenesis, hepatogenesis, plasma cell differentiation, thyroglobulin production (Christis et al., 2010), as well as proliferation of pancreatic β cells (Lee, A. H. et al., 2005; Masaki et al., 1999; Reimold et al., 2000; Reimold et al., 2001; Xu, T. et al., 2014). Metabolic genes are also upregulated by XBP1s, glycogenesis and lipid metabolism. Taken together, these studies suggest that XBP1 plays a critical role in adapting to restore ER homeostasis and promoting cell survival.

Conversely, XBP1 is also shown to affect pro-apoptotic pathway. One of genes upregulated by XBP1s is *p58*^{IPK}, an inhibitor of PERK. It has been demonstrated that high levels of p58^{IPK} can downregulate the activity of the PERK/eIF2α/ATF4 pathway (van Huizen et al., 2003), and as upregulation of p58^{IPK} by XBP1s occurs many hours after PERK activation, it is thought that p58^{IPK} signaling, under prolonged ER stress, represents the shift from the adaptation

strategy to the cell death phase (Szegezdi et al., 2006). Hence, XBP1s may play dual role in regulating cell survival and death.

Regulated Ire1-dependent decay (RIDD)

IRE1 mediated splicing of *XBP1* mRNA is a very unique pathway, as even a computational stimulation in mammals, searching for mRNAs with structural and regulatory similarity to XBP1, did not identify other mRNAs (Nekrutenko et al., 2006). However, Weisman group has identified a novel function IRE1 RNase, with broad range of mRNA substrates, using *Drosophila melanogaster* S2 cells (Hollien et al., 2006). It was shown that in response to ER stress induced by dithiothreitol (DTT), a subset of mRNA gets degraded by IRE1α endonuclease activity, depending on their ER localization and amino acid sequence. Degradation of ER-bound mRNAs may serve to limit protein influx and unfolded protein load into the ER lumen after UPR induction. This process is called Regulated IRE1-Dependent Decays (RIDD). Although, a sequence consensus CUGCAG has been proposed as a common substrate and cleavage motif for both *XBP1* mRNA splicing and RIDD (Oikawa et al., 2010), recent study suggested that IRE1 RNase has different mechanism for cleaving RIDD substrates and *XBP1* mRNA intron. It was shown that IRE1 engaged in RIDD within an IRE1 monomer/dimer whereas catalytically active IRE1 unit engaged in *HAC1/XBP1* mRNA splicing is generated within the IRE1 oligomer (Tam et al., 2014).

RIDD also occurs in mammalian cells (Han et al., 2009; Hollien et al., 2009). Interestingly, while RIDD down-regulates many RNAs by 5-10 fold in Drosophila cells, in mammals the magnitude of expression changes was two-fold or even less for many targets (Hollien et al., 2009; Hollien et al., 2006)]. More significantly, the pool of mRNAs degraded by RIDD activity was in a cell-type specific manner (Han et al., 2009; Hollien et al., 2009; Hollien

et al., 2006; So et al., 2012). In addition, it was shown that IRE1α can cleave premature microRNAs (mIRs-17, -34a, -96, and -125b), thus regulating translation of pro-apoptotic protein Caspase-2 (Upton et al., 2012).

Matter of life or death (inflammatory response vs apoptosis)

While IRE1α promotes cell survival through alleviating ER stress, it can also promote stress-induced apoptosis. Under ER stress, activated IRE1α also recruits and activates the adaptor protein tumor necrosis factor (TNF) receptor-associated factor 2 (TRAF2) to the ER membrane. This initiates the downstream activation of apoptosis signal-regulating kinase1 (ASK1), which further activates JUN N-terminal kinase (JNK) and p38 MAPK (Nishitoh et al., 2002; Urano et al., 2000). Activated JNK protein leads to phosphorylation of activation protein-1 (AP-1), which in turn transcribes its own inflammatory gene program such as TNF, interleukin (IL-)-8, and granulocyte macrophage colony-stimulating factor (GM-CSF) (Angel et al., 2001; Davis, 2000). Activation of JNK also activates pro-apoptotic Bcl2-interacting mediator of cell death (BIM) protein (Lei et al., 2003) and inhibits the survival protein Bcl-2 (Yamamoto et al., 1999; Yoneda et al., 2001). Once phosphorylated by JNK, BIM translocates to the mitochondrial membrane and promotes mitochondrial apoptosis (Chen, D. et al., 2004). In addition, inhibition of Bcl2 by activated JNK leads to oligomerization of pro-apoptotic proteins Bax and Bak, which are inserted into the mitochondrial membrane and induce mitochondrial apoptosis (Lei et al., 2003).

Moreover, the IRE1-TRAF2 complex recruits Iκ*B* kinase (IKK) protein complex, and subsequent phosphorylation of IκB leads to its degradation, upon which releases nuclear factor-κB(NF-κB) for nuclear translocation (Ghosh, S. et al., 2002). Genes targeted by NF-κB include those encoding crucial pro-inflammatory cytokines, such as TNFα and IL-6 (Li, Y. et al., 2005;

Pahl, 1999; Rius et al., 2008; Zhang, K. et al., 2008), and enzymes involved in immunomodulation, such as cyclooxygenase-2 (Hotamisligil et al., 2008; Pahl, 1999).

There are two signaling pathways that control caspase-dependent apoptosis: the intrinsic (or mitochondrial) death pathway and the extrinsic cell (or death receptor) death pathway. Alternatively, in response to ER stress, the IRE1-TRAF2 complex can also interact with pro-caspase-12 (Morishima et al., 2004) and initiate caspase cascade which results in cell apoptosis.

In mice, the pro-caspase-12 is localized on the cytoplasmic side of the ER, and mice and mouse embryonic fibroblasts (MEFs) deficient in caspase-12 were shown to be resistant to ER stress-induced apoptosis, even though their cells still undergo apoptosis in response to other death stimuli such as TNF (Nakagawa et al., 2000). In humans, caspase-4, homologous to mouse caspase-12, is cleaved specifically by ER stress, but not by other apoptotic signals. Knocking down the expression of caspase-4 was shown to decrease ER stress-induced apoptosis (Hitomi et al., 2004; Morishima et al., 2002). However, more recent study showed that caspase-12 does not instigate ER-stress induced apoptosis, but regulates inflammation response (Saleh et al., 2006).

1.2.2. PERK PATHWAY

PERK is a metazoan type I transmembrane protein (Walter et al., 2011). It is composed of an ER stress sensor in the ER lumen, with a kinase domain on its cytosolic fraction. While IRE1α and PERK share a weak sequence homology in their luminal domains, it has been demonstrated that BiP binds to the luminal domain of both of these proteins (Bertolotti et al., 2000). Hence, under inactive state, PERK retains its monomeric form in a similar manner as IRE1α. Upon sensing ER stress, BiP dissociates from the N-terminus of PERK and binds to misfolded proteins, while PERK undergoes dimerization/oligomerization and transphosphorylation (Sha et al., 2011). Activated PERK phosphorylates Serine 51 on the α-

subunit of eukaryotic translation initiation factor-2 (eIF2α), attenuating global protein translation to reduce the flux of protein entering the ER and alleviating ER stress (Harding, Zhang, et al., 2000; Harding et al., 1999; Yan et al., 2002). Although the translation of most mRNAs is inhibited in ER-stressed cells, the translation of some mRNAs, such as activating transcription factor 4 (ATF4), is preferentially upregulated under PERK-eIF2α pathway. Expression of ATF4 subsequently promotes genes involved in amino acid metabolism, anti-oxidant response, and autophagy to cells to manage pleiotropic consequences of protein misfolding in the ER (Harding, Novoa, et al., 2000; Harding et al., 2003; Okada et al., 2002; Scheuner et al., 2001).

A paradox of PERK-peIF2α-ATF4 pathway is that, while it promotes adaptive pathway to restore ER homeostasis and cell survival, it simultaneously activates pro-apoptotic pathways by inducing transcription factor C/EBP homologous protein (CHOP/GADD153) (Palam et al., 2011). CHOP is a pro-apoptotic bZiP transcription factor, which promotes both the transcription of BIM (Puthalakath et al., 2007) and the death receptor family member DR5 (Yamaguchi et al., 2004), while downregulating pro-survival protein BCL-2 expression (McCullough et al., 2001).

PERK signaling is also shown to phosphorylate transcription factor: nuclear factor erythroid 2-related factor 2 (Nrf2) and NF-κB. In unstressed cells, Nrf2 is found in the cytoplasm as a complex with Keap1, the cytoskeletal anchor. Upon ER stress, PERK phosphorylates Nrf2, independent of eIF2α phosphorylation, and dissociates from Keap1 for nuclear translocation and promotes increased cell survival after chronic ER stress (Cullinan et al., 2003).

Given the dichotomy in outcomes of PERK-eIF2α pathway, cells have adapted various pathways to regulate PERK activity. First, the cytosolic kinase domain of PERK can be inhibited by the action of the DNAJ family member $p58^{IPK}$ (van Huizen et al., 2003; Yan et al., 2002). Inhibition of PERK kinase activity relieves eIF2α phosphorylation, hence removing the

translational block. In addition to inducing cell death through apoptosis, CHOP also induces growth arrest and DNA damage-inducible 34 (GADD34) as a negative feedback. As ER stress is resolved, through activation of UPR, eIF2α is dephosphorylated by the GADD34-protein phosphatase1 (PP1) complex and restore mRNA translation (Connor et al., 2001). In addition to inducing GADD34, CHOP expression downregulates during the early time point of ER stress, for example by microRNA (mIR-211) as a pro-survival mechanism (Chitnis et al., 2012). Furthermore, the UPR stress sensor of mammalian cells have evolved to integrate the intensity of the stimulus and reflect this in the signals they transduce. For example, under a very low-level of UPR activation by stress agents, thapsigargin (TG) or tunicamycin (TM), will activate PERK, ATF6, and splicing of *XBP1* mRNA, while evading terminal UPR by not upregulating CHOP (Rutkowski et al., 2006). Hence, this mechanism also allows cells to avert death as they adapt.

1.2.3. ATF6 PATHWAY

ATF6, a third branch of UPR, is a type II transmembrane ER stress sensor. While PERK is uniquely responsible for global inhibition of translation initiation on the cytoplasmic side (Harding et al., 1999),ATF6, along with IRE1α, is concerned exclusively with activating genes which enhances the ER folding capacity (Kaufman, 2002).

Although there are two *ATF6* genes in the mammalian genome (*ATF6α* and *ATF6β*), only *ATF6α* is required to activate UPR gene expression (Wu, J. et al., 2007). Like its counterparts, IRE1α and PERK, ATF6 remains inactive in ER by binding of BiP on the luminal domain of ATF6 under normal condition. As BiP dissociates from ATF6, upon ER stress, ATF6 is packaged into transport vesicle, COP II complex, and transported to Golgi apparatus (Schindler et al., 2009).

Unlike IRE1α and PERK, ATF6 activation does not require phosphorylation of a kinase domain. Rather, once in Golgi Apparatus, ATF6 undergoes regulated intramembrane proteolysis (RIP), a process known to regulate sterole response element binding protein (SREBP) (Ye, J. et al., 2000). ATF6 is cleaved by two proteases: first, site-1 protease (S1P) removes the luminal domain and site-2 protease(S2O) subsequently removes the transmembrane anchor (Haze et al., 1999; Ye, J. et al., 2000). This process yields the mature transcription factor ATF6(N) (50 kDa) as a basic Leu zipper (bZIP) transcription factor. The liberated ATF6(N) translocates to the nucleus to activate the transcription of UPR target genes.

Once in the nucleus, ATF6(N) binds to ER stress-response element (ERSE) which consists of a tripartite structure CCAATN9CCACG (Li, M. et al., 2000). This interaction between ATF6(N) and ERSE increases chaperone activity, as well as degradation of unfolded proteins to restore ER homeostasis (Wu, J. et al., 2007; Yamamoto et al., 2007). For example, ATF6 upregulates BiP, protein disulfide isomerase (PDI), and ER degradation-enhancing alpha-mannosidase-like protein 1 (EDEM1), XBP1 and CHOP (Ron et al., 2007).

More recently, accumulating studies have added several ATF6 homologs as new members of UPR initiators: such as CREB-hepatocyte (CREBH), old astrocyte specifically-induced substance (OASIS; also known as CREB3-like-1), and LUMAN/CREB3. CREBH is a hepatocyte-specific bZIP transcription factor which is required for acute phase response, induced either by pro-inflammatory cytokines or ER stress (Zhang, K. et al., 2006). OASIS is induced at the transcriptional level during ER stress, specifically in astrocytes but not in other cell types, and consequently induced BiP and suppressed ER-stress-induced cell death (Kondo et al., 2005). LUMAN/CREB3, unlike CREBH and OASIS, is found in professional antigen presenting cells such as monocytes, and dendritic cells. Cumulative data has shown that LUMAN can induce

ERAD genes to prevent ER-stressed-induced cell death (Asada et al., 2011). Given the differences in activating stimuli, tissue distribution, and response element binding, this branch of UPR pathway may also be involved in regulating cellular physiology.

1.2.4. UPR in immunity

UPR pathways have been implicated in development and differentiation process in both adaptive and innate immunity. Among all immune cell types, the role of UPR in development and differentiation is the most explicitly studied B cells/plasma cells than any other cells. Due to large secretory demand as part of their function, plasma cells, among many other secretory cells, depend on a well-developed ER, as well as UPR which can quickly facilitate synthesis and trafficking of large volume of immunoglobulin load in ER. Plasma cell differentiation is regulated by the transcription factors interferon regulator factor 4 (*IRF4*), B lymphocyte-induced maturation protein 1 (BLIMP1; encoded by *Prdm1* gene), and its downstream target XBP1. Long-lived plasma cells, which produce large quantities of antibody, express high levels of BLIMP1, IRF4 and XBP1. Short-lived plasmablasts also express high levels of XBP1 but intermediate level of BLIMP1.

Upon stimulation by antigen, BLIMP1 initiates cascades of genes for plasma cell differentiation. First, BLIMP1 represses *MYC1* expression, allowing cells to exit cycling. Second, BLIMP1 also represses genes required for the identity of mature and germinal center B-cells, including BCL-6 and PAX5 (Lin, K. I. et al., 2002; Shaffer et al., 2002). While PAX5 is required for commitment to B cell lineage, it also represses the expression of XBP1, IgH, IgL and the immunoglobulin joining (J) chain (Reimold et al., 1996; Rinkenberger et al., 1996; Shaffer et al., 1997; Singh et al., 1993). Therefore, repression of PAX5 by BLIMP1, in turn, will de-repress expression of the IgH, the IgL, the J chain and XBP1. The induction of *XBP1*, by BLIMP1,

subsequently induces the gene expressions that are involved in ER expansion, degradation of misfolded proteins, trafficking between the endoplasmic reticulum and the Golgi, and targeting of secretory vesicles to the plasma membrane, which are all necessary for high levels of antibody production and secretion. Furthermore, increased *XBP1* expression is also shown to increase cell size, lysosome content, mitochondrial mass and function, ribosome number, and the size of the ER during plasma cell differentiation (Shaffer et al., 2004)

Early studies proposed that XBP1 induction during plasma differentiation is due to increased immunoglobulin synthesis and accumulated unfolded proteins in the ER, (Iwakoshi, Lee, & Glimcher, 2003; Iwakoshi, Lee, Vallabhajosyula, et al., 2003) and the generation of plasma cells is highly impaired in the absence of XBP1. However, subsequent studies have shown that ER expansion precedes the onset of immunoglobulin synthesis (van Anken et al., 2003)]. Additionally, studies using B cell-specific-*XBP1* knockout mice demonstrated that XBP1 is not required for the formation of plasma cells (Hu et al., 2009; Todd et al., 2009). Rather, XBP1 was associated with regulating the development stage after the expression of Syndecan-1 (CD138) and trafficking of plasma cells to the bone marrow (Hu et al., 2009; Todd et al., 2009). More recently, the decrease in immunoglobulin production in XBP1-deficient B cells were explained by hyperactivation of IRE1α, leading to increased RIDD, and subsequently degrading Igμ-heavy main mRNAs (Benhamron et al., 2014).

Interestingly, the role of two other arms of UPR, ATF6 and PERK, remains unclear. Using *in vitro* system, overexpression of a dominant-negative ATF6, a mutant in differentiating splenic B cells, diminishes IgM secretion and compromises the quality control of IgM synthesis. However, the exact timing and duration of ATF6α activation during plasma cell differentiation differs from that of IRE1-XBP1 pathway (Gunn et al., 2004). On the contrary, another study

showed ATF6α is not required for antibody secretion by antigen-activated marginal zone B cells and B1 cells, using ATF6α-deficient (Aragon et al., 2012). PERK, although partially activated in LPS-stimulated B cells (Gass et al., 2008; Ma et al., 2010), it is not required for antibody secretion (Gass et al., 2008). These data suggest that ATF6 and PERK are not activated due to lack of ER stress. Hence, plasma cells use a "physiological" UPR and not "stress-induced" UPR, by increasing *XBP1* transcription level and splicing upon plasma cell differentiation, while maintaining basal IRE1α activity.

In addition to plasma cells, IRE1α activation is seen in the progenitor cells, as early as in pro-B cells (Brunsing et al., 2008; Zhang, K. et al., 2005). While IRE1α knockout is embryonically lethal, fetal liver cells isolated from *IRE1*$^{-/-}$ mice contain CD43^{+}B220^{+} pro-B cells. However, the number of CD43^{-}B220^{+} pre-B cells and B220^{+}IgM^{+} immature B cells are reduced, suggesting a critical role of IRE1α at pro-B cell stage for further differentiation into the next step (Zhang, K. et al., 2005). IRE1α activation in pro-B cells might result from the increased secretory demand caused by synthesis of Igu heavy chain, since the expression of VDJ recombination factors recombinase activating gene 1 (*rag1*), *rag2*, and terminal deoxynucleotidyl transferase (*TdT*) are downregulated, impairing VDJ rearrangements (Zhang, K. et al., 2005). Of note, *RAG2*$^{-/-}$ mice which are reconstituted with eIF2α mutant fetal liver, exhibit mature splenic B-cell population with normal levels of serum immunoglobulin (Zhang, K. et al., 2005). While, this suggests that PERK-eIF2α can be dispensable for B cell development and antibody secretion, whether ATF6 is involved in B cell development remains inconclusive. Taken together, further studies are necessary to provide insight into understanding how different UPR signaling branches coordinate their activities in B cell development and functions.

Among three ER stress sensors, studies have demonstrated that IRE1α -XBP1 arm of the UPR is involved in dendritic cell (DC) function, as well as development. Deletion of *XBP1* in progenitor cells abrogates maturation and decreases DC survival due to increased apoptosis, which was rescued by overexpression of XBP1s (Iwakoshi et al., 2007). Conversely, in another study, XBP1 deletion in $CD11c^+$ DCs does not affect development; *XBP1* deletion, however leads to IRE1α hyper-activation, followed by RIDD of components of the MHC I, thus preventing antigen class presentation (Osorio et al., 2014).

UPR also regulates macrophage functions. In macrophage, toll-like receptor 2/4 (TLR2/4) specifically activates IRE1α-XBP1, which induces expression of pro-inflammatory cytokines such as IL-6, IL-1β, TNF and IFN-β (Martinon et al., 2010; Smith et al., 2008; Zeng et al., 2010). In addition, under saturated fatty acid induced ER stress, ATF4 activation induces *Il6* expression in macrophage (Iwasaki et al., 2014). On the contrary, TLR-TRIF signaling in macrophage inhibits translation of *Atf4* mRNA, consequently inhibiting its downstream target CHOP, while leaving PERK-eIF2α intact (Woo et al., 2009). More interestingly, ER stress-induced activation of IRE1α can induce *Il-1β* transcription through glycogen synthase kinase 3B (GSK3B), which can simultaneously inhibit *XBP1* and its downstream target *Tnf* (Kim, S. et al., 2015).

Despite the effort to understand the role of UPR in other immune cells, such as T cells and granulocytes, there are only few studies which implicate the role of UPR in these cell development and function. While, IRE1α-XBP1 pathway is one of the UPR pathways involved in eosinophil development (Bettigole et al., 2015),as well as the terminal differentiation of effector $CD8^+$ T cells (Kamimura et al., 2008), further studies are still required to understand if other arms of UPR are also involved in these immune cell types' development and functions. The critical role of UPR in immunity is also observed in *C. elegans.* During *C. elegans* development,

Xbp-1 deletion leads to larval lethality in exposure to *Pseudomonas aeruginosa* (Richardson et al., 2010).

In addition, UPR has also been implicated in the function of Paneth cells and goblet cells. Although these two types of cells are not derived specifically from common progenitor cells which produces different kinds of immune cells, these cells still play a significant role in intestinal innate immunity by producing anti-microbial peptides and mucus, respectively (Clevers et al., 2013; Kaser et al., 2008). Mice with intestinal epithelial cell (IEC)-specific *XBP1* deletion results in decreased antimicrobial function, which leads to increased susceptibility *to Listeria monocytogenes*. In addition, hyper-activation of IRE1α, under *Xbp1* deletion, induces JNK activation, which results in spontaneous enteritis in these mice (Kaser et al., 2008). Recent study also shows that mice, with *Xbp1* deletion specifically in Paneth cells, developed small intestinal enteritis (Adolph et al., 2013). Moreover, IRE1β, which is only expressed in gastrointestinal epithelium and airway, is also required to protect mice against pathogen through regulation of mucin production (Bertolotti et al., 2001; Martino et al., 2013), further validating the importance of UPR in highly secretory epithelial cells.

1.3 ENDOPLASMIC RETICULUM ASSOCIATED DEGRADATION (ERAD)

Proper folding of proteins is monitored and ensured by the quality control machinery. Accumulation of misfolded proteins, which disrupts ER homeostasis, has been associated with altered immune response, metabolic regulation, insulin resistance, tumor growth, and neuron degenerative diseases (Hetz, 2012; Sha et al., 2011). Proteins that are either terminally unfolded or misfolded are degraded via a highly-conserved quality-control machinery known as ER-associated degradation (ERAD), thereby maintaining ER homeostasis. ERAD machinery, a

highly-conserved system through yeast to mammals, proceeds through a tightly coupled steps involving: 1) substrate recognition, 2) substrate retrotranslocation/dislocation across the ER membrane, 3) ubiquitination and 4) degradation by the cytoplasmic 26S proteasome (Figure 1.2).

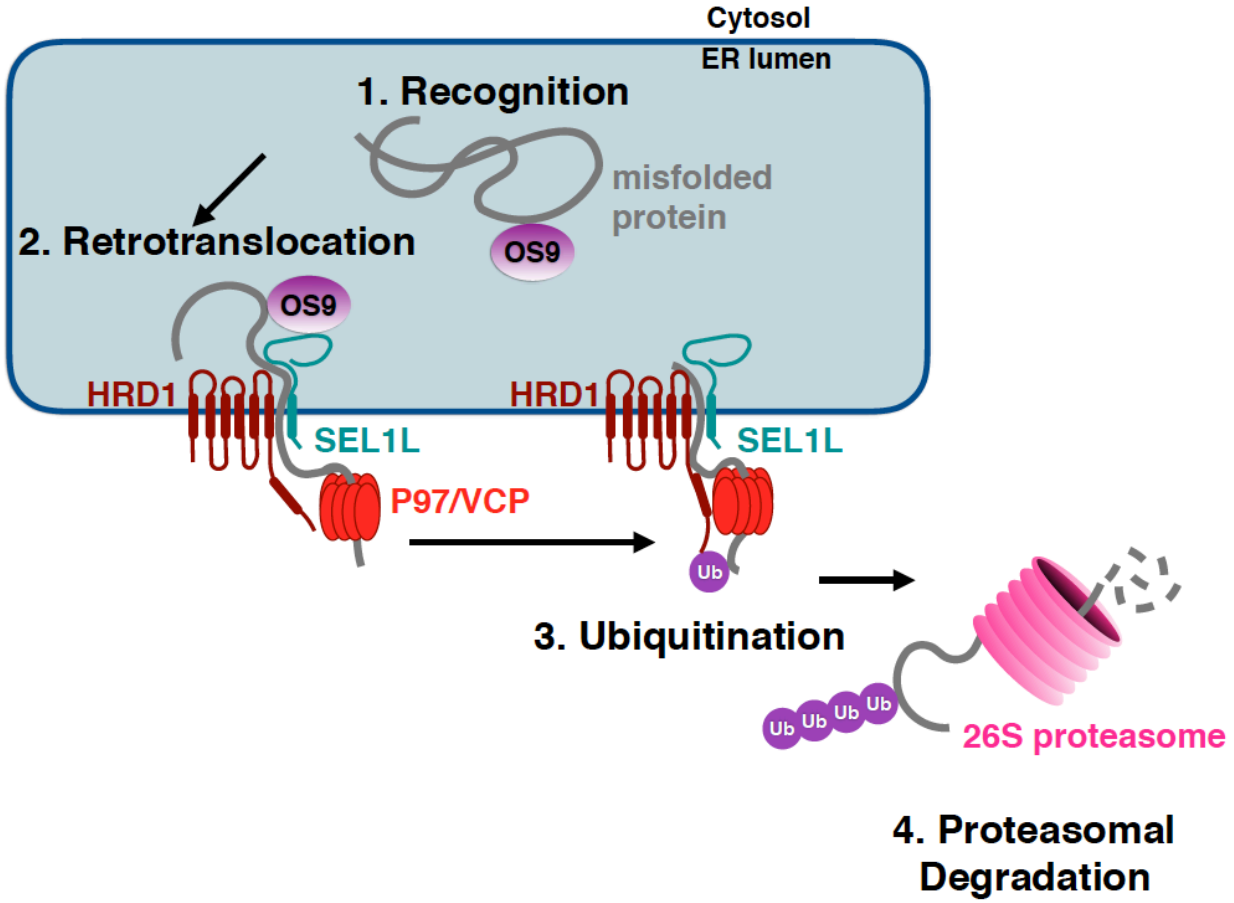

Figure 1. 2 Four major steps of mammalian ER-Associated Degradation (ERAD).
(a) Misfolded proteins are recognized by chaperones such as OS9 or BiP. (b) ERAD substrates are targeted to for retrotranslocation by the HRD1-SEL1L ERAD complex. (c) In the cytosol, the retrotranlocated proteins are ubiquitinated by the E3 ligase complex. (d) The ubiquitniated protein substrates are finally degraded by 26S proteasomes.

1.3.1. Recognition of misfolded proteins

ERAD substrate are recognized by chaperones or chaperone-like lectins, through their specific glycosylation signatures, which are generated by mannosidases that trim mannose residues from misfolded glycans. Subsequently, these misfolded glycans are further processed by the activity of EDEM1, EDEM3, and Man1c1 to remove terminal mannose residues. Demanosylated and deglucosylated substrates, which expose alpha-16 mannose, are irreversibly targeted for degradation (Aebi et al., 2010; Hosokawa et al., 2010; Lederkremer, 2009; Olivari et al., 2006; Parodi, 2000; Tremblay et al., 1999).

The substrate recognition factors, osteosarcoma amplified 9 (OS-9) and XTP3-traansactivated gene B protein (XTP3B), bind to substrates via their mannose-6-phosphate receptor homology (MRH) domains (Bernasconi et al., 2008; Christianson et al., 2008; Hosokawa et al., 2008). Both OS9 and XTP3B may recognize misfolded regions within a substrate in addition to the glycosylation signature (Hosokawa et al., 2009). These ERAD substrate receptor proteins interact with adaptor proteins, such as SEL1L, at the ER membrane and are subsequently delivered to the retrotranslocation complex (Olzmann et al., 2013).

Alternatively, OS9 and XTP3B may bind non-glycosylated substrates indirectly via an interaction with GRP94 and BiP (Bernasconi et al., 2008; Christianson et al., 2008). The disulfide isomerase (PDIs) participates in retrotranslocation of bacteria and virus antigens (Schelhaas et al., 2007; Tsai, B. et al., 2001). It has also been suggested that non-glycosylated substrates are degraded via post-translational glycosylation (Sato et al., 2012). Despite identifying different mechanisms of recognizing diverse substrates, it is still unclear as to how the cell recognizes and discriminates between terminally misfolded proteins and folding intermediates.

1.3.2. Retrotranslocation/dislocation from the ER

Following the substrate recognition, the targeted unfolded proteins, whether they are soluble within the ER lumen or membrane-integrated substrates, are directed to the retrotranslocation channel in order to be degraded by the ubiquitin/proteasome system (UPS) in the cytosol (Christianson et al., 2014). As many ERAD substrates are highly hydrophobic proteins, it is absolutely essential for the processes of dislocation and degradation to be tightly coupled. Extensive studies have identified different candidates which may be responsible for forming a retrotranslocation pore on the ER membrane: Sec61 (Pilon et al., 1997; Wiertz et al., 1996; Zhou, M. et al., 1999), transmembrane protein Derlin-1 (Lilley et al., 2004; Ye, Y. et al., 2004) and E3 ligase HRD1 (Baldridge et al., 2016; Carvalho, P. et al., 2010; Stein et al., 2014). While earlier studies proposed Sec61 as translocon, which mediates the retrotranslocation of the ERAD substrate, more recent studies demonstrated that blockage of Sec61 has no effect on *in vitro* reconstituted substrate retrotranslocation (Wahlman et al., 2007). Derlin1 associates with members of the retrotranslocation complex, such as the E3 ligase HRD1, SEL1L, and deletion of Derlin1, leads to accumulation of misfolded proteins (Mehnert et al., 2014; Ye, Y. et al., 2004). Alternatively, formation of lipid droplets has been suggested as a route for partially folded ERAD substrates to reach the cytosol (Koenig et al., 2014; Ploegh, 2007), as these substrates are too big in size to pass through a protein channel (Olzmann et al., 2013). Once a substrate emerges from the dislocon complex, it is recognized by the cytoplasmic homohexameric ATPase p97/VCP (ATPase Cdc48 in yeast), which provides the driving force for pulling the ERAD substrates through the retrotranslocation complex (Ye, Y. et al., 2001, 2003).

1.3.3. Ubiquitination and Degradation in the cytosol

Following translocation of the misfolded substrates, E3 complexes ubiquitinates the misfolded substrates and deliver them to the cytosol for degradation. The general steps of ubiquitination involve: 1) ATP-dependent activation of ubiquitin by an E1 enzyme, 2) followed by transfer of the ubiquitin to an E2-conjugating enzyme, and 3) lastly the transfer of ubiquitin to the substrate, catalyzed by an E3 ligase (Komander et al., 2012). In mammals, ubiquitination of the E3 ligase HRD1 is selectively facilitated by UBC63 (Mueller et al., 2008), an E2 ubiquitin-conjugating enzyme, and Ubc7p in yeast (Bazirgan et al., 2006).

In yeast, two ER-anchored E3s, Hrd1p and Doa10p, are responsible for most substrate ubiquitination in ERAD. Similarly, mammalian homologues of yeast E3 ligase, such as HRD1, gp78, TRC8, RMA1, and TEB4, have been shown to mediate ubiquitination of ERAD substrates. Among dozens of mammalian E3 ligases, HRD1 is a principal ER-resident E3 ligase that forms a complex with the ER-resident SEL1L. HRD1 consists of six transmembrane domains and its cytoplasmic tail has a catalytic RING finger needed for E3 ligase activity, plus a long proline-rich C terminus. SEL1L is a type 1 transmembrane protein with a large luminal domain (Sundaram et al., 1993) As a central adaptor protein of HRD1, SEL1L forms a dimer or oligomer and interacts with the N-terminal domain of HRD1 (Jeong et al., 2016). SEL1L nucleates the whole complex by interacting with multiple ERAD components, including HRD1, Derlin1/2, p97, OS-9, XTP3-B and several housekeeping chaperones (Christianson et al., 2008; Mueller et al., 2008; Mueller et al., 2006). Just like its counterpart Hrd1p in yeast, SEL1L is necessary for the stability of HRD1 (Gardner et al., 2000; Sun et al., 2014). During ERAD process, SEL1L recruits the misfolded proteins by interacting with OS9 and XTP3B. Given these functions of SEL1L, it may not be surprising that when SEL1L expression is reduced, degradation of both luminal and integral membrane substrates is impaired (Sha et al., 2014; Sun et al., 2015). Of note,

a study has shown that SEL1L is heavily glycosylated on its luminal domain, which may be necessary for association with OS9, XTP3B and mannosidase EDEM1 (Cormier et al., 2009).

In addition to its role in retrotranslocation, p97 functions to recruit other cytosolic factors during ERAD. These include deglycosylating enzyme peptide N-glycanase (PNGase), which removes the N-linked glycans from the substrates (Hirsch et al., 2003) and deubiquitination enzymes (DUBs), which were originally proposed to remove ubiquitin on the substrate prior to the insertion to the proteasome (Ernst et al., 2009). Additionally, p97 is known to be involved in fusion of ER and Golgi membrane, cell cycle progression, apoptosis regulation, and ribosome-associated degradation (Zhang, T. et al., 2014).

The subsequent degradation of the ERAD substrates is carried out by the 26S proteasome, a protein complex composed of two parts: two 19S regulatory lid particles and the 20S barrel-shaped catalytic core particles (Bar-Nun et al., 2012). The 20S core complex cleaves the ERAD substrates into a mixture of peptides of varying size, which can be further processed by cytosolic proteases to single amino acids that can be incorporated into newly generated polypeptides (Finley, 2009).

1.3.4. ERAD and Immunity

Although substantial progress has been made towards identifying the core components of the mammalian ERAD machinery and the mechanism by which the substrates are recognized for retrotranslocation and subsequent degradation, many questions remain unanswered. Furthermore, discoveries from the use of tissue-specific ERAD knockout mouse model have only begun to shed light on the physiological importance of ERAD. While ERAD has been studied as mechanism for clearance of misfolded model substrates such as mutant CFTR, emerging evidences are suggesting novel roles of ERAD in cellular regulation and functions.

Dendritic cells are one of the antigen presenting cells in the mammalian immune system. These cells are responsible for inducing immunity against pathogens, as well as regulating immune tolerance against self-antigens (Kim, S. J. et al., 2013). Once the immature dendritic cells come into contact with a presentable antigen and phagocytose it, they degrade the antigen into small pieces of peptides which becomes present on their cell surface using MHC. Both MHC class I (MHC-I) and MHC class II (MHC-II) traffics through the ER; while MHC-I presents antigen from endogenous sources to $CD8^+$ T cells, MHC-II presents peptide from exogenous sources to $CD4^+$ T cells (Adams et al., 2013; Merad et al., 2013; Rudolph et al., 2006). Earlier studies have demonstrated how HRD1 targets the misfolded MHC-I for degradation in the in vitro cultured cell lines (Burr et al., 2011; Huang et al., 2011). Recent study, using dendritic cell-specific HRD1 knockout mice, demonstrated that HRD regulates MHC-II expression by targeting BLIMP1 for proteasomal degradation. Consequently, the HRD1-null dendritic cells failed to prime $CD4^+$ T cells without affecting $CD8^+$T cell activation (Yang, H. et al., 2014).

HRD1 is also shown to regulate immunity induced by T cells. T cell proliferation and activation is initiated by the binding of antigenic peptides present by the MHC to the T-cell receptor (TCR), followed by engagement of the T-cell co-receptor CD28 with its ligand, B7, on APCs. *HRD1* deletion in developing thymocytes resulted in: 1) elevated p27Kip1 protein, an inhibitor of cyclin-dependent kinase (CDK); 2) subsequently inhibited T cell clonal expansion; 3) reduced T-cell numbers and attenuated $CD4^+$ T cell differentiation into Th1, Th17 and Treg lineages (Xu, Y. et al., 2016). These data further emphasize the unconventional role of ERAD in cellular regulation and functions.

1.4 B CELL DEVELOPMENT AND FUNCTION

1.4.1. OVERVIEW

The primary purpose of lymphocyte development is to form a functional and diverse antigen receptor repertoire against all potential pathogens. In order to fulfill this goal, the microenvironment, in which B cell develops, provides a sequence of signaling molecules that promotes a series of developmental programs and checkpoints. In mammals, the generation of B lymphocytes occurs primarily in the fetal liver prior to birth, and in the bone marrow during the postnatal life. Bone marrow becomes the major site of B lymphopoiesis, where B cell development occurs in a stepwise process. During B cell lymphopoiesis, progenitor B cells (pro-B) undergo IgH chain gene rearrangement and differentiation, into a large cycling precursor B cell (pre-B), which synthesizes cytoplasmic Igμ heavy chain and assemble into pre-B cell receptor (pre-BCR) to express on the cell's surface. The cycling large pre-B cells will, then differentiate into resting small pre-B cells to begin IgL chain gene rearrangement. Subsequently, IgL chain will assemble with μH chains and form IgM, which is expressed on the cell surface as the B cell receptor (BCR). The immature B cells will then undergo extensive selection to eliminate cells with auto-reactivity and subsequently enter the periphery via blood stream. To further enter a mature, functional compartment, immature B cells progress through multiple transitional stages of development in the periphery lymphoid tissues, such as spleen. Mature B cells can be categorized into three subsets: 1) B1 B cells found in the peritoneal and pleural cavity, such as lung and gut epithelia; 2) marginal zone (MZ) B cells found in the splenic marginal sinus, and; 3) follicular mature (FM) B cells, which circulate in most lymphoid tissues. Although B1 B cells and MZ B cells are less abundant in number, these distinct group of B cells generate a burst of short lived plasma cells with fast kinetics (Cerutti et al., 2013; Martin, F. et al., 2001). On the other hand, since FM B cells require interaction with follicular T cells to form

germinal center, it takes up to seven days to become firmly established (Jacob et al., 1991). Although FM B cell response is not as quick as MZ B cells, it provides highly specific response to protein antigens with higher affinity.

1.4.2. B CELL DEVELOPMENT IN THE BONE MARROW

B cell development is preceded by the first wave of "primitive hematopoiesis" in the mouse fetus at day E7.5 in the extra-embryonic environment of the yolk sac (Cumano et al., 2001; Foundation for Accountability, 2001; Godin et al., 2002). During this time of the development, fetal-type hemoglobin-expressing erythocytes, megakaryocytes, platelets, and special types of myeloid cells, such as microglial cells, are generated (Irion et al., 2010; Schulz et al., 2012). Subsequently, during the second and third waves of hematopoiesis at E10.5, first lymphoid cells, among other cell types derived from hematopoietic progenitor cells originating from the aorta-gonad-mesonephros (AGM) region, are generated (Cumano et al., 2001).These sets of lymphoid cells migrate through embryonic blood to the developing fetal liver, omentum, thymus, and bone marrow (Cumano et al., 2007; Ohmura et al., 2001). On day E12.5, second round of hematopoiesis wave, in fetal liver and bone marrow, generates the first-B lymphoid cells and myeloid cell (Tsuneto et al., 2013); and from day E17.5 and onward, bone marrow becomes the major site of the third wave of hematopoietis, where B cells are continuously generated from pluripotent hematopoietic stem cells (HSCs) throughout life (Hodgson et al., 1990).

While B lymphopoiesis in the fetal liver is transient due to the lack of niches that provide long-term residence, bone marrow provides niches to HSCs, along with long-lived memory T lymphocytes and plasma cells for extended period of time (Tokoyoda et al., 2010). In bone marrows, as HSCs differentiate into multipotent progenitor cells (MPPs), they lose their capacity

to self-renew and become more committed towards one lineage (Bryder et al., 2010). MPPs develop into lymphoid primed multipotent progenitors (LMPP), which has a capacity to produce granulocytes, macrophages and the classically defined lymphoid lineage, but not erythrocytes or megakaryocytes (Adolfsson et al., 2005). These LMPPs express FMS-related tyrosine kinase 3 (FLT3 or CD135) on the cell surface, which upon interaction with FLT5 ligand expressed on bone marrow stromal cells, will give rise to common lymphoid progenitors (CLPs) (Dolence et al., 2014). At this stage, cells are committed to the lymphoid lineage, defined as natural killer, B and T cells. CLPs also express high levels of FLT3, which can induce the expression of IL-7R (Borge et al., 1999). The IL-7R regulates B cell lineage commitment by regulating the expression of transcription factor early B cell factor (EBF) (Dias et al., 2005; Kikuchi et al., 2005). It has been shown that genetic deficiency in the IL-7 pathway in adult mice blocks B-cell development from the CLP stage to the pro-B cell stage (Miller et al., 2002; Peschon et al., 1994; von Freeden-Jeffry et al., 1995), underscoring the indispensable role of IL-7 in B lymphocyte development. However, CLPs do not seem to require IL-7 for survival (Miller et al., 2002). Of note, B cell development in fetal liver does not require IL-7 (Carvalho, T. L. et al., 2001).

CLPs develop into $B220^{+}CD19^{-}$ pre-pro B cells, where the expression of transcription factor paired box gene 5 (*PAX5*) is initiated. *PAX5*, which is responsible for maintaining B cell lineage commitment, is expressed at about 40 % in $B220^{+}CD19^{-}$ pre-pro B cells (Rumfelt et al., 2006) and up to 100 % in pro-B cells to mature B cells (Martin, C. H. et al., 2003), but transcriptionally silenced during plasma cell differentiation (Fuxa et al., 2007; Kallies et al., 2007). Since not all cells are committed to the B cell lineage in the pre-pro B cell stage, D to J rearrangements at the IgH locus can also be found even in T cells (Born et al., 1988).

In fully committed $CD19^+$ pro-B cells, the rearrangement of the immunoglobulin (Ig) gene segments, variable (V), diversity (D) and joining(J), of the IgH locus is initiated, where D_H to J_H rearrangements precedes V_H to D_HJ_H- rearrangements. This site-specific recombination process is regulated by several factors such as IL-7, PAX5, the recombination-activating genes RAG-1 and RAG-2, and TdT (Corfe et al., 2012; Oettinger et al., 1990). When the $V_HD_HJ_H$ rearrangements are successful, a μH chain is synthesized and binds to the ER chaperone called BiP and retained in the endoplasmic reticulum (Hendershot, L. M., 1990). The nascent μH chain pairs with surrogate light chains (SLCs), composed of VpreB and λ5 polypeptides, to form pre-BCR. A pre-BCR associates with the Igα/β heterodimers, which allows the pre-BCR to be deposited on the surface of large pre-B cells. However, studies have shown that a μH chains can be expressed on cell surface, without forming a pre-BCR with SLC, and initiate signals (Galler et al., 2004; Schuh et al., 2003).

The expression of pre-BCR on the surface of the large pre-B cell has multiple purposes. First, signaling by the pre-BCR, in combination with the IL-7R, leads to proliferation burst via phoshphoiostide-3 kinase (PI3K) and extracellular signal-regulated kinase (ERK) signaling pathways. Of note, depending on the relative fitness of the pre-BCR, the cycling large pre-B cells enter between two to five rounds of cell divisions and also grow in size (Rolink, A. G. et al., 2000). While it remains unclear whether a ligand is required at all for pre-BCR stimulation, studies have shown that the signaling is initiated either by the pre-BCR crosslinking (Anbazhagan et al., 2013) or by the bone marrow stromal cell galectin-1-(GAL1) (Gauthier et al., 2002). Second, as soon as the pre-BCR signaling is initiated, the recombination of second IgH allele is stopped by down regulating RAG expression (Philippe et al., 2008; Rolink, A. et al., 1995). This allelic exclusion prevents expression of two IgH chains by one B lineage cell

(Martensson, I. L. et al., 2010), and as a consequence, at least 98.5% B lineage cells express only one IgH chain. Moreover, the pre-BCR is believed to signal its own downregulation by silencing transcription of the genes that encode the *VpreB* and *λ5* (Karnowski et al., 2008; Parker et al., 2005), thus limiting additional pre-BCR formation. As pre-BCR signaling decreases, large pre-B cells exit the proliferation phase, and become a resting, small pre-B cells.

The pre-BCR termination is followed by upregulation of *RAG 1* and *RAG 2* genes, which initiate V_L-J_L rearrangement (Wang, Y. H. et al., 2002). However, if the rearrangement of the light chain gene fails, then it attempts to rearrange the light chain genes on the other chromosome. There are two light chain loci, *Igκ* and *Igλ* in both mice and human. During the rearrangement of IgL chain, the IgH chain loci remain closed to maintain allelic exclusion at this locus. The final product of IgL rearrangement assembles with the μH chain and forms BCR on the immature B cells. The complete BCR consists of two heavy chains, 2 light chains, and the subunits of Igα and Igβ (also known as CD79A and CD79B, respectively) (Kurosaki et al., 2010). These immature B cells remain in a resting state and migrate from the bone marrow via blood to the spleen.

In the periphery tissues, the immature B cell requires further maturation in order to become a functionally competent cell; otherwise, upon interaction with antigens, immature B cells respond with cell death, instead of activation and proliferation. While IgM is first expressed by pre-B cells in pre-B cells, IgD expression emerges at the transitional and mature B cells. After functional V-(D)-J recombination of IgH and IgL chain genes, mature naïve IgM^+IgD^+ B cells co-express IgM and IgD through alternative mRNA splicing. Depending on the stimuli, B cells can be further signaled to produce other isotypes (IgA, IgG and IgE) through alternative transcript splicing and class switch recombination. The primary role of mature B cells in the

humoral component of adaptive immunity is to generate antibody and this unique function of B cells is the basis for the vast majority of successful vaccination strategies. Antibody is produced by two subpopulations of terminally differentiated B cells known as plasmablasts and plasma cells. The cycling, short-lived plasmablasts are differentiated from B1 B cells or MZ B cells through recognition of microbial molecular patterns by TLRs, whereas quiescent, long-lived plasma cells are differentiated from follicular B cells in the germinal center.

1.4.3. Pre-BCR

Pre-BCRs and BCRs are two surface receptors expressed transiently on pre-B cell, and on immature B cells and following B cell subgroups, respectively. These two receptors contain transmembrane form of the Igμ heavy chain that is non-covalently associated with a heterodimer Igα and Igβ, and the surrogate light chain (SLC), or the immunoglobulin light chain (IgL).

The SLC of the pre-BCR is composed of λ5 and VpreB, which are homologous to J_λ-C_λ and V_λ of the BCR, respectively (Kudo et al., 1987; Sakaguchi et al., 1986). These two proteins are non-covalently associated with each other and through λ5, the SLCs form an immunoglobulin-like heterodimer with Igμ hain to form pre-BCR, which is expressed transiently on the surface of pre-B cells (Karasuyama et al., 1990; Tsubata et al., 1990). Both the λ5 and the VpreB proteins have immunoglobulin domain and non-immunoglobulin domain, also known as a unique region. In VpreB, the immunoglobulin domain appears to have a V domain-like structure, but lacks the last β-strand (β7) of a typical V domain. Unlike λ5, the unique region on the carboxyl terminal end of VpreB shows no sequence homologies to any other proteins (Melchers et al., 1999). In λ5 the immunoglobulin domain of the has a constant domain-like structure, with strong homologies to λL chains. Unlike VpreB, λ5 protein has β7-like strand near $C\lambda_5$, which has strong homology to J regions of λL chains. It has been proposed that β7-like strand of λ5 is non-

covalently associated with the β-strands of VpreB (Melchers, 2005). Interestingly, it was demonstrated that deletion of the β7 strand in λ5 protein abrogates the formation of surrogate light chain (Minegishi, Hendershot, et al., 1999), suggesting that the extra β7 strand is necessary to complement the incomplete VpreB domain to fold and assemble SLCs (Bornemann et al., 1997; Guelpa-Fonlupt et al., 1994; Minegishi, Hendershot, et al., 1999). λ5 also has unique region, towards the amino terminal region, which shows only marginal sequence homologies to Ig domain structure (Melchers et al., 1993). Interestingly, deletion of both unique regions in VpreB and λ5 did not abrogate SLC assembly (Minegishi, Hendershot, et al., 1999). However, subsequent studies demonstrated that the unique region of λ5 retains the preBCR complex in the ER and limiting expression of the surface (Fang et al., 2001; Guloglu et al., 2005; Ohnishi et al., 2003), whereas the function of VpreB unique region still remains elusive.

VpreB can associate with Igμ heavy chain (Seidl et al., 2001), while λ5 alone cannot (Minegishi, Hendershot, et al., 1999; Seidl et al., 2001). Only when λ5 is covalently associated with VpreB, does λ5 form a disulfide bridge with the first constant region (CH1) of an Igμ chain to form the pre-BCR (Melchers, 1999). Only the fully assembled SLC binds to μH chains without any structural defect (Kline et al., 1998; ten Boekel et al., 1997).

The expression of pre-BCR on the surface marks the first checkpoint in B cell development. Using pre-BCR mutant mice, the critical role of pre-BCR as a first checkpoint have been elucidated. Mice have two *VpreB* genes, *VpreB1* and *VpreB2*, and one *λ5/14.1* gene encoding the SLC components on chromosome 16 (Melchers, 2005). Interestingly, *VpreB1* is expressed in all cells that express *λ5*, whereas *VpreB2* is detected in ~30 % of these same cells; also, both gene products are functional and can form an SL chain with λ5 (Dul et al., 1996)]. In model systems that are lacking either the entire SLC or its components, i.e. λ5 mutant or *VpreB1*$^{-}$

$^{-/-}$/*Vpreb2*$^{-/-}$, exhibit a decrease of precursor B cell expansion, blocking further B cell development in bone marrow (Kitamura, Kudo, et al., 1992; Mundt et al., 2001; Shimizu et al., 2002). Of note, mice with *VpreB1* deletion exhibited partial block of the development at the transition from pro-B to large pre-B, demonstrating that VpreB2 alone is capable of supporting B cell development and give rise to mature B cells in the periphery (Martensson, A. et al., 1999). Furthermore, in the μMT mouse model and in the mouse model with a targeted deletion of λ5, the efficiency with which cells progress into proliferation pre-B cell compartment is drastically reduced (Kitamura, Kudo, et al., 1992; Kitamura et al., 1991).

While ability to deposit a pre-BCR complex on the cell surface is important for development, accumulating data also suggests regulation pre-BCR signaling cascade is also a crucial factor regulating B cell development. Mice deficient of SLP-65 (also known as BLNK or BASH), an adaptor protein activated by p-Syk, show a partial block at B cell development at the pre-B cell stage. Furthermore, due to a defect of receptor down-regulation, pre-B cells from these mice express large amount of pre-BCR on their surface (Flemming et al., 2003; Jumaa et al., 2003; Jumaa et al., 1999), while temporary extended expression of λ5 leads to delay in the progression of maturing B lineages (Martin, D. A. et al., 2007). Taken together, these data underscore the importance of SLC expression and regulation during early B lineage differentiation.

CHAPTER TWO: REVIEW- Endoplasmic reticulum quality control in cancer: Friend or Foe[a]

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(* These authors contributed equally)

2.1 ABSTRACT

Quality control systems in the endoplasmic reticulum (ER) mediated by unfolded protein response (UPR) and endoplasmic reticulum associated degradation (ERAD) ensure cellular function and organismal survival. Recent studies have suggested that ER quality-control systems in cancer cells may serve as a double-edged sword that aids progression as well as prevention of tumor growth in a context-dependent manner. Here we review recent advances in our understanding of the complex relationship between ER proteostasis and cancer pathology, with a focus on the two most conserved ER quality control mechanisms – the IRE1α-XBP1 pathway of the UPR and SEL1L-HRD1 complex of the ERAD.

2.2 INTRODUCTION

In eukaryotic cells, approximately one third of the total proteome is folded to maturity in the endoplasmic reticulum (ER) prior to transportation to various subcellular or extracellular compartments. A myriad of chaperones, folding enzymes and nascent proteins crowd the molecular environment of the ER lumen all the while maintaining a delicate homeostasis in its protein folding machinery. Various perturbations to this equilibrium, including both physiological and pathological stimuli, can lead to an accumulation of misfolded proteins inside the ER, subjecting the cell to a condition called “ER stress” and activating a series of adaptive mechanisms to alleviate the stress and restore ER homeostasis. These mechanisms consist of two major ER quality control machineries, including unfolded protein response (UPR) and ER-associated degradation (ERAD) (Hetz et al., 2013; Ron et al., 2007; Tsai, Y. C. et al., 2010).

Originally discovered as a response to nutrient depletion, autophagy is a cellular process involved in the lysosomal degradation of cellular components and in the maintenance of energy homeostasis through recycling of amino acids and nutrients (Ryter et al., 2014). Several studies

suggest that autophagy is activated as an adaptive mechanism in cells experiencing ER stress and may play a role in the maintenance of ER homeostasis in cancer (Kroemer et al., 2010; Ogata et al., 2006). However, as the role of autophagy goes beyond the ER (Levine et al., 2008), whether the effect of autophagy in cancer is related to its function in the ER remains to be established. Hence, as the role of autophagy in cancer has recently been extensively reviewed (Gewirtz, 2014; Nagelkerke et al., 2015; Perez-Mancera et al., 2014), it will not be the focus here.

Owing to a high proliferation rate, cancer cells often experience impaired ATP generation, hypoxia, hypoglycemia and specific mutations which may perturb ER homeostasis and trigger the activation of UPR (Tsai, Y. C. et al., 2010). Persistent ER stress often activates pathways that lead to cell death, effectively eliminating cells with a potential to go rogue. On the other hand, tumor cells may hijack the ER quality control machineries to provide survival signals required for neoplasm growth and eventually avoid cell death (Croft et al., 2014). Researchers have considered targeting various components of UPR and ERAD as potent therapeutic means to specifically modulate the survival of cancer cells (Clarke et al., 2014). In this review, we will discuss the involvement of two most highly conserved branches of the ER quality control systems – the IRE1α signaling pathway of the UPR and the SEL1L-HRD1 complex of the ERAD – in cancer pathogenesis.

2.3 The IRE1α signaling pathway

IRE1 is a type-1 ER-resident membrane protein with bifunctional cytosolic kinase and endoribonuclease (RNase) domains (Cox et al., 1993; Mori et al., 1993). In mammals, IRE1 exists in two isoforms, IRE1α (Tirasophon et al., 1998) and IRE1β (Wang, X. Z. et al., 1998). IRE1α is ubiquitously expressed and global knockout of the gene results in early embryonic lethality (Iwawaki et al., 2009; Zhang, K. et al., 2005). In contrast, IRE1β expression is limited to

the gastrointestinal epithelial cells (Bertolotti et al., 2001) and has no RNase activity towards the classical IRE1α substrate X-box binding protein 1 (*Xbp1*) mRNA (Tsuru et al., 2013). While IRE1β knockout mice are viable, they are hypersensitive to experimental colitis (Bertolotti et al., 2001), which may be in part due to reduced mucin biosynthesis (Tsuru et al., 2013).

Upon ER stress, IRE1α undergoes dimerization and/or oligomerization and trans-autophosphorylation, which triggers conformational change and activation of its RNase domain. Activated IRE1α splices 26 nucleotides from *Xbp1* mRNA, leading to translational frameshift and the generation of an active transcription factor XBP1s. Subsequently, XBP1s enters the nucleus, where it transactivates various target genes, including those involved in protein folding, ERAD, protein trafficking, and lipid biosynthesis (Figure 2.1) (Sha et al., 2011). Additionally, IRE1α has been shown to degrade a subset of mRNAs via a process called Regulated IRE1-Dependent Decay (RIDD) (Figure 2.1) (Hollien et al., 2009; Hollien et al., 2006; Sakaki et al., 2012; So et al., 2012). Moreover, IRE1α cleaves some premature microRNAs as a means of regulating apoptosis (Upton et al., 2012) as well as its own mRNA level (Dai et al., 2013; Maurel & Chevet, 2013). The physiological significance of these extra-*Xbp1* activities of IRE1α in vivo remains poorly characterized.

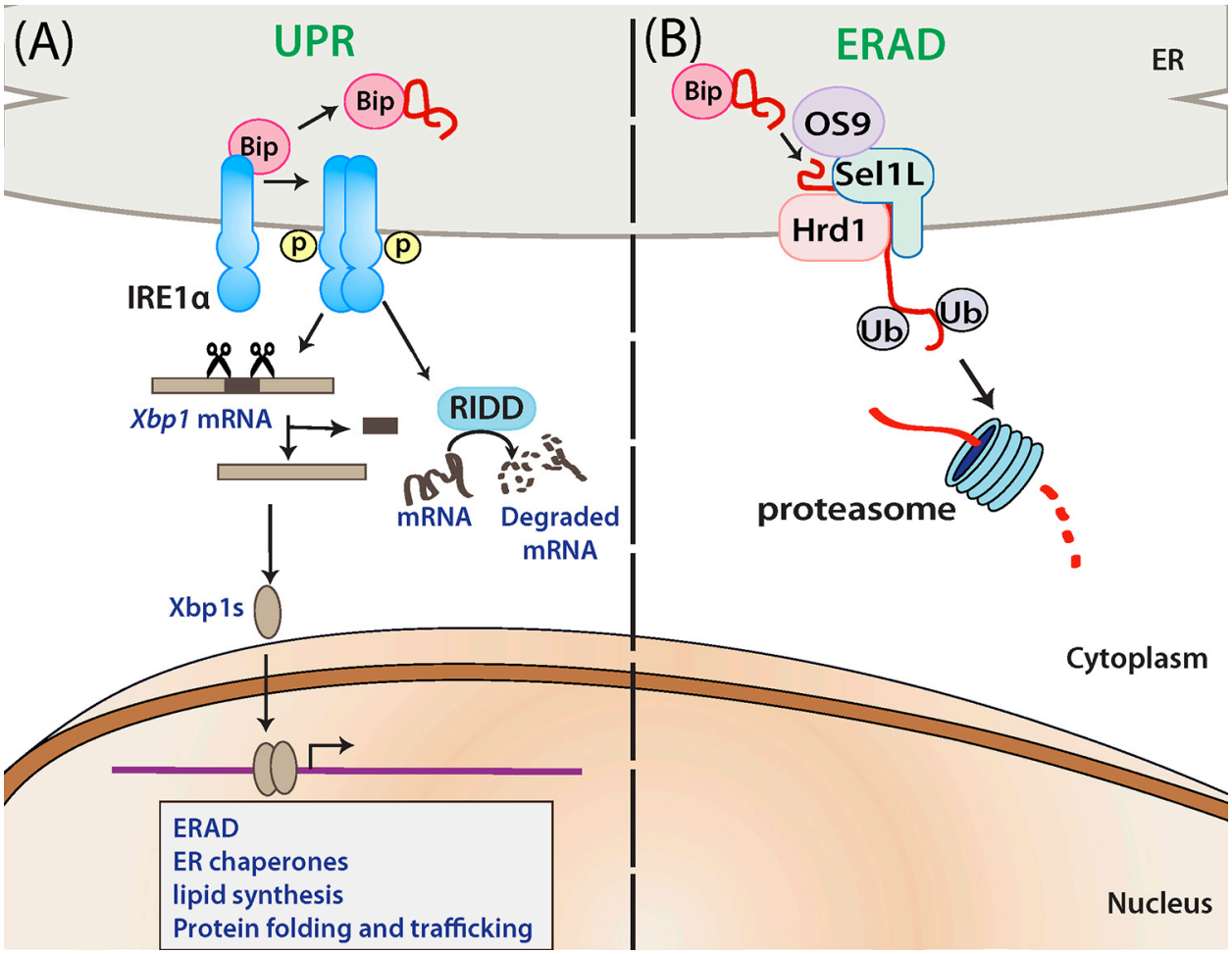

Figure 2. 1 Schematic diagrams depicting the roles of IRE1α in UPR and SEL1L-HRD1 in ERAD.

Upon sensing ER stress, IRE1α undergoes dimerization or oligomerization, and trans-autophosphorylation, activating its cytosolic endonuclease activity. Subsequently, IRE1α alternatively splices *Xbp1* mRNA to generate Xbp1s which translocates into the nucleus and regulates different genes. Furthermore, activated IRE1α can selectively degrade particular mRNAs by a process called regulated IRE1-dependent decay (RIDD). Unlike IRE1α-XBP1 pathway, physiological significance of other IRE1α pathways are not well established. (B) Misfolded proteins in the ER lumen are recognized, ubiquitinated and retrotranslocated by the

HRD1-SEL1L ERAD complex to the cytosol for proteasomal degradation. BiP and OS9 may be involved in the recognition of misfolded substrates.

Similar to IRE1α-deficient mice, global deletion of XBP1 leads to embryonically lethal in mice (Iwawaki et al., 2009; Reimold et al., 2000; Zhang, K. et al., 2005). Using cell type-specific knockout mouse models, studies have demonstrated a critical role of IRE1α-XBP1 pathway in secretory cells, most notably B cell-derived plasma cells and pancreatic β cells. Mice with B cell-specific *Xbp1* deficiency show a profound defect in plasma cell production, along with decreased levels of antigen-specific immunoglobulin (Reimold et al., 2001; Shaffer et al., 2004; Todd et al., 2009). Intriguingly, IRE1α deficiency in B cells affects not only plasma cell differentiation, but also early stage of B cell development (Zhang, K. et al., 2005). While VDJ rearrangement occurs normally in XBP1$^{-/-}$ B cells (Reimold et al., 2001), this event is severely defective in the pro-B cell stage of IREα$^{-/-}$ B cells (Zhang, K. et al., 2005). The authors propose that the cytoplasmic domain of IREα may directly regulate transcriptional activation of genes involved in VDJ recombination such as *Rag1* (recombination-activating gene 1), *Rag2* (recombination-activating gene 2), and *TdT* (terminal deoxynucleotidyl transferase).

In vitro, IRE1α can be activated by glucose in a concentration-dependent manner (Lipson et al., 2006) and hyperactivation of IRE1α by high glucose may lead to insulin mRNA degradation in pancreatic β cells (Lipson et al., 2008). Intriguingly, β cell-specific deletion of *Xbp1* in mice results in islet atrophy and hyperglycemia associated with impaired β cell proliferation, insulin maturation and secretion at basal level (Lee, A. H. et al., 2011). Moreover, deficiency of XBP1 caused constitutive hyper-activation of IRE1α, leading to attenuation of *insulin* mRNA via RIDD.

On the other hand, while IRE1α deficiency in β cells causes disruption in glucose homeostasis and impairs β cell proliferation under metabolic stress, it did not affect pancreatic structure or islet area (Xu, T. et al., 2014). These differential phenotypes observed in β cell

specific IRE1α- and XBP1- null mice suggest that each component of this pathway may have its own unique function in cellular physiology. Alternatively, it points to a possible role of the unspliced form of XBP1u, whose physiological role awaits further investigation. Taken together these studies highlight the indispensable role of the IRE1α -XBP1 pathway in ER expansion and survival of highly secretory cell types.

2.4 THE ROLE OF IRE1A-XBP1S SIGNALING PATHWAY IN CANCER

Table 1 and **Figure 2.2** depict various possible molecular mechanisms underlying the role of IRE1α in cancer. The role of IRE1α in cancer is best illustrated and characterized in multiple myeloma (MM). MM is a malignant proliferation of plasma cells in the bone mar-row and share phenotypical characteristics with long-lived plasma cells. Due to abundant synthesis of secretory proteins in the ER, MM cells are hypersensitive to the activation of UPR that aggravates as the disease advances (Nakamura et al., 2006). Thus, these cells require a large capacity of folding and disposal in the ER and are particularly sensitive to compounds targeting proteostasis. IRE1α activation can contribute to cancer progression in several pathways mediated by its substrate XBP1s, which is highly expressed in MM (Bagratuni et al., 2010). Blocking of IRE1α RNase activity by IRE1 inhibitors such as STF-083010 or 4μ8C or similarly reducing XBP1 expression by proteasome inhibitor or toyocamycin, an XBP1 inhibitor, attenuates the growth of MM cells, via apoptosis (Cross et al., 2012; Lee, A. H., Iwakoshi, Anderson, et al., 2003; Papandreou et al., 2011; Ri et al., 2012). Conversely, forced expression of XBP1s in B cells promotes multiple characteristics of myeloma pathogenesis with lytic bone lesions, plasmacytosis and increased monoclonal antibodies (Carrasco et al., 2007). More than 1000 genes are upregulated in XBP1s-transgenic myeloma cells compared to non-transgenic B cells, including Cyclin D1, Cyclin D2, MAF and MAFB, many of which are known to be involved in

human MM pathogenesis. In clinical studies, human MM patients with high ratio of *Xbp1s* mRNA to *Xbp1u* mRNA have a significantly lower survival rate (Bagratuni et al., 2010). Collectively, these studies suggest a potential causative role of XBP1s in diseases pathogenesis in some MM patients and implicate IRE1α-XBP1 axis as a potential therapeutic target in MM.

Table 1 The role of IRE1α in various types of cancer.

Type of cancer	System	Mechanism	Reference
Multiple myeloma	B cell specific Xbp1s transgenic mice and patient samples	Aiding in secretory maturation of plasma cells and resistance to anti-cancer drugs	(Alexanian et al., 2012; Bagratuni et al., 2010; Carrasco et al., 2007; Leung-Hagesteijn et al., 2013)
Triple negative breast cancer	Breast cancer lines and mice with mammary glands injected with tumor cells	High Xbp1s levels aid in sustaining hypoxia (co-localizing with hypoxia markers), promoting angiogenesis and invasion in collaboration with HIF1α via transcriptional regulation of various genes including VEGFA, PDK1, GLUT1, DDIT4	(Chen, X. et al., 2014)
Colon cancer	Eleven patient primary tumor samples	High levels of Xbp1 expression in colorectal carcinoma and adenoma	(Fujimoto et al., 2007)
Glioma	Human U87 glioma cell line; mouse orthotopic brain model; chick chorio-allantoic membrane	Supports tumor vascularity, blood vessel cooption and invasiveness by promoting pro-angiogenic VEGFA, IL-6, IL-8, IL-1β, and by inhibiting anti-angiogenic thrombospondin1, decorin.	(Auf et al., 2010)
Several cancers	Cell lines	IRE1α mutations identified in tumor cells are defective in signaling	(Ghosh, R. et al., 2014; Xue et al., 2011)
Liver cancer	Hepatoma HuH7 cell line	Downregulates GPC3 (mediator of tumor growth via canonical Wnt signaling) by RIDD; itself silenced in cancer cells by oncomiR miR-1291	(Maurel, Dejeans, et al., 2013)
Colon cancer	In vivo Xbp1 intestine-specific-null mouse model	Protects against colitis-associated-cancer and Apc-mediated colorectal cancer; depletion of Xbp1 predisposes intestinal epithelium to inflammatory diseases and tumorigenesis	(Niederreiter et al., 2013)
Glioma	Human U87 glioma cell line	Suppresses attachment and migration properties by downregulating RhoA and SPARC (a matrix protein that retards cell cycle, induces cell shape change and promotes invasiveness)	(Dejeans et al., 2012)

Abbreviations – Apc: Adenomatous polyposis coli; DDIT4: DNA-damage-inducible transcript 4; GLUT1: Glucose transporter 1; GPC3: Glypican 3; HIF1α: Hypoxia inducible factor 1 alpha; IL-6, -8, -1β: Interleukin 6, 8, 1beta; IRE1α: Inositol requiring enzyme 1 alpha; miR: microRNA; PDK1: Phosphoinositide-dependent kinase 1; RIDD: Regulated IRE1-dependent decay; RhoA:

Ras homolog gene family member A; SPARC: Secreted protein acidic and rich in cysteine; VEGFA: Vascular endothelial growth factor A; XBP1s: Xbox binding protein 1 spliced

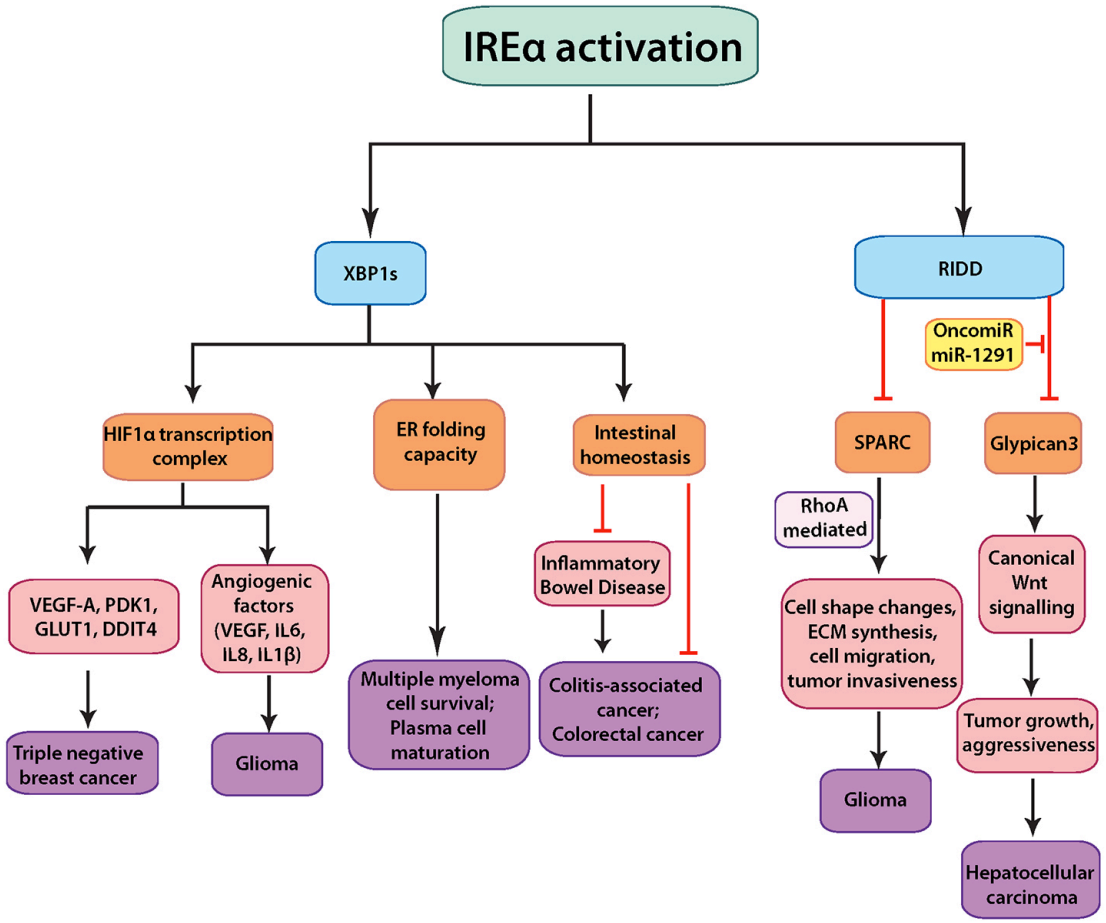

Figure 2. 2 The role of IRE1α-mediated signaling pathways in cancer pathogenesis.

IRE1α can exert XBP1s–dependent and –independent functions in cancer cells. In XBP1s-dependent pathways, IRE1α activation can trigger plasma cell maturation and multiple myeloma cell survival; protection from colon cancer via maintenance of intestinal homeostasis; induction of transcription of angiogenic factors and other tumor promoting components in complex formation with HIF1α, as discovered in gliomas and breast cancer. Via RIDD, IRE1α downregulates SPARC mRNA thereby preventing RhoA-mediated increase in glioma invasiveness. In liver cancer, the oncogenic miR-1291 downregulates IRE1α, thereby allowing

its otherwise RIDD substrate Glypican-3-to promote tumor growth and aggressiveness via canonical Wnt signal.

In addition to MM, the IRE1α-XBP1 signaling pathway has been implicated in colon and breast cancers. As substantial evidence is lacking in most cases, we will discuss these studies in brief. XBP1has been implicated in colon carcinogenesis in a 2007 clinical study, where higher levels of total *Xbp1* mRNA and protein assessed via RT-PCR and immunohistochemistry, respectively, were found in colorectal polyps, colon carcinomas and colon cancer cell lines as compared to normal and stromal tissue (Fujimoto et al., 2007). It should be pointed out that this study was limited by a small dataset of only 11 patients. In mice, loss of *Xbp1* in the intestinal epithelium leads to an increase in intestinal stem cell numbers in an IRE1α-dependent manner and Stat3-dependent hyper-proliferation of intestinal epithelial cells (Niederreiter et al., 2013). Consequently, these Xbp1 null mice are more susceptible to colitis-associated cancer as well as genetic-induced colorectal cancer associated with the mutation of adenomatous polyposis coli (Apc-min).

In addition to its role in ER maintenance, the -XBP1signaling pathway may aid in the regulation of hypoxia in highly aggressive triple-negative breast cancer (TNBC) (Chen, X. et al., 2014). In breast cancer cell lines and xenograft models, loss of Xbp1 reduces tumor growth and metastasis due to impaired angiogenesis, independently of cell proliferation or apoptosis. ChIP-seq analysis coupled with co-IP experiments reveals that XBP1s and HIF1α may function within the same transcriptional complex to regulate the expression of genes involved in survival and angiogenesis such as *VEGFA* (vascular endothelial growth factor A), *PDK1* (phosphoinositide-dependent kinase 1), *GLUT1* (glucose trans-porter 1), *DDIT4* (DNA-damage-inducible transcript 4) via the recruitment of RNA polymerase II (Chen, X. et al., 2014). In line with this notion, inhibition of IRE1α in gliomas reduces the expression of pro-angiogenic genes such as VEGF-A, IL-6, IL-8 and IL-1β, while having an opposite effect on anti-angiogenic factors and matrix

proteins such as *thrombospondin-1, decorin and osteonectin* (also known as *secreted protein acidic and rich in cysteine* or *SPARC*) (Auf et al., 2010; Drogat et al., 2007). Indeed, in tumors expressing a dominant negative IRE1α, where IRE1α transmembrane and luminal domains (aa 1–555) is fused upstream to full length Nck-1 (non-catalytic region of tyrosine kinase adaptor protein 1), there is a marked decrease in angiogenesis, tumor vascular density and growth (Drogat et al., 2007; Nguyen et al., 2004). Taken together, these studies point to a critical role of IRE1α in mediating hypoxia and angiogenesis in tumor growth and identify the IRE1α-XBP1s signaling pathway as a candidate for therapeutic intervention in targeting the angiogenic switch in tumor development.

IRE1α may be involved in cancer pathogenesis via *Xbp1*-independent pathways as well (Figure 2.2). Various RIDD targets have recently been implicated in pathways promoting tumor growth and metastasis. Among them lies SPARC, a matrix-associated protein that retards cell-cycle progression, triggers changes in cell shape and induces synthesis of extracellular matrix, thereby promoting tumor cell invasiveness. (Gaddam et al., 2013; Hollien et al., 2009). IRE1α, via its RNase activity, leads to a downregulation of *Sparc* mRNA levels as shown in a rat glioma model. Expression of the same aforementioned dominant negative IRE1α transgene leads to an increase in tumor cell attachment and migration along with upregulation of SPARC and activation of its mediator RhoA, a cytoskeleton regulator protein (Dejeans et al., 2012; Nguyen et al., 2004).

Glypican-3 (GPC3) is an RIDD substrate as its mRNA is cleaved at the 3' UTR by IRE1α in an ER stress-independent manner (Maurel, Dejeans, et al., 2013). GPC3 is a glycosylphosphatidylinositol (GPI)-anchored membrane protein that has been associated in diseases such as Wilms tumors and Simpson–Golabi–Behmel syndrome, and is highly

overexpressed in hepatoblastoma and hepatocellular carcinoma (HCC) (Jakubovic et al., 2007). GPC3 stimulates canonical Wnt signaling by forming complexes with Wnts, thereby promoting aggressiveness, tumor growth and poor prognosis (Capurro et al., 2005). In the GPC3^{high}HCC subgroups, the oncogenic microRNA, miR-1291, is particularly up-regulated (Maurel, Dejeans, et al., 2013). Intriguingly, miR-1291 downregulates IRE1α via destabilization of its mRNA by targeting a site in its 5' UTR region (Maurel, Dejeans, et al., 2013). This regulatory circuit whereby the oncomiR-1291 downregulates IRE1α leading to high levels of GPC3 may promote tumor growth and invasiveness via stimulation of canonical Wnt signaling in liver cancer cells.

Finally, IRE1α has also been implicated in the pathogenesis of several cancers via mechanisms yet unknown. Studies conducted using glioblastoma, serous ovarian cancer and lung adenocarcinoma models have demonstrated that mutations often accrue in IRE1α genomic loci (Korennykh et al., 2009). We recently analyzed the activities of some cancer-associated IRE1α mutants (P830L, S769F, L474R, and R635W) and found that a highly conserved proline residue at position 830 (Pro830) is crucial for maintaining IRE1α structural integrity (Xue et al., 2011). This residue seems to act as a structural linker with adjacent tyrosine (Tyr945) and tryptophan (Trp833) residues to link the kinase and RNase domains of IRE1α. The P830L mutation destabilizes IRE1α and renders both kinase and RNase domains of IRE1α inactive. Similarly, the Ser769Phe mutation abolishes IRE1α activation and signaling, although the mechanism remains unclear (Xue et al., 2011). It is possible that cancer cells may accrue these loss-of-function IRE1α mutations to attenuate the pro-apoptotic function of IRE1α as recently proposed (Ghosh, R. et al., 2014). However, as whether or not IRE1α signaling exerts pro- and/or anti-apoptotic effects remains controversial (Ghosh, R. et al., 2014; Lu et al., 2014), further investigations are required to delineate physiological significance of IRE1α mutations in tumorigenesis.

2.5 ERAD

ERAD is responsible for the recognition, retrotranslocation and ubiquitination of misfolded proteins in the ER for proteasomal degradation in the cytosol (Olzmann et al., 2013). The ERAD system revolves around transmembrane ubiquitin E3 ligase proteins that connect together the substrate recognition machinery in the ER lumen and the ubiquitin-proteasome system in the cytosol. Failure to remove misfolded ER proteins may result in their accumulation and aggregation, which may account for the pathogenesis of various diseases such as cystic fibrosis, α1-antitrypsin deficiency and type-1 diabetes (Chiti et al., 2006; Guerriero et al., 2012).

There are two principle E3 ERAD complexes in yeast (Hrd1p and Doa10p), and at least half a dozen in metazoans, each of which recognizes a subset of misfolded proteins in the ER (Olzmann et al., 2013). Hrd1p forms a complex with Hrd3p in yeast (Gardner et al., 2000; Hampton et al., 1996) and with Suppressor/Enhancer of Lin-12-like (SEL1L) in mammals (Mueller et al., 2008; Mueller et al., 2006). As shown in Figure 1, SEL1L nucleates the HRD1 ERAD complex by interacting with multiple ERAD components such as HRD1, Der-lin1/2, p97, OS9, and E2 enzyme UBC6e (Christianson et al., 2011; Mueller et al., 2008; Mueller et al., 2006). A recent proteomic analysis has implicated both Sel1L-dependent and –independent HRD1-mediated degradation, which may be dictated by substrate topology or accessibility of specific E3 ligases (Christianson et al., 2011).

Physiological importance of SEL1L and HRD1 in vivo is recently emerging. Global deletion of *Sel1L* causes embryonic lethality in mice (Francisco et al., 2010; Sun et al., 2014). In the absence of *Sel1L*, the development of embryonic pancreatic epithelial cell was blocked (Li, S. et al., 2010). Using inducible and adipocyte-specific *Sel1L* -deficient mouse and cell models, we

recently demonstrated that *Sel1L* plays a critical role in the stabilization of HRD1 protein in mammals (Sha et al., 2014; Sun et al., 2014) and that the SEL1L –HRD1 complex plays a critical role in mammalian ERAD, ER homeostasis and survival in vivo (Sun et al., 2014). Acute loss of *Sel1L* in adult mice causes premature lethality and severe pathologies of secretory tissues with striking abnormalities of the ER structure integrity, suggesting a crucial role of *Sel1L* in secretory cell types in particular. On the other hand, loss of *Sel1L* in adipocytes leads to resistance to diet-induced obesity and postprandial hypertriglyceridemia due to the ER retention of lipoprotein lipase (Sha et al., 2014). In addition, variants in the *Sel1L* gene have also been identified in humans with Alzheimer's diseases (Saltini et al., 2006) and SEL1L mutations have been linked to early-onset cerebellar ataxia in canines (Kyostila et al., 2012), pointing to a possible role of SEL1L in maintaining homeostasis in neuronal/glial cells.

Global deletion of HRD1, also known as Synoviolin (encoded by the gene Syvn1), also causes embryonic lethality in mice (Yagishita et al., 2005). Loss of Hrd1 in the liver upregulates the expression of *Nrf2* (nuclear factor (erythroid-derived 2)-like 2) protein and its target genes *Nqo1* (NAD(P)H quinone oxidoreductase 1) and *Gclm* (glutamate-cysteine ligase, modifier subunit). This study demonstrated that Nrf2 is a substrate for Hrd1-mediated proteasomal degradation in the pathogenesis of liver cirrhosis. In this context, ER and oxidative stress response signaling pathways converge as ubiquitination of Nrf2 by HRD1 results in downregulation of the Nrf2-mediatedantioxidant response pathway (Wu, T. et al., 2014). In dendritic cells (DC), HRD1 seems to regulate the expression of MHC (major histocompatibility complex) class II via regulating the protein turnover of a key transcriptional repressor BLIMP1 (B lymphocyte induced maturation protein 1) (Yang, H. et al., 2014). Loss of *Hrd1* causes the accumulation of BLIMP1, which represses gene transcription of MHC class II. Dendritic cell

(DC)-specific *Hrd1* knockout mice exhibit splenomegaly with increased B cell numbers and defects in $CD4^+$ T cell priming in the autoimmune inflammatory response (Yang, H. et al., 2014). How HRD1 mediates the degradation of nuclear transcription factor and whether this function of HRD1 is SEL1L dependent remain to be demonstrated.

2.6 THE ROLE OF SEL1L-HRD1 ERAD IN CANCER

While the role of ERAD in cancer remains largely unknown, SEL1L has been implicated in cancer pathogenesis. Ectopic SEL1L induction in pancreatic cancer cells leads to G1 phase cell cycle arrest via the induction of PTEN – a phosphoinositide-3-phosphatase and well-known tumor suppressor that normally inhibits cell proliferation, growth and motility. High levels of SEL1L in these cells also lead to reduction in invasiveness possibly via negative modulation of genes encoding matrix metalloproteinase inhibitors (Cattaneo et al., 2005). Furthermore, a 2012 study discovered that the SEL1L SNP (rs12435998) shares a close association with the age at diagnosis of pancreatic ductal adenocarcinoma and the patient survival time with or without pancreaticoduodenectomy by analysis of DNA obtained from Caucasian (non-smoker) patients (Liu, Q. et al., 2012).

In the context of colorectal cancer, while basal SEL1L expression level in normal mucosa of the epithelial lining is low, it is elevated in adenoma and adenocarcinoma cells (Ashktorab et al., 2012). Nonetheless, SEL1L expression pattern has so far been found to lack correlation with patient survival and the grade of colon cancer. In an investigation involving glioma stem cell lines and valproic acid (VPA), a histone deacetylase inhibitor, the same group reported that SEL1L downregulation in glioma stem cell lines leads to an impairment of neurosphere size and proliferative rate, while inducing differentiation toward a neuronal fate via Notch1 signaling (Cardano et al., 2011). VPA, a promising therapeutic agent owing to its anti-cancer and

minimally toxic properties, was found to upregulate the expression of SEL1L and other UPR genes in glioma stem cells. siRNA-mediated SEL1L knockdown in these cells negatively affects their self-renewal potential and exacerbates the cytotoxic effects of VPA. These data suggest that SEL1L may protect against VPA-mediated cytotoxicity via the maintenance of cancer stem cell properties (Cattaneo et al., 2014). In addition, correlations between low SEL1L protein levels (detected by mono-clonal antibody staining) and poor prognosis have been reported in breast carcinoma patients (Orlandi et al., 2002). In the context of esophageal cancers, while absent in normal cells, SEL1L is expressed in early neoplastic events and persists in later stages of esophageal cancer (Granelli et al., 2004).

It should be noted that, as most of these studies implicating SEL1L in cancer pathogenesis are based on association studies, interpretation of their findings should proceed with caution. Many outstanding questions remain, for example, what is the mechanism by which SEL1L is involved in tumorigenesis? How do changes in SEL1L level in tumor cells affect ER homeostasis or specific ERAD substrates? How ER homeostasis affects tumorigenesis? Nonetheless, these studies are important because they have opened avenues for more definitive future investigations using animal models. Another well-studied ERAD component is the lectin protein osteosarcoma amplified-9 (OS9) involved in the recognition and recruitment of misfolded glycoprotein or non-glycoproteins to the ERAD complex (Christianson et al., 2008; Hosokawa et al., 2009). Under hypoxic conditions, OS-9-mediated ubiquitination and subsequent degradation of HIF-1α, aided by an E3 ligase and tumor suppressor von Hippel–Lindau (VHL), is instrumental in downregulating genes that promote cell survival, proliferation, invasion, angiogenesis and metastasis (Baek et al., 2005; Ivan et al., 2001). A recently identified gene CIM (Cancer Invasion or Metastasis-related) also known as ERLEC1 (Endoplasmic Reticulum Lectin-

1) has been found to sequester OS-9 away from the HIF-1α complex in lung cancer cells, causing HIF-1α stabilization and accumulation thereby aiding tumor growth and metastasis (Yanagisawa et al., 2010). This study proposes OS9 to be an important link between hypoxia regulation and cancer progression. Intriguingly, we have recently shown that in the absence of Sel1L, OS9 accumulates and is stabilized (Sha et al., 2014; Sun et al., 2014), suggesting that OS9 is a substrate of the Sel1L–Hrd1 ERAD complex. Thus, the Sel1L–Hrd1 ERAD complex, along with other ERAD components (such as VHL) may exert its role in tumorigenesis via the regulation of either ER homeostasis in general or specific substrates such as OS9.

2.7 THERAPEUTICS

Maintaining ER proteostasis assumes high importance in cancer cells due to the increased pressure on protein folding owing to their enhanced metabolic needs, as is evident from the high basal level of expression of UPR markers in these cells. Consequently, developing interventions that aim to sensitize tumor cells to various anti-cancer agents by selectively inhibiting UPR has become a popular therapeutic strategy of late.

A recent endeavor that tested the efficacy of several IRE1α inhibitors (STF-083010, 3-ethoxy-5,6-dibromosalicylaldehyde, 2-hydroxy-1-naphthaldehyde, toyocamycin, etc.) in a dosage and time-dependent manner on 14 pancreatic cancer cell lines observed growth retardation due to either cell cycle arrest or induced apoptosis as well as reduction in invasiveness demonstrated by soft agar assays and xenograft experiments (Chien et al., 2014). Irestatin is another IRE1α endonuclease activity inhibitor that has been seen to impair proliferation survival under starvation conditions of malignant myeloma cells (Li, X. et al., 2011). Interestingly, while there is considerable variability in cellular responses to these IRE1α

inhibitors, synergistic effects have been observed when using various drug treatments in combination (Li, X. et al., 2011).

The use of oncolytic virus therapy (OVT) – viral induction of tumor cell lysis and recruitment of the immune system to the infected tissue – is quickly rising in popularity owing to its selectivity in targeting malignancies based on the inherent abnormalities of cancer cells. A major drawback of this approach lies in the great variation in the response rates of these viruses on patients. In this context, a genome-wide RNAi screen recently identified various ER stress pathway components including IRE1α whose inhibition results in preconditioning of cancer cells to undergo apoptosis when challenged with rhabdoviral oncolysis. This sensitization to caspase-2-dependent cell death occurs via pro-apoptotic factors such as MCL1 (myeloid cell leukemia 1), RAIDD (RIP-Associated ICH1/CED3-Homologous Protein With Death Domain) and PIDD (p53-induced death domain) (Mahoney et al., 2011). This "one-two punch" tactic was validated using primary patient samples and can be useful in combating cancers that are otherwise individually resistant to UPR inhibition and oncolytic viruses.

Other means of sensitizing tumor cells toward proteotoxicity involve drugs that block proteasomal activity. Popular among these are the following – Bortezomib (BTZ) which leads to abrogation of NF-κB function and increases sensitivity to TNFα-related and caspase-mediated apoptosis; Nelfinavir which blocks cellular proteasomal activity by virtue of its own protease property and elicits pro-apoptotic effects marked by amassing of polyubiquitinated proteins (Schonthal, 2012); Eeyarestatin I (EerI) which is an agent that targets p97 (ATPase functioning in the transportation of ubiquitinated proteins) as a means to blocking ERAD (Wang, Q. et al., 2010). Most of these therapeutic agents and their modes of action in combating cancer have been summarized in Table 2.

Table 2 Therapeutic interventions in cancer targeting IRE1α and ERAD.

Agent	Mechanism	Effect	Functional partners	Reference
STF-083010, 3-Ethoxy-5,6-dibromosalicylaldehyde, 2-Hydroxy-1-naphthaldehyde, toyocamycin	IRE1α inhibition	Growth retardation owing to cell cycle arrest or induced apoptosis; reduction in invasiveness	Synergistic effects seen when used in combination or with gemcitabine/bortezomib	(Chien et al., 2014)
Rhabdovirus	Oncolytic virus therapy plus IRE1α inhibition	IRE1α inhibition preconditions cancer cells specifically and sensitizes them to apoptosis following rhabdovirus infection – called “one-two punch”	shRNA or small molecule inhibitors targeting IRE1a	(Mahoney et al., 2011)
Bortezomib	Blocks proteasome activity	Leads to abrogation of NF-κB function, increased sensitivity to apoptosis, thereby pushing tumor cells towards proteotoxicity	TNFα and caspase (mediating apoptosis)	(San Miguel et al., 2008)
Nelfinavir	Blocks proteasome activity	Exercises protease property in blocking cellular proteasome activity and inducing pro-apoptotic effects via amassing polyubiquitinated proteins	BiP/GRP78, CHOP and caspase activation induced	(Gupta et al., 2007)
Eeyarestatin I	Targeting p97 ATPase	Blocks ERAD by preventing de-ubiquitination of ERAD substrates and preferentially pushing cancer cells towards cytotoxicity		(Cross et al., 2009)
Irestatin	IRE1α RNase inhibitor	Blocks UPR by abrogating XBP1s transcription, thereby impairing proliferation or inhibiting tumor cell survival in oxygen-starvation conditions		(Perry et al., 2012)

Abbreviations – BiP: Binding immunoglobulin protein; CHOP: C/EBP homologous protein; ERAD: Endoplasmic reticulum associated degradation; IRE1α: Inositol requiring enzyme 1 alpha; GRP78: 78 kDa glucose-regulated protein; NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells; shRNA: short hairpin ribonucleic acid; TNFα: Tumor necrosis factor alpha; UPR: Unfolded protein response; XBP1s: Xbox binding protein 1 spliced.

A 2003 study by Lee et al. (Lee, A. H., Iwakoshi, Anderson, et al., 2003) using MM cell line demonstrated XBP1 to be an important therapeutic target in cancer as proteasome inhibition in MM cells suppresses IRE1α RNase activity and XBP1s generation, resulting in an increased apoptotic cell death (Lee, A. H., Iwakoshi, Anderson, et al., 2003). However, despite recent advances in therapy with proteasome inhibitors (e.g. bortezomib, carfilzomib), MM remains incurable due to the resistance to most drugs (Alexanian et al., 2012). A recent study by Leung-Hagesteijn and colleagues demonstrated that silencing of IRE1α or XBP1 in MM cell lines confers resistance to proteasome inhibitors (Leung-Hagesteijn et al., 2013). The loss of XBP1 in MM results in attenuation of Ig production and a decline in ER stress and ERAD function, which reduces ER stress hypersensitivity in MM cells and accounts for the resistance to proteasomal inhibitors. Moreover, a subset of Xbp1s-negativeMM cells lacks plasma cell features (Leung-Hagesteijn et al., 2013). These findings may explain the inability of proteasomal inhibitors in treating MM patients, while underscoring the importance of targeting both the committed plasma cells and the progenitors in therapeutic treatment to overcome drug resistance.

2.8 CONCLUSIONS

When normal cells experience stress due to the accumulation of misfolded proteins inside the ER, a series of adaptive mechanisms are initiated, namely, UPR and ERAD. These pathways lead to global translational attenuation, the induction of specific signaling cascades and clearance of misfolded proteins in the ER aimed at restoring ER homeostasis in the cell. However, in the context of cancer cells, insults from hypoxia, nutrient deprivation, genetic mutations, and enhanced metabolic needs, can often lead to a gross accumulation of faulty proteins in the ER. Therefore, the survival of tumor cells may become especially dependent, relative to normal cells, on the adaptation to stressful conditions via UPR and ERAD. This can potentially serve as the

Achilles heel of cancer cells and allow us to view UPR and ERAD components as lucrative therapeutic targets for cancer treatment. On the other hand, it is the same UPR activation that is wielded by the tumor cells in order to mount resistance to various anti-cancer drugs, thus becoming a double edged sword in tumor cell survival. Linking ER homeostasis to cancer progression may shed further light on the cell-intrinsic processes in tumor cells and allow us to potentially target them specifically over surrounding normal cells. However, most of the studies implicating various UPR and ERAD components in cancer pathogenesis are limited by their model system of study (cell culture based experiments) and small sample sizes (sampling of patient tissue). Hence our understanding of the mechanisms by which the ER quality control systems contribute to the function and survival of cancer cells still warrants further thoughtful investigations.

2.9 PERSPECTIVES

Although ER stress is thought to occur in many physiological and pathological conditions, what is lacking in most studies to date is the direct and accurate measurement of stress levels in the ER. Since cancer cells probably have an elevated protein turnover rate and are likely to accost this enhanced metabolic need by adaption via UPR and ERAD, it is quite possible that the basal levels of various components of ER chaperones are higher in tumor cells. Therefore, the induction or inhibition of these so-called "UPR markers" such as GRP78 and CHOP may not serve as a reliable indicator of ER stress. Thus, although many studies have suggested various possible roles of ER stress and IRE1α signaling pathways in tumor progression, we still lack a deep and accurate understanding of exactly what the status of ER stress and the contribution of UPR pathways in cancer pathogenesis are. Hence we would like to emphasize the urgent need to directly quantitate ER stress at the level of UPR sensors such as IRE1α and PERK activation.

Using the phos-tag-based approach, one can directly measure and quantitate the extent of IRE1α phosphorylation (Figure. 2.3), which we have shown to correlate with the stress level in the ER. As this method is very sensitive and can detect mild ER stress under physiological and pathological conditions (He et al., 2012; Sha et al., 2009; Sha et al., 2014; Sun et al., 2014; Xue et al., 2011; Yang, L. et al., 2010) it promises to provide insights into several outstanding questions, including when and to what extent UPR and IRE1α are activated during tumorigenesis, how small molecules affect IRE1α signaling and ER homeostasis in tumors, and how perturbation of ER homeostasis affects the survival and death of cancer cells in humans.

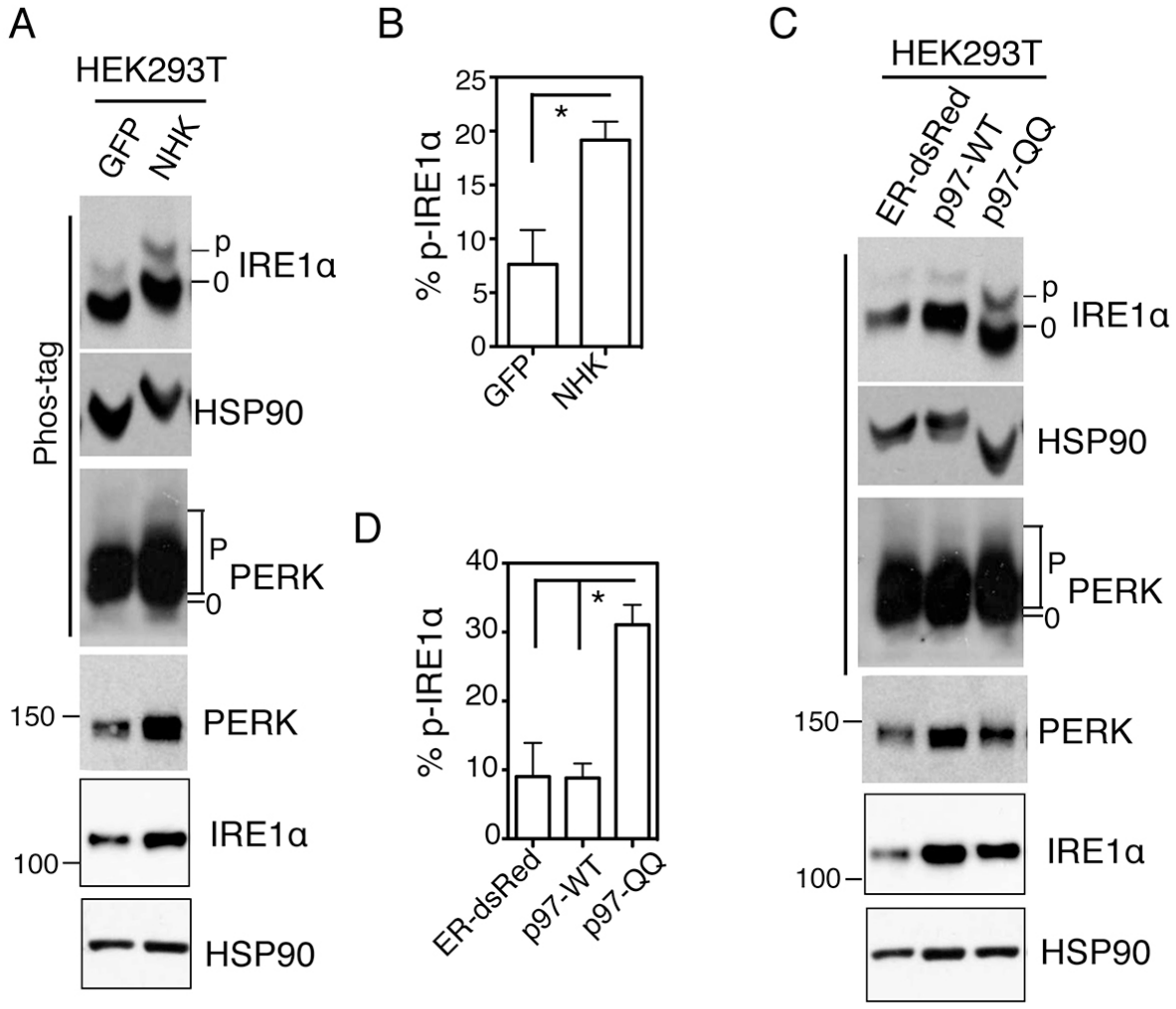

Figure 2. 3 Methods for quantitation of IRE1α activation and stress levels in the ER. (A and C)

Immunoblots of IRE1α and PERK in HEK293T cells transfected with the indicated plasmids for 24 h. NHK, the unfolded form of a1-antitrypsin; p97-QQ, dominant negative form of p97-WT. ER-dsRed and GFP, negative control plasmids. HSP90, a position and loading control. (B and D) Quantitation of percent of phosphorylated IRE1α in total IRE1α protein in Phos-tag gels shown in A, C. Values are mean ± SEM *, $P < 0.05$ using unpaired two-tailed Student's t-test. This data is taken from (Yang, L. et al., 2010)

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CHAPTER THREE: The Sel1L-Hrd1 Endoplasmic Reticulum-Associated Degradation complex manages a key checkpoint in B cell development[b]

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(* These authors contributed equally)

3.1. ABSTRACT

Endoplasmic reticulum (ER)-associated degradation (ERAD) is a principle mechanism that targets ER-associated proteins for proteasomal degradation. Using various knockout mouse models, we demonstrate a critical, cell-intrinsic role of the Sel1L-Hrd1 complex of ERAD in early B cell development. Mechanistically, we show that Sel1L-Hrd1 ERAD selectively recognizes and targets the pre-B cell receptor (pre-BCR), and surprisingly not the BCR, for proteasomal degradation in a BiP-dependent manner. Loss of ERAD in B cell precursors leads to the accumulation of pre-BCR complex, persistent pre-BCR signaling and a severe developmental block at the transition from the large to small pre-B cells. Thus, this study implicates the Sel1L-Hrd1 ERAD as a key regulator of B cell development and reveals the molecular machinery and mechanism underpinning the transient nature of pre-BCR signaling.

3.2. INTRODUCTION

Proteins destined for secretion or membrane localization are folded in the endoplasmic reticulum (ER). Protein folding is a complex, error-prone process that often results in the generation of irreparable misfolded, often toxic, protein by-products. A stringent quality control system is coupled to the protein folding process, ensuring that only correctly folded proteins exit the ER to proceed to their destination. ER-associated degradation (ERAD) is a fundamental quality control mechanism responsible for the recognition and translocation of terminally misfolded or unfolded proteins in the ER, targeting them for cytosolic proteasomal degradation (Olzmann et al., 2013). The use of artificial and disease-related mutant proteins as ERAD substrates has shed important insights into ERAD biology (Guerriero et al., 2012); however, very little is known about the physiological importance of ERAD and the nature of its endogenous substrates.

The best-characterized ERAD machinery in mammals is the highly conserved Sel1L-Hrd1 complex consisting of the E3 ubiquitin ligase Hrd1 and its adaptor protein Sel1L (Olzmann et al., 2013). While many biochemical insights into ERAD biology have been gained from the degradation of model substrates such as immunoglobulin (Ig) light and heavy chains, the current challenges are to identify the nature of its endogenous protein substrates and to delineate the physiological relevance and significance of ERAD in individual cell types in vivo. Using global inducible Sel1L-deficient mouse models, we recently demonstrated that Sel1L is a key component of mammalian Hrd1 ERAD machinery by stabilizing the Hrd1 protein and that the Sel1L-Hrd1 complex plays a critical role in the maintenance of ER homeostasis and animal survival (Sun et al., 2014). While Sel1L-deficient mice are embryonic lethal (Francisco et al., 2010), acute Sel1L deletion in adult mice results in pancreatic atrophy and premature lethality (Sun et al., 2014). In adipocytes, Sel1L deficiency causes intracellular retention of lipoprotein lipase, leading to hypertriglyceridemia (Sha et al., 2014). In the small intestine, epithelial Sel1L-Hrd1 ERAD is indispensable for the secretory function of Paneth cells and protects mice from pathogen-induced ileitis (Sun et al., 2016), whereas in the colon, it modulates the progression of experimental colitis in part via the degradation of IRE1α, the sensor of unfolded protein response (UPR) (Sun et al., 2015). However, the physiological importance of Sel1L-Hrd1 ERAD in cell differentiation and development has not been explored to date.

To this end, we chose to investigate B cell development as a model system because of its complicated yet well-defined and developmentally restricted expression of many growth factors and cell surface-associated receptors required for different stages of development. We reasoned that ERAD may help reshape the membrane receptor proteome in the B cell development. To reach the antibody-producing mature stage, B cell precursors undergo a stepwise differentiation

process in the bone marrow (BM) from pro-B cells, cycling large pre-B cells, resting small pre-B cells, and finally to immature B cells. This process requires a productive and sequential rearrangement of the genes encoding Ig heavy (IgH) and light (IgL) chains that form B cell receptor (BCR). Prior to IgL chain rearrangement, developing B cells express a set of B lineage-specific genes called λ5 (CD179b) and VpreB (CD179a), which form an IgL-chain-like structure known as the surrogate light chain (SLC) to pair with Igμ heavy chain to form the "pre-BCR" complex (Clark et al., 2014; Herzog et al., 2009; Lee, Y. K. et al., 1999; Pillai et al., 1987).

3.3. EXPERIMENTAL PROCEDURES

Mice

Sel1L$^{f/f}$ mice on the C57B6/L background have been recently described (Sun et al., 2014) and crossed with CD19 promoter-driven Cre (*CD19-Cre*) mice from Jackson laboratory (B6.129P2(C)-Cd19tm1(cre)Cgn/J) to generate *Sel1L*$^{f/f}$; *CD19-Cre*+ (*Sel1L*^{CD19}) and control *Sel1L*$^{f/f}$*; CD19-Cre-* (*Sel1L*$^{f/f}$) littermates at 1:1 ratio. *Sel1L*$^{f/+}$*; CD19-Cre*+ (*Sel1L*$^{CD19/+}$) were generated to determine the effect of Sel1L heterozygosity and CD19-Cre effect on B cell development. Adult 6-8-week-old mice were used in most studies. In addition, *Sel1L*^{CD19} mice were crossed with *Chop*$^{-/-}$ mice (B6.129S(Cg)-Ddit3 tm2.1Dron/J, JAX 005530) to generate *Sel1L*$^{flox/flox}$*; CD19Cre+; Chop*$^{-/-}$ double knockout (*Sel1L*^{CD19}*; Chop*$^{-/-}$) mice. Inducible Sel1L-deficient (*Sel1L*^{ERCre}) mice expressing estrogen-receptor-Cre (*ER*^{Cre}) fusion protein were previously reported (Sun et al., 2014). *Sel1L*$^{f/f}$*; ER*$^{Cre-}$ and S*el1L*$^{f/f}$*; ER*$^{Cre+}$ (*Sel1L*^{ERCre}) littermates were used in the study. In all the studies, the CD19-Cre allele were maintained as heterozygous. Mice were housed under specific pathogen-free conditions. Cohoused age- and gender-matched littermates were used in all in vivo experiments. All animals were sacrificed by cervical

dislocation and tissues were immediately harvested. Frozen tissues were stored at −80 °C. All animal procedures have been approved by the Cornell IACUC (#2007-0051).

Flow Cytometry, Intracullar Staining, BrdU Labeling and Antibodies

Flow cytometric analysis of peripheral immune cells was performed as we previously described (Ji et al., 2014; Ji et al., 2012). To isolate BM, tibia and femurs were isolated from mice and BM was flushed using cold PBS supplemented with 5 % FBS and 1 % penicillin/streptomycin. The RBCs in the BM suspension was lysed and resuspended in cold PBS with 5 % FBS and 1 % penicillin/streptomycin. Cell suspension were stained with either 1:100 or 1:200 fluorochrome- or biotin conjugated antibodies against CD4 (GK1.5), CD8 (YTS169), Gr-1 (RB6-8C5), CD11b (M1/70), CD45 (30-F11), CD19 (6D5), CD138 (281-2), CD2 (RM2-5), IgM (RMM-1), IgD (11-26c.2a), CD5 (53-7.3), λ5 (C-16), CD43 (1B11), VpreB (R3), Pre-BCR (SL156), strepavidin-PerCP and isotype control antibodies (BioLegend or BD Biosciences). Annexin V, propidium iodide and 7-AAD were purchased from BioLegend and used per manufacturer's protocol. Secondary antibodies such as anti-IgG FITC and anti-Igμ PE were from Jackson Immunoresearch. For intracellular staining, cells were fixed and permeabilized by BD Cytofix/Cytoperm Fixation/Permeabilization Kit per manufacturer's protocol. For BrdU labeling, mice were injected intraperitoneally with BrdU (Sigma, 0.6 mg/10 g of body weight) at 2 hours prior to sacrifice. BM cells were collected, labeled with cell surface antibodies, and then fixed in cold 70 % ethanol at −20 °C overnight. The samples were washed and digested with Dnase I (Roche, 50 U/ml in 4.2 Mm MgCl2 and 150 mM NaCl) at room temperature for 30 min. The rest of the procedures were performed as the regular flow cytometric analysis using BrdU-FITC (PRB-1, BD Biosciences). Samples were analyzed using BD LSR cell analyzer at the Flow

Cytometry Core Facility at Cornell University. Data were analyzed using the CellQuest software (BD Biosciences) and Flowjo (Flowjo.com).

Western Blot and Antibodies

Preparation of whole cell lysates, endoH treatment and Western blots were performed as previously described (Sha et al., 2014; Sun et al., 2015). Antibodies used in this study were: Igμ-peroxidase (goat, 1:5,000) from Sigma; HSP90 (rabbit, 1:6,000), BiP (goat, 1:1,000) and α-Tubulin (mouse, 1:2000) from Santa Cruz; Sel1L (rabbit, 1:2,000) and OS9 (rabbit, 1:10,000) from Abcam; phospho-Syk and Syk (rabbit, 1:1000) from Cell Signaling; and Calnexin (rabbit, 1:8,000) from Assay Design. Hrd1-specific antibody (rabbit, 1:200) was from Dr. Richard Wojcikiewicz at SUNY Upstate Medical University (Pearce et al., 2007). Antibodies for Bag6 (rabbit, 1:10,000) and H2A (rabbit, 1:10,000) were a kind gift from Dr. Yihong Ye (NIDDK). Band density was quantitated using the Image Lab software on the ChemiDOC XRS+ system (Bio-Rad). Protein levels were normalized to HSP90 and are presented as mean ± SEM unless otherwise specified.

Fluorescence-activated cell sorting (FACS)

Tibia and femurs from 6-week-old Sel1LCD19 and *Sel1L*$^{f/f}$ mice were flushed with cold PBS (5 % FBS, 1 % penicillin/streptomycin) using a 10 ml syringe and 27 G1/2 needle (BD) to obtain single cell suspension. Red blood cells were lysed using RBC lysis buffer (Biolegend) and washed with cold PBS and filtered through 70 μm cell strainer. Cells were incubated with 1:100 dilution of PE anti-mouse CD43, FITC anti-mouse IgM, APC-Cy7 anti-mouse B220 and APC anti-mouse CD2 in cold PBS with 5 % FBS for 40 min at 4 °C. Cells were washed and resuspended in cold PBS and sorted using BD FACS Aria at Flow Cytometry Core Facility at Cornell University. Pro-, large pre-, small pre-, immature and mature B were gated as

$B220^{low}/IgM^{-}/CD43^{high}/CD2^{-}$, $B220^{low}/IgM^{-}/CD43^{low}/CD2^{-}$, $B220^{low}/IgM^{-}/CD2^{+}$, $B220^{low}/IgM^{+}$ and $B220^{high}/IgM^{+}$ cells, respectively.

Isolation of peritoneal lymphocytes

Using scissors and forceps, the outer skin of the peritoneum of an anesthetized mouse was gently pulled back to expose the inner skin lining the peritoneal cavity. Using 27 G needle, 5 ml of ice cold PBS (5 % FBS) was injected into the peritoneal cavity followed by gentle massaging to dislodge any attached cells. The fluid in the peritoneum was collected using a 25 G needle attached to a 5 ml syringe. The collected cell suspension was centrifuged at 1500 rpm for 5 min.

Magnetic-activated cell sorting (MACS) and Plasma cell differentiation

Splenocytes were prepared as described above. Isolated splenocytes were stained with biotin-conjugated anti-mouse CD19 antibody (1:100, BioLegend) for 40 min at 4 °C. Cells were washed and incubated with Streptavidin particle plus (1:6, BD Biosciences) for 30 min at 4 °C. $CD19^{+}$ B cells were purified using Cell Separation Magnet (BD Biosciences) per manufacturer's protocol. Purified B cells were diluted in RPMI-1640 medium supplemented with 10 % heat-inactivated FBS, 50 μM β- mercaptoethanol (Invitrogen), 1 % penicillin/streptomycin (Cellgro), and 2 mM L-glutamine (Cellgro). To induce plasma cell differentiation, purified B cells were stimulated with 20 μg/ml LPS and 20 ng/ml murine IL-4 (PeproTech) for 72 hours. Cell lines Pre-B cell line 70Z/3 (ATCC) was cultured in complete RPMI supplemented with 10 % heat-inactivated FBS (Life Technologies), 50 μM β-mercaptoethanol (Invitrogen), 100 U/ml penicillin and 100 μg/ml streptomycin (Cellgro), 2 mM L-glutamine (Cellgro), 1 mM sodium pyruvate and 10 mM HEPES.

Generation of Sel1L knockdown cells

Sel1L RNAi knockdown targeting sequences #7 (gatccccGGTTACACTGTGGCTAGAAttcaagagaTTCTAGCCACAGTGTAACCtttttc, tcgagaaaaaGGTTACACTGTGGCTAGAAtctcttgaaTTCTAGCCACAGTGTAACCggg) was cloned into pSuper/retro vector (H1 promoter) as previously described (Sha et al., 2009). Control RNAi were against the firefly luciferase.

Design of Hrd1 single guide RNA (sgRNA) expression Vectors

We used the lentiviral CRISPR-Cas9 vector from the Zhang lab, plentiCRISPRv2, which expresses the sgRNA, Cas9 protein, and puromycin resistance gene from one vector. The HRD1 sgRNA was designed using the Zhang lab software available at http://crispr.mit.edu. According to Zhang lab protocols (http://www.genome-engineering.org/crispr/?page_id=23), DNA oligonucleotides for the sgRNA and reverse complement sequence plus adapters needed for ligation were synthesized and cloned into the plentiCRISPRv2 vector (HRD1: forward, 5'-CACCGATCCATGCGGCATGTCGGGC-3', reverse, 5' AAACGCCCGACATGCCGCATGGATC-3'). Empty p-lentiCRISPRv2 plasmid was used as control vector.

Generation of CRISPR-based knockout cell lines

To generate lentiviral supernatant, HEK293T cells were co-transfected with 2.3 μg of sgRNA plasmid or empty plentiCRISPRv2 plasmid, 1.5 μg of pRRE(gag-pol), 0.8 μg of pVSV-G and 0.57 μg of pREV in the presence of the Lipofectamine 2000 (Life Technologies), per manufacturer's instruction. After 12 h of incubation, the medium was changed to fresh virus collecting medium (DMEM supplemented with 10 % heat-inactivated FBS and 1 % penicillin/streptomycin). 24 hours later, the viral supernatant was harvested by centrifugation and

ready to be used or stored in -80 °C. To generate knockout cells, 70Z/3 cells were infected with HRD1 CRISPR-Cas9 or empty CRISPR-Cas9 lentiviruses. 24 hours post infection, cells were selected with 2 μg/ml puromycin (Invitrogen) for 1 week. The knockout efficiency was assessed by Western blot analysis.

Retroviral transduction

The cDNAs encoding mouse WT or JC λ5 (Fang et al., 2001) were subcloned into the murine retroviral vector MiG where GFP was linked to the cDNA of interest via an internal ribosome entry site. JC λ5, the truncated form of λ5, was generated by deletion of its non-Ig region and replaced with the leader sequence of conventional light chain λ1 (Guloglu et al., 2006). Retroviruses were made by co-transfection of HEK293 cells with MiG plasmids plus pψECO-encoding ecotropic helper functions. Viral supernatants and polybrene (5 μg/ml) were added to cells and incubated for 2 h, followed by centrifugation at 2500 rpm at 37 °C for 1 h. 2 weeks after infection, cells were sorted using BD FACS Aria for stably-transfected GFP+ cells for further analysis.

Crosslink of pre-BCR

Pre-BCR signaling was activated as previously reported (Guo et al., 2000). 70Z/3 cells were directly stimulated with 10 μg/ml of anti-μ antibody (Jackson ImmunoResearch) at 37 ºC for the indicated times. Stimulation was stopped by the addition of ice-cold PBS. Cell were pelleted by centrifugation and stored in -80 °C for Western blot analysis.

In vitro IL-7 stimulation

This experiment was performed as previously described (Flemming et al., 2003). For quantitation of the absolute number, MACS sorted BM $CD19^+$ B cells were cultured in RPMI-1640 medium supplemented with 10 % heat-inactivated FBS, 50 μM β-mercaptoethanol

(Invitrogen), 1 % penicillin/streptomycin (Cellgro), and 2 mM L-glutamine (Cellgro). Purified recombinant IL-7 (PeproTech) was used at 0.1–10 ng/ml for 4 days. Viable cells were counted and normalized to the day 0 cell number. For BrdU incorporation, FACS sorted BM pro-/large pre- B cells were treated with IL-7 (1 ng/ml) for 2 days, and BrdU (10 μM) was added for 4 hours followed by Flow Cytometric analysis as descried above.

Immunoprecipitation (IP) using primary antibodies

Cells were incubated with 20 mM Nethylmaleimide (NEM) in PBS for 10 min on ice, snap frozen and lysed in lysis buffer (150 mM NaCl, 1 mM EDTA, 50 mM Tris HCl, pH 7.5, protease inhibitor cocktail (Sigma), 10 mM NEM) supplemented with either 1 % Triton X-100 or 1 % Nonidet P-40 (NP-40) and incubated on ice for 15 min. Cells were centrifuged at 12,000 g at 4 °C for 10 min, supernatants were collected and protein concentration was measured using the Bradford assay. The protein lysates were incubated with Igμ-agarose beads (Sigma) for 16 h at 4 °C with gentle rocking, followed by five washes with IP lysis buffer. Immunocomplexes were eluted by boiling for 5 min in 5X SDS sample buffer followed by SDS-PAGE and Western blot analysis.

Sucrose gradient sedimentation analysis

Confluent WT and Sel1L KD 70z/3 cells in two 10 cm plates were either mock-treated or treated with 20 μg/ml lipopolysaccharide (LPS) for 18 hours, harvested and lysed in 0.5 ml of 1 % NP40 lysis buffer as described in Immunoprecipitation. Extracts were centrifuged through 20~50 % sucrose gradients in 150 mM NaCl, 1 mM EDTA, 50 mM Tris HCl pH 7.5 and protease inhibitor, which were prepared freshly by layering higher to lower density sucrose fractions in 5 % increments in poly-allomer tubes of 11x3x60 mm (Beckman Coulter, Brea, CA, USA). Extracts were centrifuged at 58,000 rpm for 14.5 hours at 4 °C using an SW 60 Ti rotor

(Beckman Coulter). Each 4 ml gradient was divided evenly into 8 fractions (500 µl each), and aliquots of fractions 2-8 were subjected to Western blot analyses under denaturing or non-denaturing conditions.

SDS-PAGE

In non-reducing SDS-PAGE, cells were lysed in 1 % NP-40 lysis buffer without DTT. The cell lysates, diluted immunoprecipitates and sucrose gradient sedimentations were prepared in non-denaturing sample buffer with a final concentration of 20 mM pH 6.8 Tris-Cl, 0.8 % SDS, 0.04 % bromophenol blue and 4 % glycerol. Samples were heated for 10 min at 50 °C followed by Western blot analysis. For denaturing SDS-PAGE, the lysates were prepared in 5x denaturing sample buffer (50 mM Tris HCl pH 6.8, 0.2 % SDS, 10 % glycerol, 0.28 M β-mercaptoethanol and 0.01 % bromophenyl blue) and boiled for 5 min prior to be separated on a SDS-PAGE gel. In vitro drug treatment Cycloheximide (Millipore, Billerica, MA) was dissolved in ethanol and used at 50 µg/ml. MG132 (Millipore, Billerica, MA) was dissolved in DMSO and used at 25 µM. LPS was purchased from Sigma and was dissolved in water and used at 20 µg/ml.

BiP depletion

SubAB and its catalytic inactive mutant form $SubA_{A272}B$ were used as previously described (Paton et al., 2006). 70Z/3 cells treated with either 0.5 µg/ml $SubA_{A272}B$ or SubAB WT for 45 min were lysed in lysis buffer (150 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl pH 8.0, protease inhibitor and protein phosphatase inhibitor and 10 mM NEM) supplemented with 1 % NP-40. A total of ~3 mg protein lysates was pre-cleared with agarose A (Invitrogen) and then incubated with Igµ-agarose overnight at 4 °C with gentle rocking. Igµ-precipitated complexes were washed and eluted by boiling for 5 min in SDS sample buffer, and subjected to Western blot analyses. To measure Igµ decay following BiP depletion, 70Z/3 cells were treated by 50

μg/ml cycloheximide together with either 0.5 μg/ml $SubA_{A272}B$ or SubAB WT for 0, 30, 60 and 90 min. Cells were subsequently lysed in lysis buffer supplemented with 1 % NP-40. Protein levels of Igμ at different time points were measured by Western blot.

Pulse labeling

For each time point, 4 x 10^6 70z/3 cells were used. Cells were cultured in cysteine and methionine-free medium (Invitrogen, 21013024) at 37 °C for 15 min and metabolically labeled with 50 μCi (35S)-cysteine and methionine (EasyTag, PerkinElmer) at 37 °C for 10 min. The pulse was stopped by the addition of 5 volumes of cold chase medium (pre-B cell culture medium supplemented with 5 mM L-methionine (Sigma, M5308) and 5 mM L-cystine (Sigma, C7352). Then cells were quickly spin down and cultured in chase medium at 37 °C for the indicated times. Cells were spin down, snap-frozen and lysed in 0.5 % NP40 lysis buffer with NEM (20 mM). Lysates were subjected to immunoprecipitation with Igμ-agarose beads (Sigma). Immunoprecipitates were separated on a 7 % SDS-PAGE gel, which was subsequently incubated with the neutralizing buffer (30 % (v/v) methanol in PBS) for 10 min followed by the enhancer buffer (1.5 M sodium salicylate (Sigma) in 30 % (v/v) methanol) for 10 min. The gel was then dried at 80 °C for 2 hours using a gel drier (Model 583, Bio-Rad) and then subjected to autoradiography with X-film (Kodak).

Limited proteolysis

WT and Sel1L KD 70z/3 cells were harvested and lysed in lysis buffer containing 50 mM HEPES (pH=7.5), 150 mM NaCl and 1 % Triton-X 100 on ice for 15 min. Aliquots of the lysates were mixed with varying concentrations of trypsin and incubated on ice for 30 min. Reaction was quenched by the addition of 5x SDS sample buffer and immediately heated at 95 ºC for 10 min followed by Western blot analysis. NP-40 solubility assay WT and Sel1L KD 70z/3 cells

were incubated in the absence or presence of 20 μg/ml LPS for 18 hours, harvested, and incubated in NP-40 lysis buffer (50 mM Tris·HCl pH 8.0, 0.5 % NP-40, 150 mM NaCl, 5 mM MgCl2) supplemented with protease inhibitor (Sigma). The lysate was centrifuged at 12,000 g for 10 min and the supernatant was collected as NP-40S fraction. The pellet was then resuspended in 1x SDS sample buffer with the volume normalized to initial cell weight, heated at 95 °C for 30 min and collected as the NP-40P fraction. The NP-40S and NP-40P fractions were subsequently analyzed by Western blot.

Immunofluorescent staining

For pre-B cell line staining, OP9 cells were plated on poly-L-Lysine-coated coverslips in 24-well plates and incubated for 18 hours at 37 °C. OP9 cells were washed with PBS and co-incubated with either WT or Hrd1$^{-/-}$ 70/z3 cells in RPMI supplemented with 10 % heat-inactivated FBS (Sigma), 50 μM β-mercaptoethanol (Invitrogen), 100 U/ml penicillin/streptomycin (Cellgro), and 2 mM L-glutamine for additional 3 hours. Cells were washed with PBS, fixed with 4 % paraformaldehyde for 20 min and permeabilized with 1 % Triton X-100 in PBS for 5 min. Cells were blocked with 5 % normal donkey serum in 0.1 % Triton X-100/PBS, then labeled with PE-anti-Igμ (1:100, Goat, Jackson ImmunoResearch) and a polyclonal rabbit anti-rodent BiP antiserum (Lee, Y. K. et al., 1999) (1:500, from Dr. Linda Hendershot at St. Jude Children's Research Hospital) for 18 hours at 4 °C. After two washes, cells were incubated with donkey anti-rabbit AF488 for 1 hour at room temperature, washed and mounted with Vectashield Mounting Medium with DAPI (Vector Laboratories, Inc.). For spleen staining, spleens were collected from *Sel1L$^{f/f}$* and *Sel1L^{CD19}* mice and fixed in 10 % neutral buffered formalin overnight at 4 °C, followed by washing in 15 % sucrose overnight at 4 °C. Spleens were embedded in optical cutting temperature (O.C.T.) compound (Tissue-Tek) and

sectioned at 8 μm thickness with a cryostat and mounted on the Superfrost slides (FISHER). The sections were washed twice with PBS and blocked in PBS with 5 % normal donkey serum and 0.5 % Tween 20 at room temperature for 20 min. The tissue sections were stained overnight with PE-anti-mouse B220 (BioLegend) at 4 °C. After two washes, the sections were washed and mounted with Prolong Gold Antifade Reagent with DAPI (Invitrogen). Fluorescent microscopic images were taken under a Zeiss LSM710 Confocal Microscope at Cornell Biotechnology Resource Center Imaging Facility.

RNA Extraction, Quantitative (Q), and Reverse-transcription (RT)-PCR

RNA extraction from cells and murine tissues, and Q-PCR were carried out as previously described using Trizol (Invitrogen) (Sha et al., 2014). Q-PCR data collected on the Roche LightCycler 480 were normalized to ribosomal *l32* gene in the corresponding sample.

Primers

Genotyping:

Sel1L (CTGACTGAGGAAGGGTCTC; GCTAAAAACATTACAAAGGGGCA) (325 bp as wild-type allele, 286 bp as floxed allele)

CD19-CRE (GCGGTCTGGCAGTAAAAACTATC; GTG AAACAGCATTGCTGTCACTT)

CD19-WT (CCTCTCCCTGTCTCCTTCCT; TGGTCTGAGACATTGACAATCA)

$Chop^{-/-}$ (AACGCCAGGGTTTTCCCAGTCA)

$Chop^{WT}$ (GCAGGGTCAAGAGTAGTG)

RT-PCR/Q-PCR:

VpreB1 (CGTCTGTCCTGCTCATGCTGC; ACGGCACAGTAATACACAGCC),

Igμ (TGTGTGTACTGTGACTCACAGGGA; AGGGAGACATTGTACAGTGTGGGT),

λ5 (GTTGGGTCTAGTGGATGGTGT; TTGGTCTGTTTGGAGGGTTGG),

L32 (GAGCAACAAGAAAACCAAGCA; TGCACACAAGCCATCTACTCA),

BiP (TGTGGTACCCACCAAGAAGTC, TTCAGCTGTCACTCGGAGAAT),

Os9 (GCTCACGCCTACTACCTCAAA, GCCAGACAAGTCTCTGTGACG),

Chop (TATCTCATCCCCAGGAAACG, GGGCACTGACCACTCTGTTT),

Erdj4 (CTTAGGTGTGCCAAAGTCTGC, GGCATCCGAGAGTGTTTCATA),

Edem1 (GGGACCAAGAGGAAAAGTTTG, GAGGTGAGCAGGTCAAATCAA),

The PCR conditions are: 94 °C for 5 min, 94 °C for 1 min, 58 °C for 20 sec and 72 °C for 30 sec repeated for 25 to 30 cycles according to individual template, followed by 70 °C for 10 min.

Statistical Analysis

Results are expressed as mean ± SEM. Comparisons between groups were made by unpaired two-tailed Student's t test, except by one-way ANOVA with Tukey post-test in Figure 3.13. All experiments were repeated at least twice or performed with independent samples.

3.4. RESULTS

3.4.1. Reduced peripheral B cells in B cell-specific Sel1L-deficient mice.

Expression of *Sel1L* and *Hrd1* genes was induced around the pre-B cell stage in developing lymphocytes, preceding that of ER chaperones BiP and Edem1 (Figure 3.1.A), suggesting a possible role of Sel1L-Hrd1 ERAD in early lymphopoiesis. To investigate whether Sel1L-Hrd1 ERAD plays a role in B cell development, we crossed $Sel1L^{flox/flox}$ ($Sel1L^{f/f}$) mice on the C57BL/6 background (Figure 3.2A) with CD19-Cre mice to generate B cell-specific Sel1L-deficient mice ($Sel1L^{CD19}$). The CD19 promoter is active specifically in B cells and throughout B cell development from the pro-B cell stage (Zhou, L. J. et al., 1991). The $Sel1L^{CD19}$ mice and their control $Sel1L^{f/f}$ littermates were born in a normal Mendelian ratio (not shown) and appeared healthy with no obvious growth defects (Figure 3.1B). Immunoblot analysis confirmed the

deletion of the Sel1L protein and reduction of Hrd1 protein in the BM-derived B cells (Figure 3.1C). Spleen weights were significantly reduced in *Sel1L*^{CD19} mice vs. controls (Figure 3.1D and Figure 3.2B). The percentage of peripheral B cells in spleen, blood, and lymph nodes was reduced by 60-75 % (Figure 3.1E) and the absolute numbers of splenic B cells were >75 % lower in *Sel1L*^{CD19} mice compared to controls, while other cell types, including CD4^{+}, CD8^{+} T and myeloid cells were not affected (Figure 3.1F-G and Figure 3.2C). The reduction in B cells was not due to elevated cell death (Figure 3.2D). Similar to the conventional BM B cells (also known as B-2 cells), the percentage of peritoneal CD5^{+} B-1 cells was decreased in *Sel1L*^{CD19} mice vs. *Sel1L*$^{f/f}$ controls (Figure 3.2E). Of note, there were ~20 % residual peripheral B cells in the *Sel1L*^{CD19} mice, which is in line with the fact that CD19-Cre-mediated deletion efficacy is approximately 75-80 % in BM (Rickert et al., 1997). Hence, we concluded that Sel1L in B cells is required for B cell development.

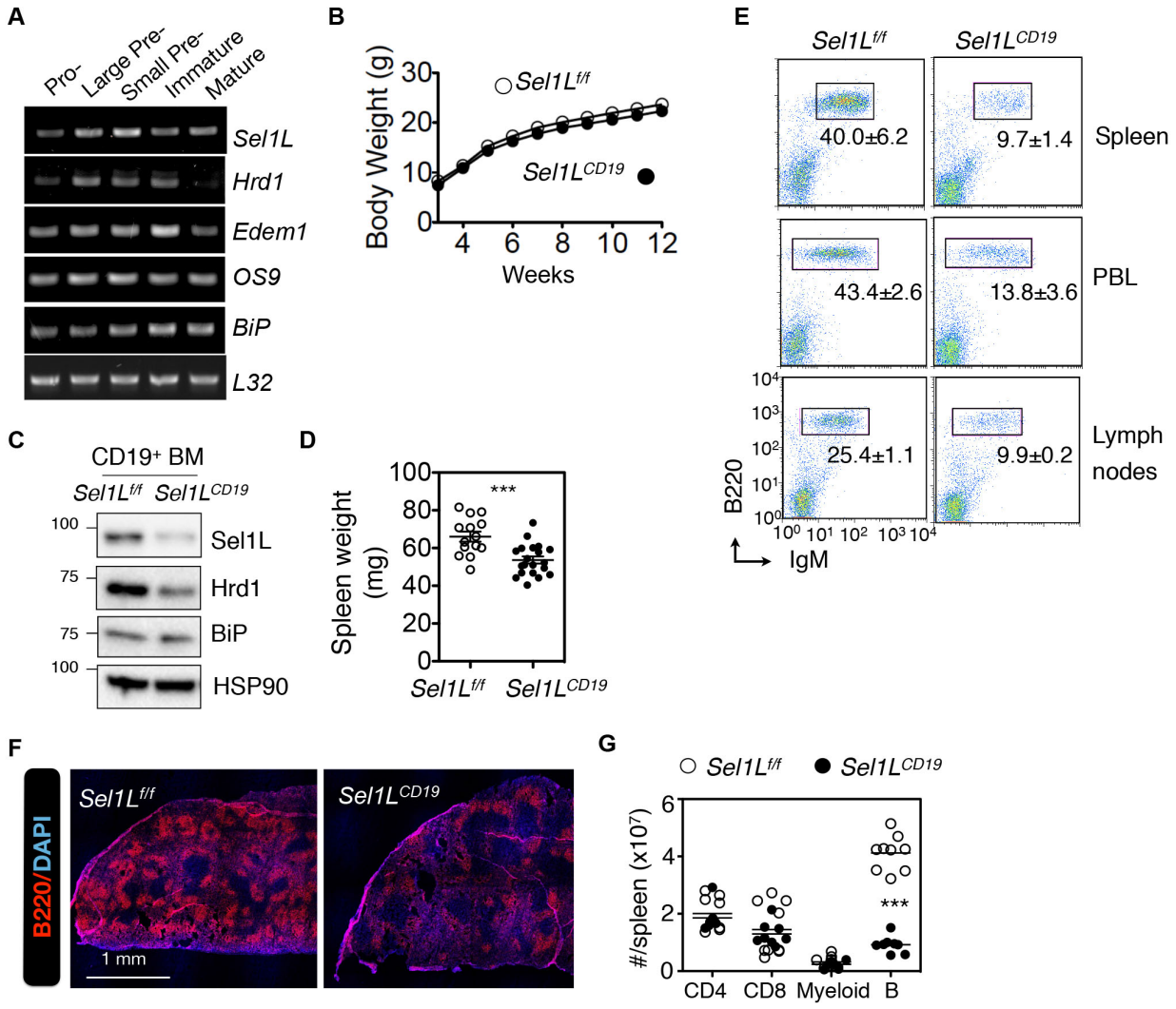

Figure 3. 1 Reduced peripheral B lymphocyte cellularity in *Sel1L*^{CD19} mice.

(A) RT-PCR analysis of ERAD genes in B cell subpopulations from bone marrows (BM) of C57BL/6 mice. (B) Growth curves for male littermates. (C) Immunoblots of Sel1L, Hrd1, and BiP in CD19^{+} BM cells from *Sel1L*$^{f/f}$ and *Sel1L*^{CD19} mice. (D) Spleen mass. (E) Flow cytometric analysis of mature B cells (B220^{+}/IgM^{+}) in spleen, peripheral blood (PBL), and lymph nodes. (F) Representative confocal microscopic images of B cells (red) in the spleen. (G) Absolute numbers of splenic CD4^{+}, CD8^{+} T, myeloid, and mature B cells. Data are representative of two (A,C,F) or three (E) independent experiments. n=8 C57BL/6 mice (A), 18 mice each (B), 3 mice each (C),

15 *Sel1L*$^{f/f}$ mice and 19 *Sel1L*^{CD19} mice (D), 3 mice each (F), and 8-9 mice each (E,G). Values shown as mean ± s.e.m.; N.S., not significant; *P<0.05, ***P<0.001 by two-tailed Student's t-test

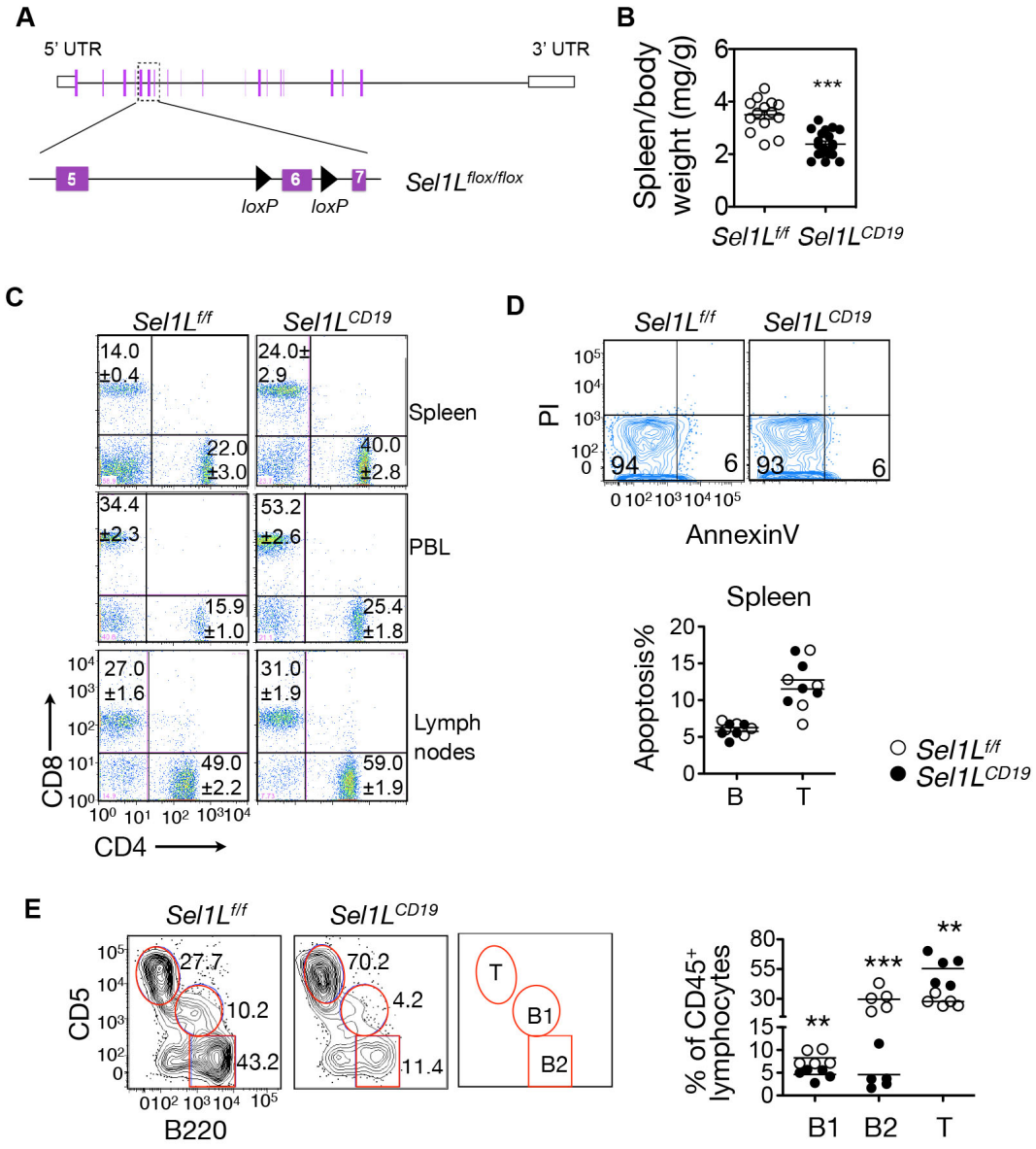

Figure 3. 2 Generation of *Sel1L*^{CD19} mice and the reduction of mature B cells.

(A) Schematic diagram of *Sel1L*$^{f/f}$ mice on the C57BL/6N background with the exon 6 flanked by two loxP sites. (B) Spleen mass normalized to body weight of *Sel1L*$^{f/f}$ and *Sel1L*^{CD19} littermates. (C) Flow cytometric analysis of lymphocytes from peripheral blood (PBL), spleen and lymph nodes of *Sel1L*$^{f/f}$ and *Sel1L*^{CD19} mice with quantitation shown in each quadrant. (D) Flow cytometric analysis of apoptotic splenocytes from *Sel1L*$^{f/f}$ and *Sel1L*^{CD19} mice using

Annexin-V and propidium iodide staining (PI), with quantitation shown on the right. (E) Flow cytometric analysis of lymphocytes from peritoneal cavity of *Sel1L*$^{f/f}$ and *Sel1L*^{CD19} mice with quantitation shown on the right. Data are representative of two independent experiments in (B-E), n= 14 *Sel1L*$^{f/f}$ and 20 *Sel1L*^{CD19} mice (B), 9 *Sel1L*$^{f/f}$ and 8 *Sel1L*^{CD19} mice (C), 5 mice each (D-E). Values shown as mean ± s.e.m.; **P< 0.01, ***P<0.001 by two-tailed Student's t-test.

3.4.2. Developmental defect at the transition from the large to the small pre-B cell stage in *Sel1L^{CD19}* mice.

In adult BM, B lymphocytes develop from pluripotent hematopoietic stem cells through an ordered differentiation process, where IgH and IgL genes are sequentially rearranged and the productive rearrangement of each establishes two key checkpoints that test the functionality of each Ig component (Figure 3.3A). At the first checkpoint, the IgH (or Igμ) must form a signaling competent pre- BCR with the SLC; at the second checkpoint, a functional BCR must be formed between IgH and a nascent IgL after SLC expression is extinguished (Figure 3.3A). Consistent with the reduced peripheral B cellularity in *Sel1L^{CD19}* mice, B220^{high}/IgM^{+} recirculating mature and B220^{low}/IgM^{+} immature B cells were significantly decreased in the BM of *Sel1L^{CD19}* mice vs. *Sel1L$^{f/f}$* controls (Figure 3.4A-B). On the other hand, the percentage of B220^{low} CD43^{hi} pro-B cells was slightly elevated in *Sel1L^{CD19}* mice while that of pre-B cells was comparable to controls (Figure 3.4A-B). These data point to a developmental defect at the pro-/pre-B cell stage in *Sel1L^{CD19}* mice. Pro-/pre-B cells can be further classified based on the expression of SLC and the differentiation marker CD2 (Figure 3.3A). Within the pro-/pre- B cell compartment of the BM, SLC^{+}/CD2^{-} pre-B I and large pre-B (II) cells were twice as many, whereas SLC^{-}/CD2^{+} small pre-B cells were reduced by 50 % in the *Sel1L^{CD19}* mice compared to *Sel1L$^{f/f}$* littermates (Figure 3.3C and Figure 3.4B). Cell death did not account for the changes in B cell populations of *Sel1L^{CD19}*mice as apoptotic rates for various precursor populations were comparable between the cohorts **(**Figure 3.3C). Both Sel1L heterozygosity and CD19-Cre expression itself as in *Sel1L$^{f/+}$; CD19-Cre* (*Sel1L$^{CD19/+}$*) mice had no effect on B cell development (Figure 3.3D). Thus, B cell-specific Sel1L deficiency results in a severe developmental defect at the transition from the large to small pre-B cells.

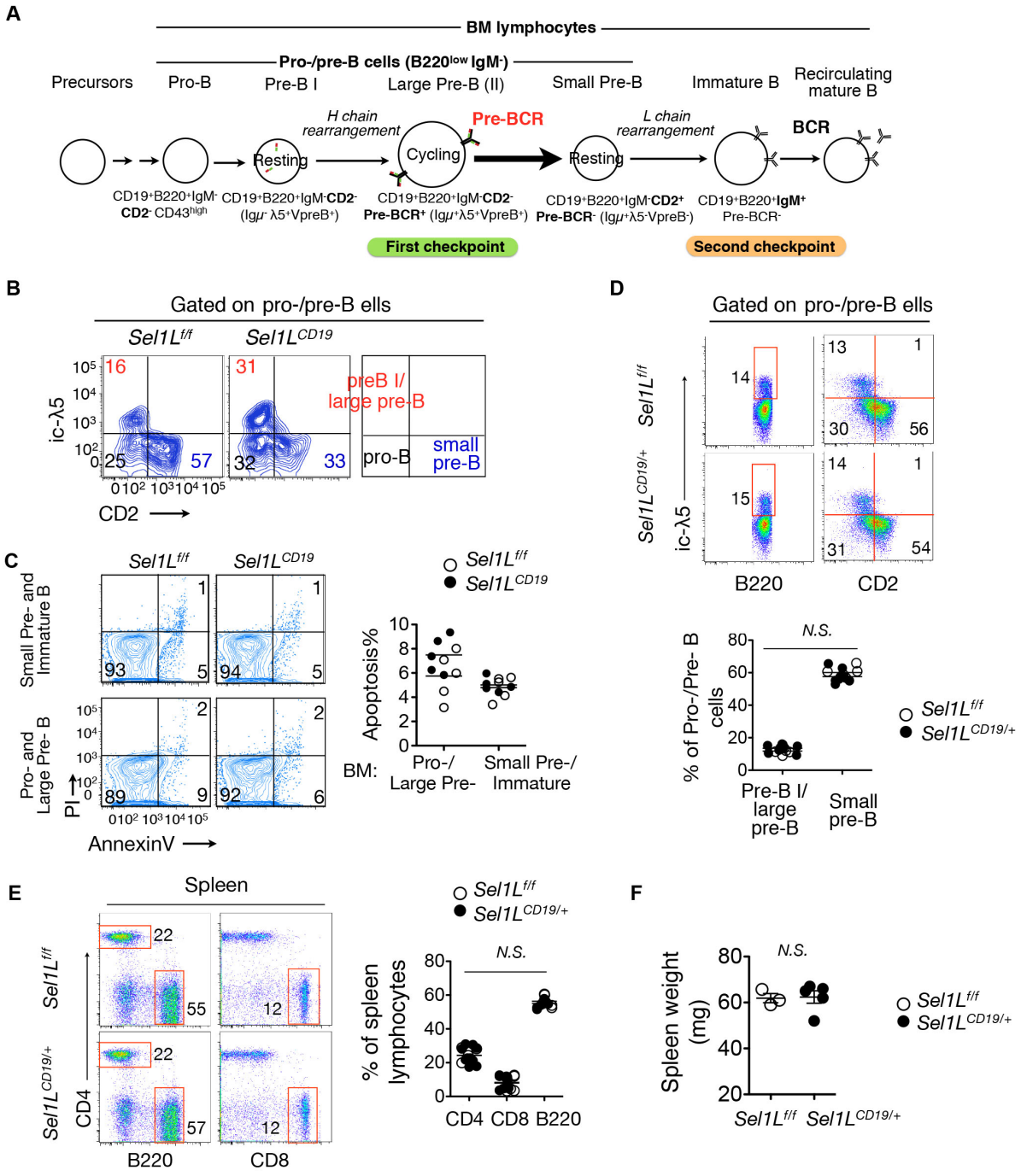

Figure 3. 3 B cell developmental defects in the absence of Sel1L.

(A) Schematic of B cell development in bone marrow (BM). Two checkpoints are highlighted in green and orange (B) Flow cytometric analysis of various cell populations in BM of *Sel1L*$^{f/f}$ and

Sel1L^{CD19} mice stained for CD2 and intracellular λ5 (ic-λ5), gated on B220^{low}IgM^{-} pro-/pre-B cells, with quantitation shown in Figure 3.3C. (C) Flow cytometric analysis of apoptotic BM progenitors from *Sel1L*$^{f/f}$ and *Sel1L*^{CD19} mice using annexin-V and propidium iodide staining (PI) with quantitation on the right. (D-E) Flow cytometric analysis of (D) pro-/pre-B BM cells, stained for B220 and intracellular (ic) λ5, and (E) of splenocytes of *Sel1L*$^{f/f}$ and *Sel1L*$^{CD19/+}$ mice. (F) Spleen weights of *Sel1L*$^{f/f}$ and *Sel1L*$^{CD19/+}$ mice. Data are representative of two independent experiments.

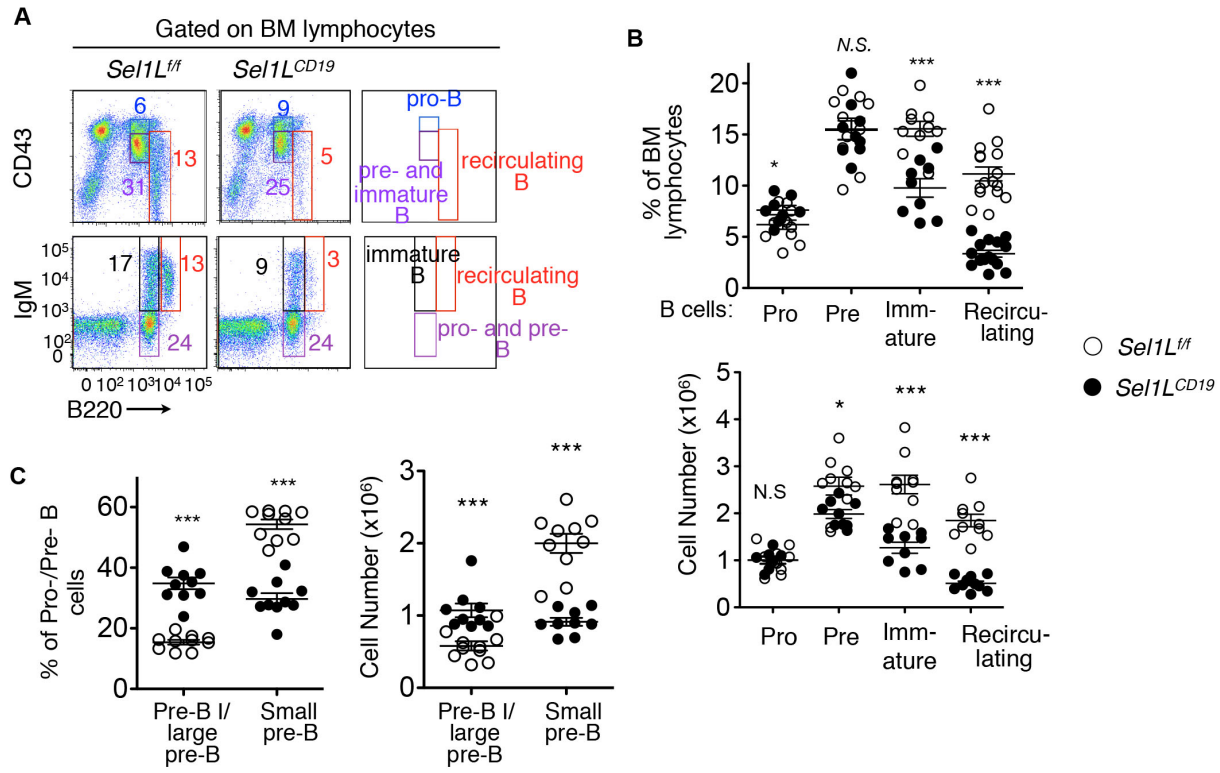

Figure 3. 4 Developmental blockade at the transition from the large to small pre-B cells in *Sel1L^{CD19}* mice.

(A) Flow cytometric analysis of B220-CD43 (upper) and B220-IgM (lower) in BM cells from *Sel1L$^{f/f}$* and *Sel1L^{CD19}* mice, with quantitation in percentage and absolute cell number shown in (B). (C) Quantitation of flow cytometric analysis of various pro-/pre-B cell populations in BM of *Sel1L$^{f/f}$* and *Sel1L^{CD19}* mice. Data are representative of three independent experiments with n= 9-10 mice each. Values shown as mean ± s.e.m.; *P<0.05, ***P<0.001 by two-tailed Student's t-test.

3.4.3. The developmental defect of *Sel1L*^{CD19} mice is independent of Chop.

We hypothesized that impairment of an ER quality control system like ERAD could activate a stress response, and that might account for the developmental block observed. Indeed, a subset of ER stress-responsive genes such as BiP and Chop were moderately elevated in the large pre-B cells of *Sel1L*^{CD19} mice (Figure 3.5A). To determine more directly the contribution of ER stress in B cell development of *Sel1L*^{CD19} mice, we examined the role of Chop in *Sel1L*^{CD19} mice by generating *Sel1L*^{CD19}; *Chop*$^{-/-}$ mice. However, *Chop* deficiency had no impact on the B cell developmental defects associated with the loss of Sel1L, in terms of low spleen weight (Figure 3.5B), paucity of the B cell compartment within the peripheral lymphocyte pool (Figure 3.5C), and the developmental block at the large pre-B cell stage in the BM (Figure 3.5D-G). Thus, B cell-specific Sel1L deficiency results in a developmental block in a Chop-independent manner.

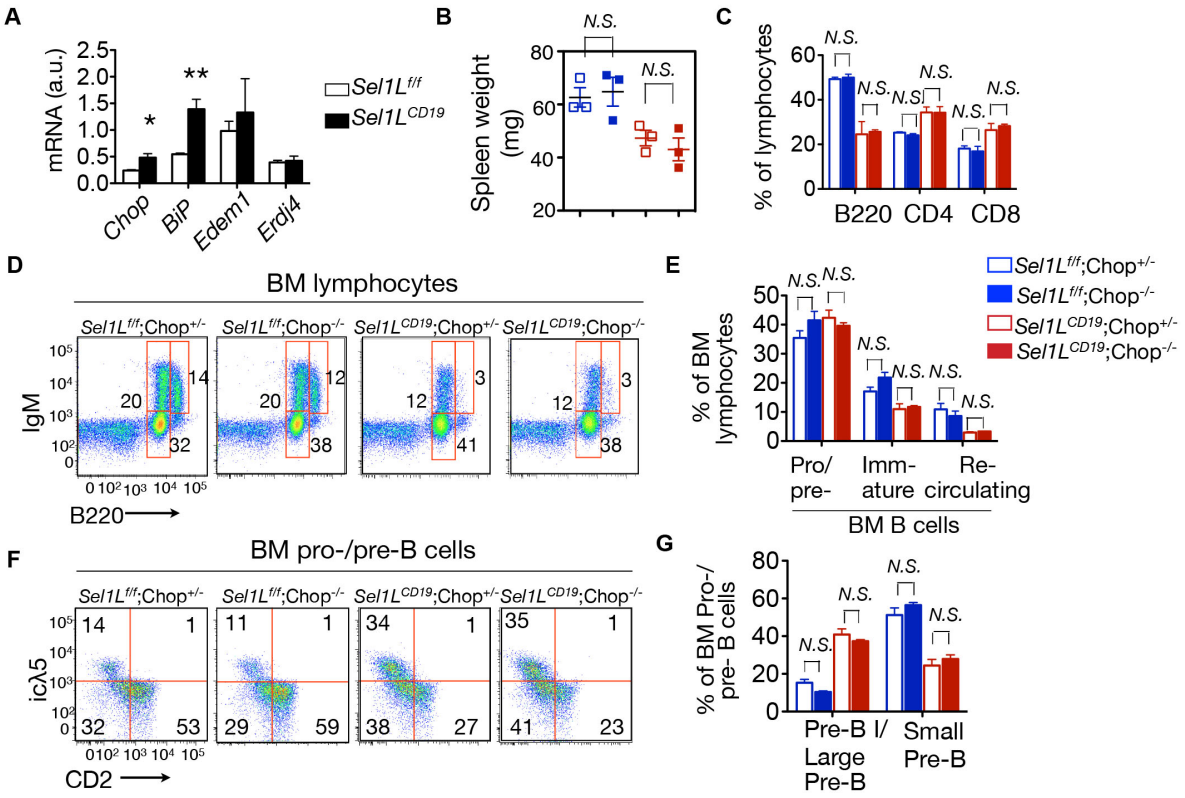

Figure 3. 5 B cell developmental defect in *Sel1L^{CD19}* mice is independent of Chop.

(A) Q-PCR analyses of BiP, Chop, Erdj4 and Edem1 mRNA levels in sorted large pre-B cells from *Sel1L$^{f/f}$* and *Sel1L^{CD19}* mice. (B) Spleen weight of WT (*Sel1L$^{f/f}$*; Chop$^{+/-}$), *Chop$^{-/-}$* (*Sel1L$^{f/f}$*; *Chop$^{-/-}$*), *Sel1L^{CD19}* (*Sel1L^{CD19}*; *Chop$^{+/-}$*) and double knockout (*Sel1L^{CD19}*; *Chop$^{-/-}$*) mice. (C) Frequency of various lymphocytes in spleens from the cohorts in (B). (D) Flow cytometric analysis of pro-/pre- B cells (B220^{+}IgM^{-}), immature B cells (B220^{+}IgM^{+}) and recirculating B cells (B220^{hi}IgM^{+}) in BM with quantitation shown in (E). (F) Flow cytometric analysis of pre-B I/large pre-B cells (ic-λ5^{+}/CD2^{-}) and small pre-B cell (ic-λ5^{-}/CD2^{-}) populations in the BM with quantitation shown in (G). Data are representative of 2 independent experiments (n= 8 *Sel1L$^{f/f}$*

and 4 *Sel1L*^{CD19} mice (A), 3 mice each (B) and 5 mice each (C-G)). Values shown as mean ± s.e.m.; N.S., not significant; *P<0.05, **P<0.01, by two-tailed Student's t-test.

3.4.4. Selective accumulation of the pre-BCR in large pre-B cells.

To explore the possible mechanism, we measured the protein levels of various key factors involved in B cell development at the pre-B cell stage, including c-Kit, IL-7Rα, CD19, and the pre-BCR complex (Clark et al., 2014; Herzog et al., 2009). All of these factors are transmembrane proteins synthesized in the ER (Figure 3.6A). While total levels (intracellular and surface) of c-Kit and IL-7Rα protein were comparable, protein levels of three main components of the pre-BCR complex were dramatically increased in the pro-/pre-B cells of *Sel1L*^{CD19} mice (Figure 3.6B). The percentage of SLC^{+} cells was significantly increased in *Sel1L*^{CD19} BM (Figure 3.6C and Figure 3.7A). Accumulation of the two SLC components VpreB and λ5 occurred within the same cells (Figure 3.6D). Moreover, as expression of SLC precedes that of the Igμ heavy chain (Kudo et al., 1992; Lassoued et al., 1996), there is a λ5-single positive pre-B I cell stage in addition to a λ5^{+}/Igμ^{+} large pre-B cells (Figure 3.3A). Strikingly, we observed a 3-fold increase in the percent of λ5^{+}/Igμ^{+} large pre-B cells in *Sel1L*^{CD19} mice compared to that of *Sel1L*$^{f/f}$ mice, while the percent of λ5^{+} Igμ^{-} pre-B I cells was not affected by ERAD deficiency (Figure 3.6E). In line with this finding, measurement of λ5 and Igμ at different developmental stages revealed their accumulation only in large pre-B cells when both were co-expressed (Figure 3.6F). These data demonstrate that Sel1L-Hrd1 ERAD recognizes and degrades the pre-BCR complex, rather than its individual components. Indeed, using a pre-BCR complex-specific antibody, we found that the proportion of pre-BCR complex-positive cells was doubled in the *Sel1L*^{CD19} BM (Figure 3.7B). Quantitative and RT-PCR analyses revealed comparable transcript levels of VpreB, λ5, and Igμ genes (Figure 3.7C-D), suggesting that pre-BCR protein accumulation is a result of post-transcriptional regulation. Hence, our data identify the pre-BCR complex, rather than its individual components, as the possible Sel1L-Hrd1 ERAD substrate in developing B cells.

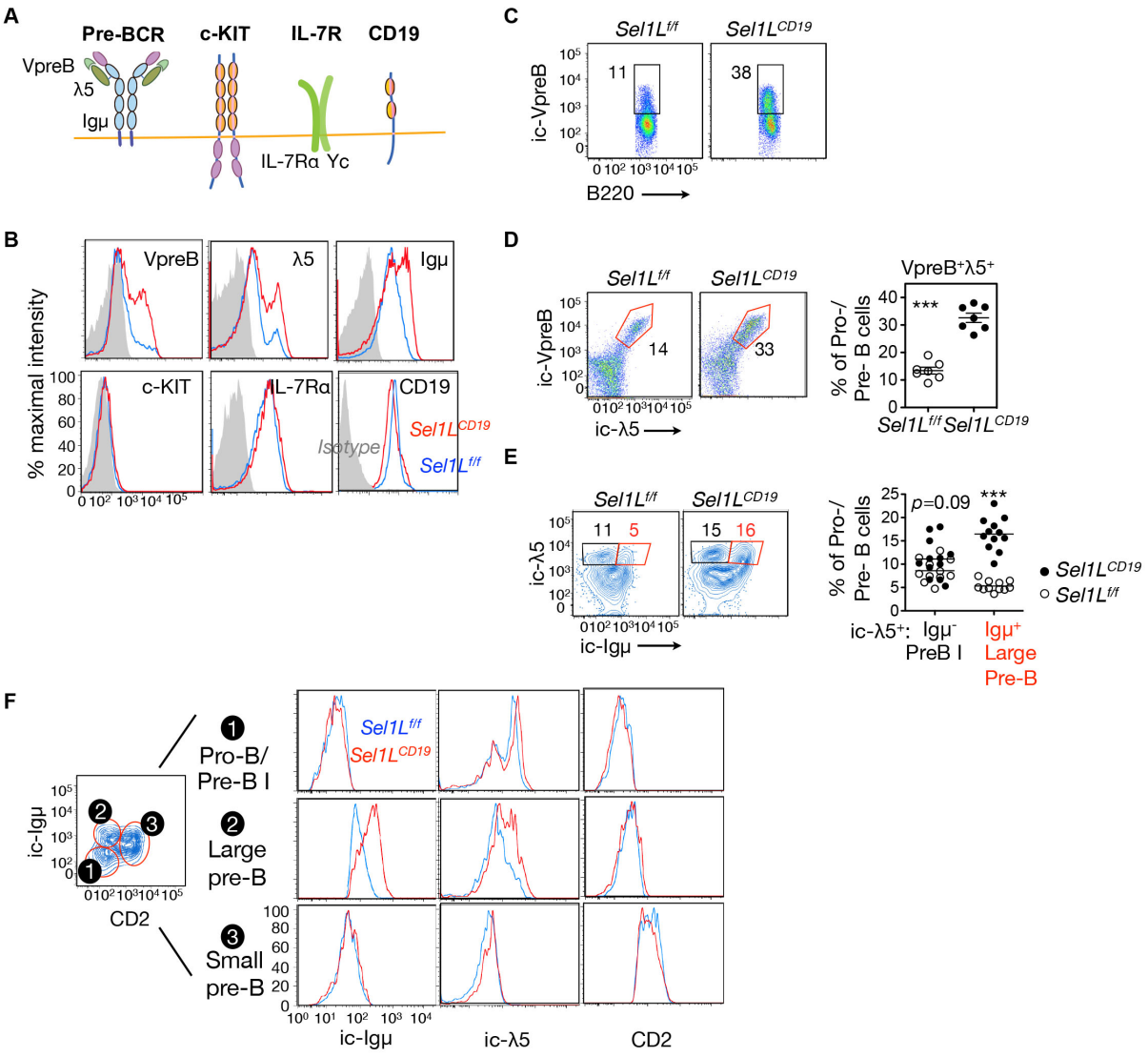

Figure 3. 6 Accumulation of the pre-BCR complex in Sel1L-deficient large pre-B cells.

(A) Schematic diagram of various membrane receptors involved in early B cell development. (B) Flow cytometric histogram analysis of BM cells from *Sel1L*$^{f/f}$ and *Sel1L*^{CD19} mice, stained for total (surface and intracellular) levels of indicated proteins, gated on B220lowIgM- pro-/pre-B cells. Gray-shaded area indicates isotype control. (C) Flow cytometric analysis of BM cells from *Sel1L*$^{f/f}$ and *Sel1L*^{CD19} mice stained for B220 and intracellular (ic) VpreB, gated on pro-/pre-B cells. (D) Flow cytometric analysis of BM for ic-λ5 and ic-VpreB in pro-/pre-B cells with

quantitation shown on the right. (E) Flow cytometric analysis of BM for ic-λ5 and ic-Igμ in pro-/pre-B cells with quantitation shown on the right. (F) Flow cytometric histogram analysis of CD2, ic-Igμ, and ic-λ5 in various early B cell populations in BM. Gating strategy for each population (1-3) is shown on the left. Data are representative of four (B) or two (C-F) independent experiments. n=18 mice each (B), 7 mice each (C), 7 mice each (D), 11-12 mice each (E), and 9 mice each (F). Values shown as mean ± s.e.m.; ***P<0.001 by two-tailed Student's t-test.

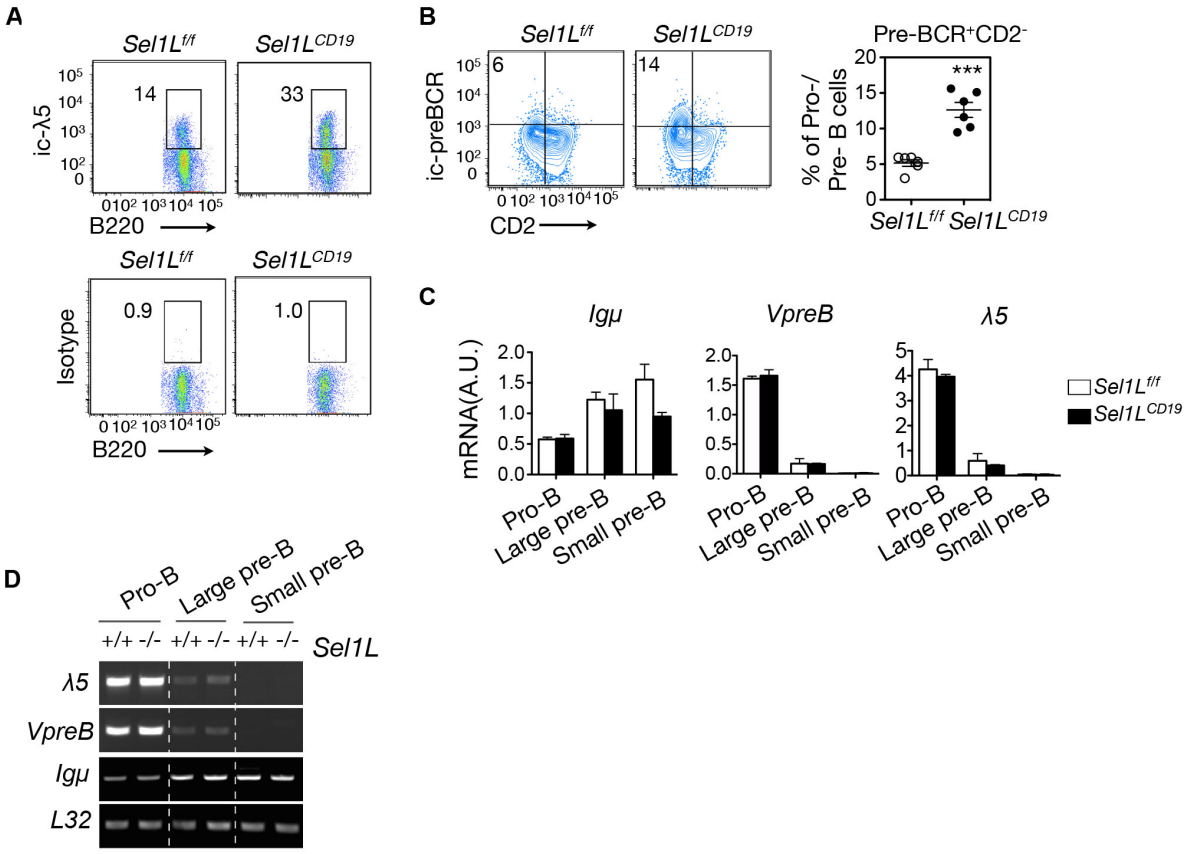

Figure 3. 7 Accumulation of pre-BCR components in Sel1L-deficient large pre-B cells, (A) Flow cytometric analysis of BM pro/pre-B cells from *Sel1L*$^{f/f}$ and *Sel1L*^{CD19} mice. The numbers next to the outlined area indicate the percentage of double positive cells. Representative data shown (n=7 mice each). (B) Flow cytometric analysis of CD2 and ic-preBCR in pro-/pre-B cells of *Sel1L*$^{f/f}$ and *Sel1L*^{CD19} mice. Quantitation is shown on the right. (C) Q-PCR analyses of pre-BCR components in various B cell progenitors isolated from BM of *Sel1L*$^{f/f}$ and *Sel1L*^{CD19} mice. (D) RT-PCR analysis of pre-BCR components in various B cell progenitors isolated from *Sel1L*$^{f/f}$ and *Sel1L*^{CD19} mice. *L32* is a loading control. Data are representative of two independent experiments. (B) 6 mice each, and (C-D) pooled cells from n=8 mice each. Values shown as mean ± s.e.m.

3.4.5. The pre-BCR complex is an endogenous Sel1L-Hrd1 ERAD substrate.

We next directly tested whether the pre-BCR is an ERAD substrate in pre-B cells. Protein levels of Igμ, λ5, and VpreB were significantly stabilized in the BM of *Sel1L*^{CD19} mice compared to *Sel1L*$^{f/f}$ mice (Figure 3.8A and Figure 3.9A). By contrast, IL-7Rα protein stability was not affected (Figure 3.8A and Figure 3.9B). To further corroborate these findings in vitro, we generated a Sel1L and Hrd1-deficient pre-BCR expressing pre-B cells, 70z/3 (Paige et al., 1978), using the RNAi and CRISPR/cas9 systems, respectively (Figure 3.8B-C). Loss of either Sel1L or Hrd1 in the 70z/3 cells increased steady-state protein levels of all three components of the pre-BCR complex (Figure 3.8B-C), and led to Igμ protein stabilization, with its half-life increasing from approximately 60 min to more than 120 min (Figure 3.8D). Inhibition of proteasomal activity using MG132 or bortezomib significantly increased Igμ protein stability in 70z/3 cells (Figure 3.8E and data not shown), implicating the proteasome in pre-BCR degradation. Furthermore, ubiquitination of Igμ protein was reduced dramatically in *Sel1L*$^{-/-}$ and *Hrd1*$^{-/-}$ 70z/3 cells (Figure 3.8F and not shown), pointing to the indispensable role of Sel1L-Hrd1 ERAD in pre-BCR ubiquitination and degradation. Thus, we concluded that Sel1L-Hrd1 ERAD recognizes and targets the pre-BCR complex for ubiquitination and proteasomal degradation and that Sel1L-Hrd1 ERAD deficiency leads to intracellular accumulation of the pre-BCR.

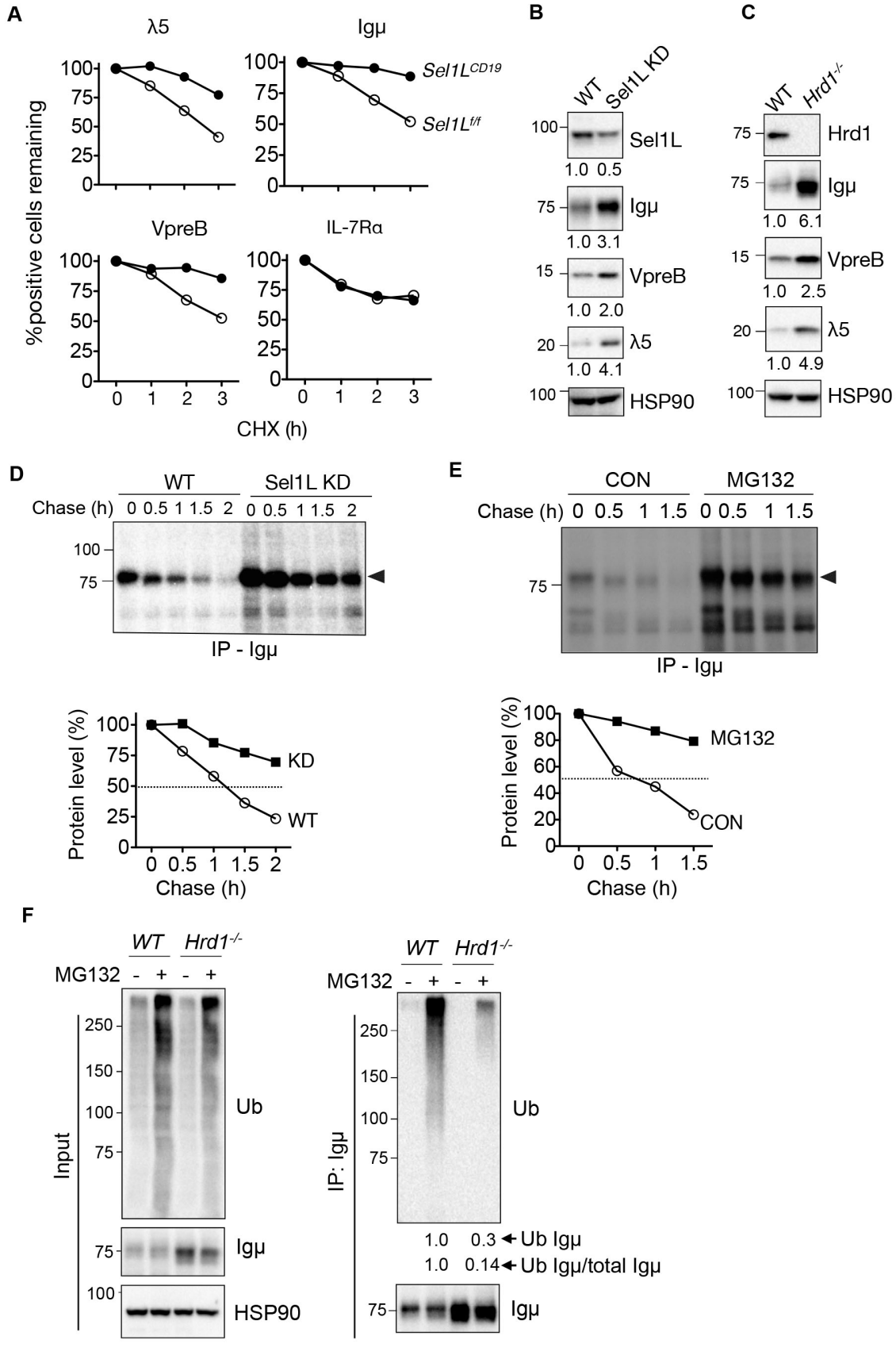
A
λ5
Igμ
VpreB
IL-7Rα
Sel1L^CD19
Sel1L^f/f
%positive cells remaining
CHX (h)
B
WT
Sel1L KD
Sel1L
Igμ
VpreB
λ5
HSP90
C
WT
Hrd1-/-
Hrd1
Igμ
VpreB
λ5
HSP90
D
WT
Sel1L KD
Chase (h)
IP - Igμ
Protein level (%)
KD
WT
Chase (h)
E
CON
MG132
Chase (h)
IP - Igμ
Protein level (%)
MG132
CON
Chase (h)
F
WT
Hrd1-/-
MG132
Input
Ub
Igμ
HSP90
IP: Igμ
Ub
1.0 0.3 Ub Igμ
1.0 0.14 Ub Igμ/total Igμ
Igμ

Figure 3. 8 The pre-BCR complex is an endogenous Sel1L-Hrd1 ERAD substrate.
(A) Protein turnover for intracellular λ5, VpreB and Igμ expression in fresh BM of *Sel1L*$^{f/f}$ and *Sel1L*^{CD19} mice treated with 50 μg/ml cycloheximide (CHX) for the indicated times. IL-7R, a control membrane protein. (B-C) Immunoblot analysis of WT, Sel1L KD (KD) (B) and *Hrd1*$^{-/-}$ (C) 70Z/3 pre-B cells with quantitation (normalized to HSP90) shown below each gel. (D) Pulse-chase analysis showing Igμ decay in WT and KD pre-B cells with quantitation shown on the right. (E) Pulse-chase analysis showing Igμ decay in WT pre-B cells treated with or without 25 μM MG132 for 0.5 h with quantitation shown on the right. (F) Immunoblot analysis of Igμ immunoprecipitates in WT and *Hrd1*$^{-/-}$ pre-B cells treated with or without 25 μM MG132 for 3 h. Quantitation of Igμ ubiquitination (with or without normalization to total Igμ) shown below the blot. Data are representative of two (A,D,E), three (B-C), and four (F) independent experiments. n=3 mice each (A).

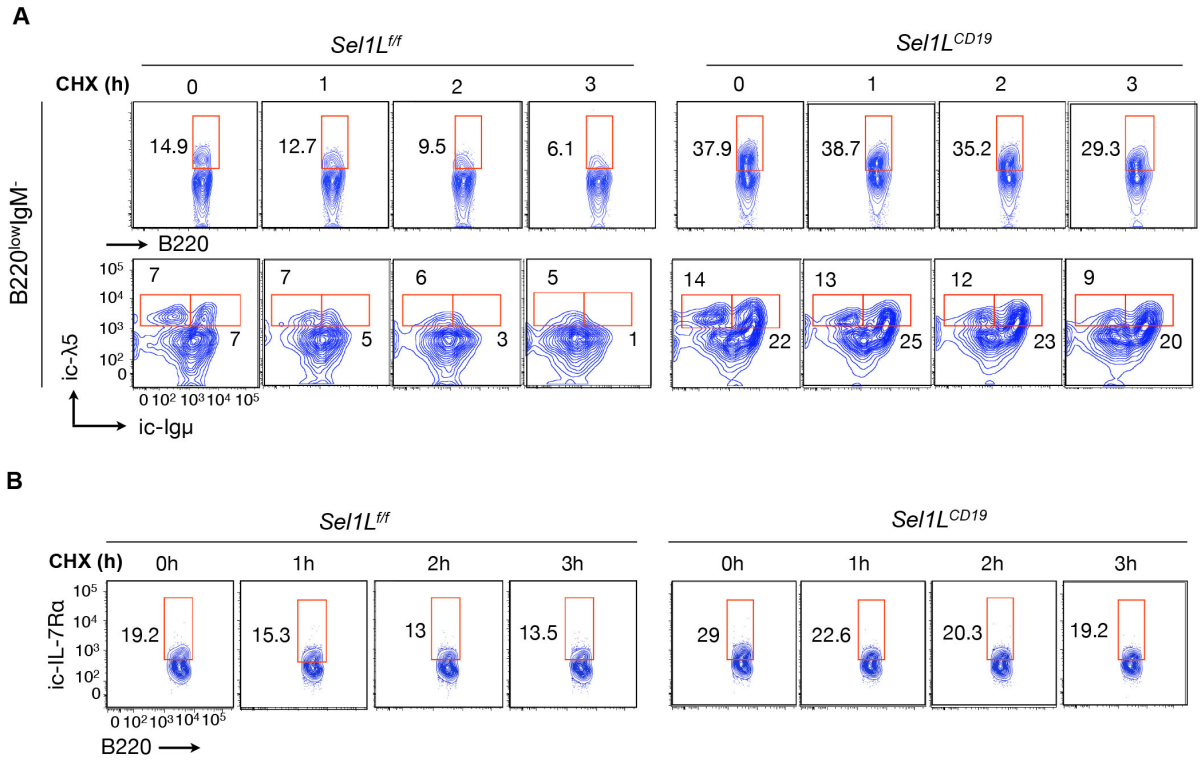

Figure 3. 9 Accumulation and stabilization of pre-BCR components in Sel1L-deficient large pre-B cells.

(A) Flow cytometric analysis for intracellular λ5 and Igμ expression in fresh BM of *Sel1L*$^{f/f}$ and *Sel1L*^{CD19} mice treated with 50 μg/ml cycloheximide (CHX) for the indicated times with quantitation of the percent of positive cells shown in Figure 3.8A. (B) Flow cytometric analysis of intracellular IL-7Rα level in BM cells of *Sel1L*$^{f/f}$ and *Sel1L*^{CD19} mice post 50 μg/mL cyclohexamide (CHX) treatment, gated on pro-/pre-B cells. Representative data from 2 independent experiments shown.

3.4.6. Enhanced ER exit of the pre-BCR in Sel1L-deficient pre-B cells.

We next determined the effect of Sel1L-Hrd1 ERAD deficiency on pre-BCR complex formation and intracellular trafficking. The accumulated pre-BCRs remained largely soluble (Figure 3.10A), colocalized with ER chaperone BiP (Figure 3.10B) and exhibited similar sensitivity to trypsin digestion in ERAD-deficient 70z/3 cells (Figure 3.11A). These data suggest that, in the case of pre-BCR, Sel1L-Hrd1 ERAD deficiency does not trigger a dramatic conformational change and hence protein aggregation. Moreover, accumulation of Igμ and SLCs in ERAD-deficient 70z/3 pre-B cells increased the abundance of the high molecular weight pre-BCR complexes, as revealed by non-reducing SDSPAGE (left two lanes, Figure 3.11B) and sucrose density gradient fractionation analyses (Figure 3.10C). Moreover, while the majority of Igμ protein in WT pre-B cells was retained in the ER and exhibited high-mannose endoglycosidase (Endo) H-sensitivity as previously reported (Brouns et al., 1996), Sel1L deficiency resulted in a nearly 3-fold increase in endoH-resistant and peptide-N-glycosidase F (PNGase F)-sensitive Igμ protein, indicative of increased ER exit of the pre-BCR complex in the absence of Sel1L-Hrd1 ERAD (Figure 3.10D). Consistently, more surface pre-BCR complexes were detected in ERAD-deficient pre-B cells (Figure 3.10E) and pre-B cells treated with the proteasome inhibitor MG132 (Figure 3.10F). Thus, impaired pre-BCR degradation by Sel1L-Hrd1 ERAD leads to elevated surface pre-BCR in pre-B cells.

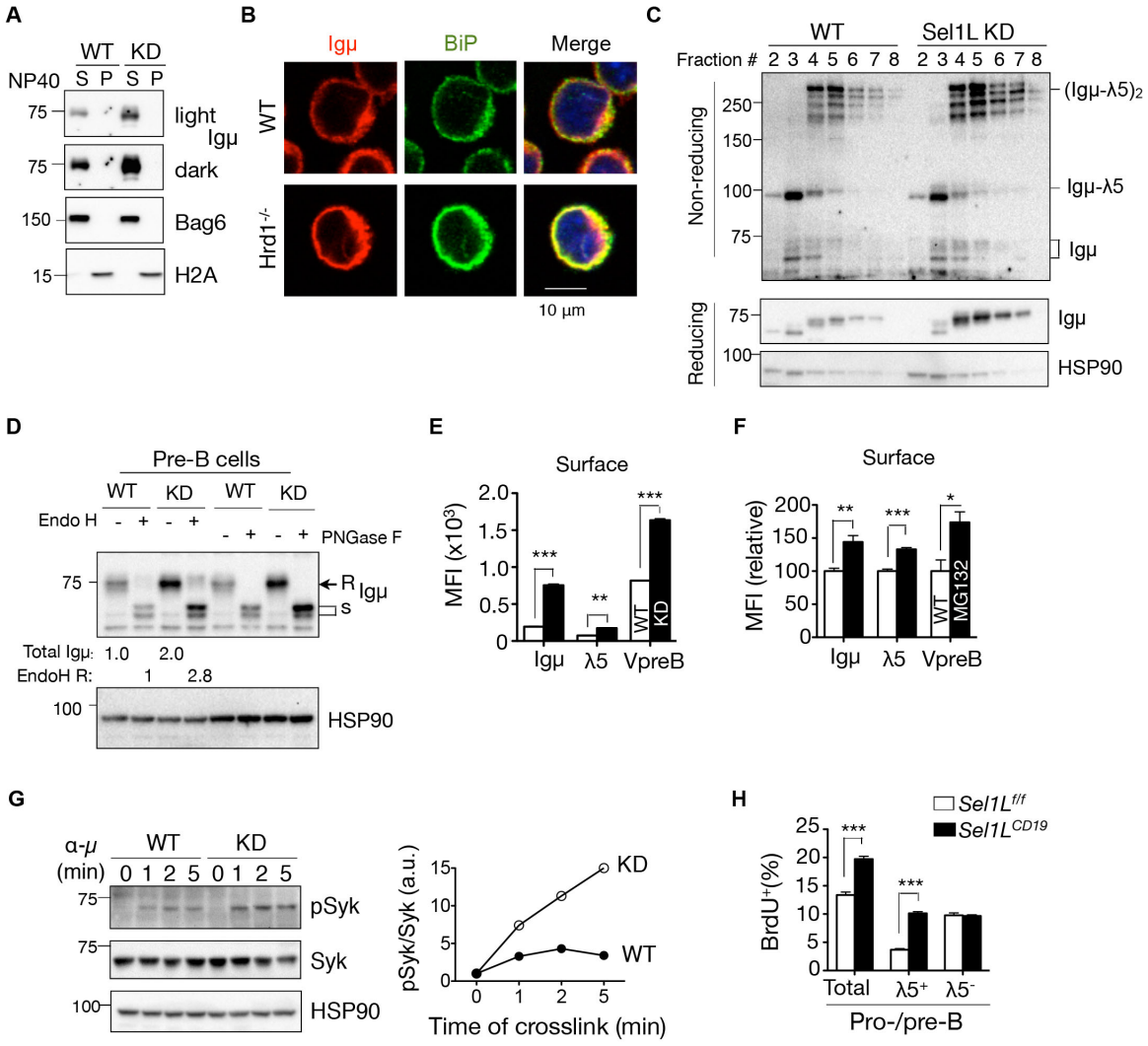

Figure 3. 10 ERAD is indispensable for the termination of pre-BCR signaling in developing B cells.

(A) Immunoblot analysis of Igμ in the NP-40 soluble (S) and insoluble (P) fractions of the WT and Sel1L KD pre-B cells. The distribution of Bag6 and H2A marks the S and P fractions, respectively. (B) Confocal microscopic images of Igμ (red) and BiP (green) staining in *WT* and *Hrd1*$^{-/-}$ 70z/3 cells counterstained with DAPI. (C) Sucrose gradient followed by immunoblot analysis of Igμ in WT and KD pre-B cells under non-reducing and reducing conditions. (D) Immunoblot analysis of Igμ in WT and KD pre-B cell lysates treated with EndoH or PNGase F.

(r, s= EndoH resistant, sensitive). Quantitation of total Igμ and EndoH (r) Igμ shown below the blot. (E-F) Quantitation of mean fluorescent intensity (MFI) of surface pre-BCR components in (E) WT and KD pre-B cells and (F) WT pre-B cells treated with or without 25 μM MG132 for 2 h. (G) Western blot analysis of phospho-(pSyk) and total Syk (Syk) of WT and KD cells crosslinked with α-Igμ (10 μg/ml) for the indicated times with quantitation shown on the right. (H) Quantitation of flow cytometric analysis of BrdU incorporation in pro-/pre-B cells and $\lambda5^+$ or $\lambda5^-$ pro-/pre-B cells of *Sel1L*$^{f/f}$ and *Sel1L*^{CD19} mice with original flow data shown in Figure 3.11D. Data are representative of 2-3 independent experiments. n=6 mice each for (H). Values shown as mean ± s.e.m.; *P< 0.05, **P<0.01, ***P<0.001 by two-tailed Student's t-test.

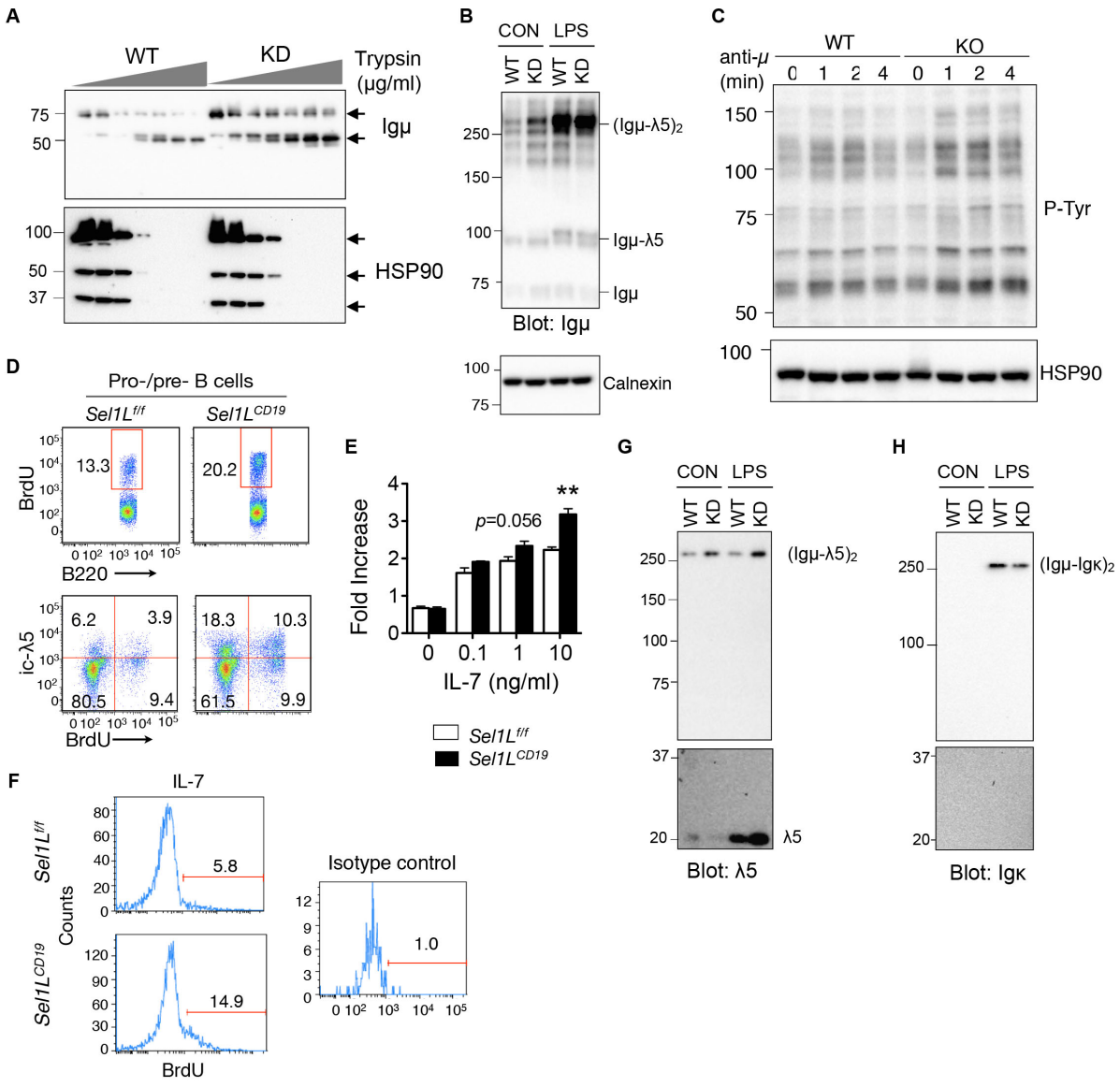

Figure 3. 11 Consequence of ERAD deficiency on pre-BCR conformation and complex formation

(A) Limited proteolysis digestion of WT and Sel1L KD cell lysates with varying concentrations of trypsin treatment, followed by Western blot analysis. (B, G, H) Western blot analysis of pre-BCR complex formation in mock- or LPS- (20 μg/mL, 18h) treated WT and KD cells under non-reducing conditions. Same cell lysates were blotted for Igμ (B), λ5 (G) and Igκ (H). Representative data from 3 independent experiments shown. (C) Immunoblot analysis of overall

tyrosine phosphorylation in *WT* and *Hrd1*$^{-/-}$ pre-B cells cross-linked with α-Igμ (15 μg/ml) at indicated times. (D) Flow cytometric analysis of BrdU incorporation in pro/pre-B cells (upper) and λ5^{+} or λ5^{-} pro-/pre-B cells (lower) of *Sel1L*$^{f/f}$ and *Sel1L*^{CD19} mice (n= 6 mice each), with quantitation shown in Figure 3.10H. Calnexin and HSP90, loading controls. (E-F) Effect of the IL-7 on proliferation of pro/pre-B cells in culture: (E) MACS sorted BM CD19^{+} cells were cultured with various concentrations of IL-7 for 4 days. Absolute number of viable cells was counted and shown as cell number relative to starting point (fold increase). (F) FACS sorted BM large pre-B cells (B220^{low}IgM^{-}CD43^{low}CD2^{-}) were treated with IL-7 (1 ng/ml) for 2 days and BrdU incorporation in each population were analyzed.

3.4.7. Persistent pre-BCR signaling and pre-B cell cycling in *Sel1L*^{CD19} mice.

We next tested whether elevated surface pre-BCR results in augmented pre-BCR signaling, consequently inducing a burst of proliferation of large pre-B cells. Phosphorylation of the pre-BCR key downstream effector, spleen tyrosine kinase (Syk) (Herzog et al., 2009), was significantly increased in ERAD-deficient pre-B cells (Figure 3.10G), as was overall tyrosine phosphorylation (Figure 3.11C). Furthermore, the total number of BrdU-positive cycling pro-/pre-B cells in BM was increased by more than 50 % in *Sel1L*^{CD19} mice compared to *Sel1L*$^{f/f}$ mice (Figure 3.10H and Figure 3.11D). Among them, only $\lambda5^+$ large pre-B cells were highly proliferative in *Sel1L*^{CD19} mice, increasing more than 2.5 fold; λ5- pro-B cell proliferation did not increase in *Sel1L*^{CD19} mice. Thus, Sel1L deficiency in B cells leads to elevated and sustained pre-BCR signaling and persistent proliferation of large pre-B cells. In aggregate, these findings demonstrate that the Sel1L-Hrd1 ERAD is required to attenuate pre-BCR signaling by promoting its protein turnover.

3.4.8. Sel1L-Hrd1 ERAD degrades the pre-BCR, not the BCR, complex.

In the pre-BCR, SLC and Igμ assemble into a quaternary structure reminiscent of the BCR with VpreB and λ5 equivalent to the variable and constant regions (VL and CL), respectively, of IgL (Figure 3.12A). We compared how Sel1L-Hrd1 ERAD targets pre-BCR for degradation by comparing the effect of ERAD on the pre-BCR vs. the BCR both in vitro and in vivo. Upon overnight LPS stimulation, 70z/3 pre-B cells differentiated into mature B cells expressing BCR with conventional κ chain replacing the SLC (Figure 3.11E-F), as previously reported (Paige et al., 1978). This unique feature allowed us to determine how ERAD affects the trafficking and stability of the pre-BCR vs. BCR complexes containing the same Igμ heavy chain in the same cell. Unlike Igμ in the pre-BCR, neither the steady-state protein level or stability of

Igμ in the BCR complex, nor its endoH sensitivity (i.e. ER exit), were affected by Sel1L-Hrd1 ERAD deficiency in mature B cells (Figure 3.12B-D). This was not due to the lack of expression of ERAD components in mature B cells, as Sel1L and Hrd1 expression levels were comparable between pre- and mature B cells (Figure 3.12E).

To further test this in vivo, we analyzed mature splenic B cells from Sel1L-inducible knockout (*Sel1L*^{ERCre}) mice expressing estrogen receptor-Cre recombinase fusion protein (ERCre) under the control of the actin promoter (Sun et al., 2014) (Figure 3.12F). Consistent with our in vitro observations, both intracellular Igμ and surface BCR (IgM) levels were comparable between *Sel1L*$^{f/f}$ and *Sel1L*^{ERCre} mature B cells (Figure 3.12G). Moreover, plasma cell differentiation, cell size and total IgG levels in LPS/IL-4-treated mature primary B cells (*Sel1L*$^{f/f}$ and *Sel1L*^{ERCre}) were not affected by Sel1L deficiency (Figure 3.13A-C). Indeed, Igμ interacted with several components of the ERAD machinery, including Sel1L and OS9, to a greater extent in pre-B cells compared to mature B cells (Figure 3.12H). Therefore, we concluded that the pre-BCR, but not the BCR, complex is an endogenous Sel1L-Hrd1 ERAD substrate**.**

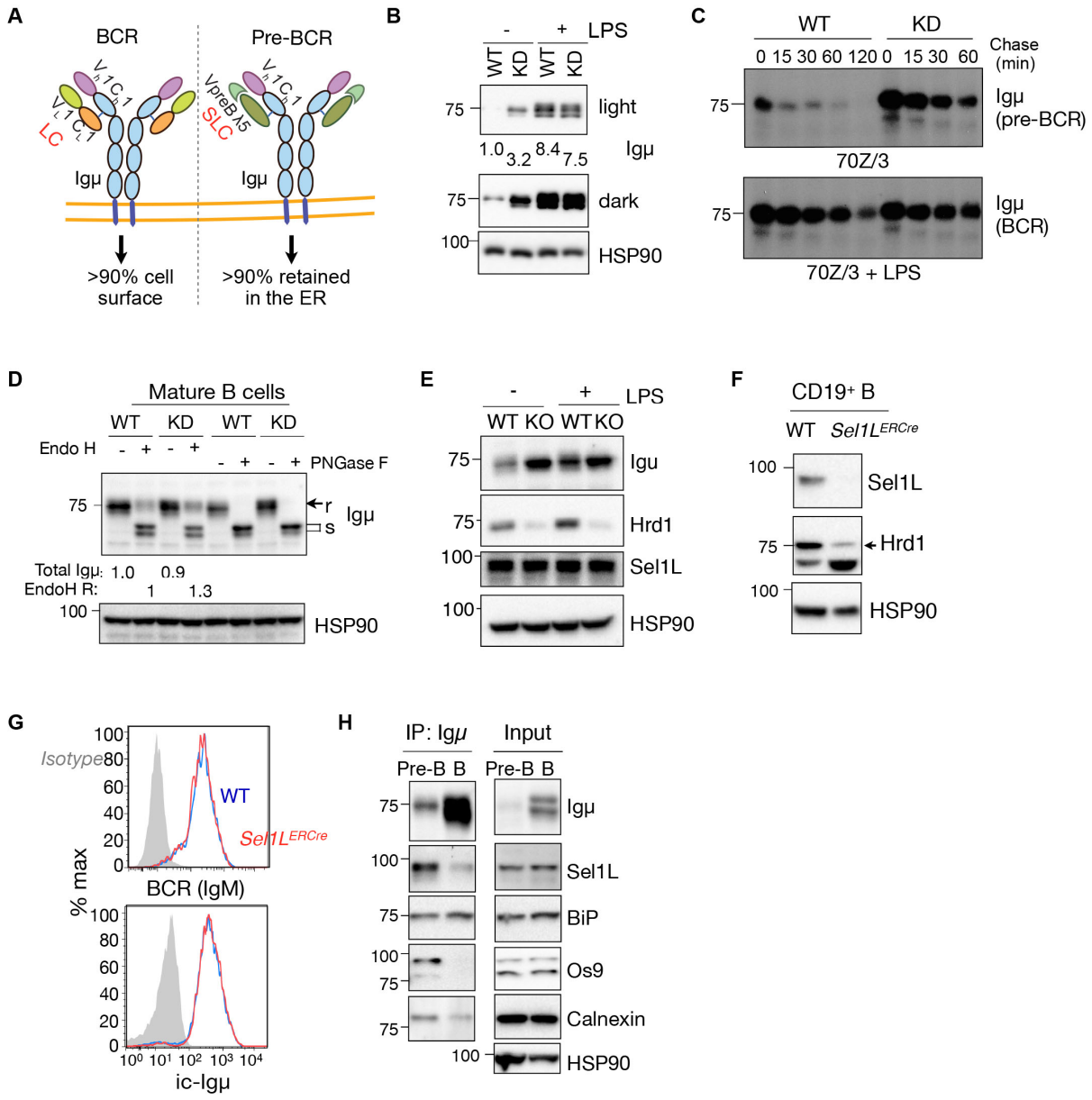

Figure 3. 12 Sel1L-Hrd1 ERAD degrades the pre-BCR, but not the BCR complex.

(A) Diagram depicting the difference between BCR and pre-BCR complexes. (B) Immunoblot analysis of Igμ in WT and Sel1L KD cells (KD) treated with or without 20 μg/mL of lipopolysaccharide (LPS) for 18 h, with quantitation shown below. (C) Pulse-chase analysis showing Igμ decay in WT and KD 70Z/3 cells treated with or without 20 μg/mL of LPS for 18 h. (D) Immunoblot analysis of Igμ in WT and KD B cell lysates treated with EndoH or PNGase F.

(r/s= EndoH resistant/sensitive). Quantitation of total Igμ and EndoH resistant (R) Igμ shown below the blot. (E) Immunoblot analysis of ERAD proteins in *WT* and *Hrd1*$^{-/-}$ 70z/3 pre-B cells treated with or without 20 μg/mL LPS for 18 h. (F) Western blot analysis of CD19^{+} splenic B cells isolated from WT and *Sel1l*-inducible knockout (*Sel1L*^{ERCre}) mice. (G) Histogram analysis of surface IgM and intracellular (ic) Igμ in splenic B cells of WT and Sel1LERCre mice (N=3 mice each). (H) Immunoblot analysis of immunoprecipitates of Igμ-agarose in pre-B 70z/3 and mature B cells. Representative of two independent experiments shown.

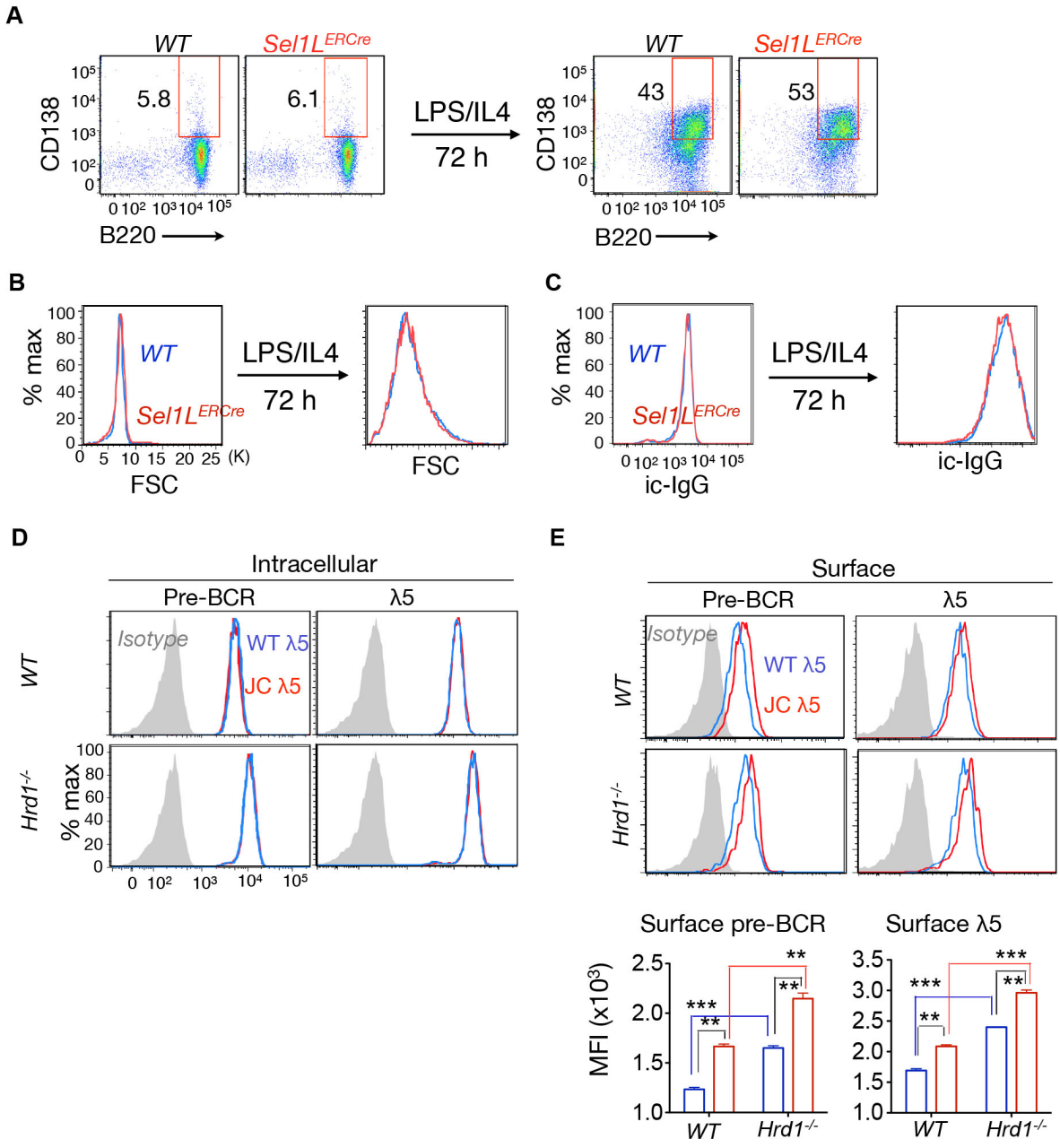

Figure 3. 13 Sel1L-Hrd1 ERAD does not control the turnover of BCR, and ERAD-mediated pre-BCR degradation is independent of the unique region of λ5.

(A) Flow cytometric analysis of splenic B cells of WT and Sel1l-inducible knockout (*Sel1L*^{ERCre}) mice before and after LPS/IL-4 stimulation. (B-C) Histogram analysis of cell size (B) and

intracellular (ic) IgG (C) in splenic B cells of WT and *Sel1L*^{ERCre} mice before and after LPS/IL-4 stimulation. (D-E) Flow cytometric analysis of intracellular (D) and surface (E) λ5 and pre-BCR in *WT* and *Hrd1*$^{-/-}$ 70z/3 pre-B cells expressing WT or JC λ5, with quantitation shown in Figure 3.14E and below respectively. Data are representative of two to three independent experiments (A-E), with n=6 mice each in (A-C). Values shown as mean ± s.e.m.; **P<0.01, ***P<0.001 by one-way ANOVA with Tukey post-test.

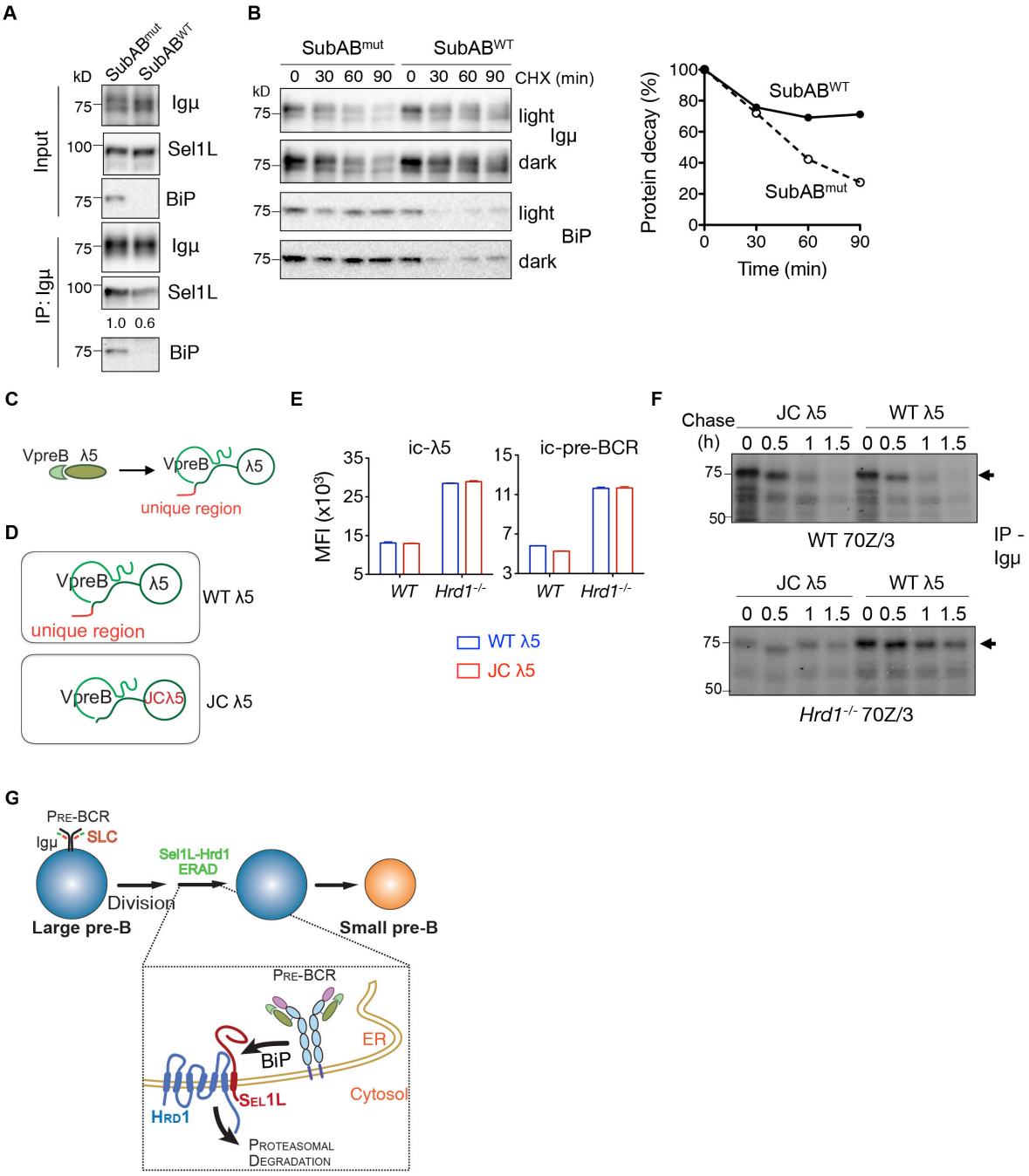

Figure 3. 14 Sel1L-Hrd1 ERAD-mediated pre-BCR degradation is BiP-dependent but λ5 unique region-independent.

(A) Immunoblot analysis of Igμ immunoprecipitates in 70Z/3 cells treated with 0.5 μg/ml SubAB^{mut} (SubA$_{A272}$B) or SubAB^{WT} for 45 min. (B) Translation shut-off assay of Igμ in 70Z/3

cells treated by 50 μg/ml cycloheximide (CHX) together with 0.5 μg/ml $SubAB^{mut}$ or $SubAB^{WT}$ for the indicated times with quantitation shown on the right. (C) Schematic diagram depicting surrogate light chains (SLC). (D) Schematic diagram depicting SLC containing WT and mutant λ5 (JC λ5) lacking the unique region. (E) Quantitation of mean fluorescent intensity (MFI) of intracellular levels of λ5 (ic-λ5) and pre-BCR in *WT* and *Hrd1*$^{-/-}$ 70z/3 pre-B cells expressing WT or JC λ5. Values shown as mean ± s.e.m. (F) Pulse-chase analysis showing Igμ decay in *WT* and *Hrd1*$^{-/-}$ pre-B cells expressing WT or JC λ5. Representative of two independent experiments shown. (G) The model: The Sel1L-Hrd1 ERAD manages the checkpoint in B cell development by targeting the pre-BCR for cytosolic proteasomal degradation (via BiP), which attenuates pre-BCR signaling and allows further B cell differentiation.

3.4.9. Pre-BCR ERAD requires BiP but is independent of the unique region of λ5.

As BiP is involved in the folding and intracellular trafficking of the pre-BCR complex (Minegishi, Hendershot, et al., 1999), we first tested whether BiP is required for pre-BCR ERAD. Depletion of BiP with the subtilase cytotoxin SubAB (Paton et al., 2006) significantly reduced the interaction between Igμ and Sel1L (Figure 3.14A) and stabilized Igμ protein (Figure 3.14B). Moreover, given that the pre-BCR and BCR complexes share the common Igμ heavy chain, we speculated that a key determinant in pre-BCR recognition by ERAD may lie in the binding of SLC to Igμ. We noted previous reports demonstrating that the unique region of λ5 (Figure 3.14C), which is a non-Ig portion and does not pair with either VpreB or Igμ (Clark et al., 2014; Herzog et al., 2009), is known to retain pre-BCR complex in the ER (Fang et al., 2001; Minegishi, Hendershot, et al., 1999; Ohnishi et al., 2003). To determine the role of the unique region of λ5 in pre-BCR degradation, we expressed wildtype (WT λ5) and mutant λ5 lacking the unique region (JC λ5, with only J and C parts of λ5) (Ohnishi et al., 2003) in *WT* and *Hrd1*$^{-/-}$ pre-B cells (Figure 3.14D).

Surprisingly, JC λ5 expression had no effect on total steady state intracellular levels of the pre-BCR complex in both *WT* and *Hrd1*$^{-/-}$ pre-B cells compared to those expressing WT λ5 (Figure 3.14E and Figure 3.13D). Moreover, total pre-BCR complex levels were higher in *Hrd1*$^{-/-}$ pre-B cells than those of WT cells expressing JC λ5 (Figure 3.14E), suggesting that the JC λ5-containing pre-BCR complex remains to be an ERAD substrate. Consistently, JC λ5 expression failed to affect Igμ protein half-life (Figure 3.14F). On the other hand, JC λ5 increased surface pre-BCR in WT pre-B cells as previously reported (Fang et al., 2001; Minegishi, Hendershot, et al., 1999; Ohnishi et al., 2003) (Figure 3.13E). Hence, we concluded that BiP, but not the unique region of λ5, is required for the recognition of the pre-BCR complex as an ERAD substrate in pre-B cells.

3.5. DISCUSSION

This study shows that Sel1L-Hrd1 ERAD manages a key checkpoint in B cell development by selectively targeting the pre-BCR complex for proteasomal degradation. Indeed, Sel1L-Hrd1 ERAD complex is indispensable for the termination of pre-BCR signaling. B-cell-specific deficiency of the Sel1L-Hrd1 ERAD system blocks developmental progression at the large to small pre-B cell transition. Surprisingly, we show that the pre-BCR, not BCR, complex is an endogenous Sel1L-Hrd1 ERAD substrate in B cells and that Sel1L-Hrd1 ERAD has no obvious effect on plasma cell differentiation. Lastly, we show that ERAD-mediated pre-BCR degradation requires BiP, but independently of the unique region of λ5 known to be important for the ER retention of the pre-BCR complex.

Pre-BCR signaling marks a critical checkpoint in B cell development. The pre-BCR signal induces a burst of proliferation of large pre-B cells to ensure expansion of B cell precursors with a functional Igμ chain, a process known as "pre-BCR-dependent positive selection" (Hess et al., 2001; van Loo et al., 2007); and importantly, it downregulates its own expression via silencing the SLC genes, while upregulating recombination activating gene (Rag) expression and IgL rearrangement. Deficiency in VpreB (Mundt et al., 2001), λ5 (Kitamura, Kudo, et al., 1992), the transmembrane region of Igμ (Kitamura & Rajewsky, 1992), Igα (Kitamura & Rajewsky, 1992) and Igβ (Gong et al., 1996) all prevents entry into the proliferating large pre-B cell stage and leads to a marked enrichment of pro-B cells in the BM, resulting in a profound decrease in mature peripheral B cells (Herzog et al., 2009). As Sel1L deficiency leads to a B cell developmental block at a later stage, the molecular mechanism underlying the defect in the *Sel1L*^{CD19} mice is unlikely due to a loss-of-function of the pre-BCR. Instead, our data (Wang, Y. H. et al., 2002)pre-BCR signaling. Persistent pre-BCR signaling, when the expression

of λ5 is extended (Martin, D. A. et al., 2007) or downstream negative effectors such as SLP-65 are inactivated (Jumaa et al., 1999), leads to elevated surface pre-BCR and continued cycling of large pre-B cells, preventing further differentiation. Similarly, Sel1L-deficiency in B cells results in intracellular accumulation and elevated surface pre BCR and signaling. Consequently, $Sel1L^{CD19}$ mice exhibit continued proliferation of large pre-B cells and decreased cellularity of small pre-B cells and mature B cells.

Expression and signaling from the pre-BCR complex are transient at the transition from the pro-B to the pre-B cell stage. Downregulation of pre-BCR signaling is a prerequisite for cell cycle exit and further differentiation to the resting small pre-B cell stage, such that IgL gene segment rearrangement can commence (Flemming et al., 2003; Kitamura, Kudo, et al., 1992; Melchers, 2005; Minegishi et al., 1998; Mundt et al., 2001; Parker et al., 2005; Shimizu et al., 2002). Self-limiting mechanisms include transcriptional silencing of SLC and dilution out of pre-BCRs due to large pre-B cell cycling. Our data show that the Sel1L-Hrd1 ERAD complex plays a key role in the termination of pre-BCR signaling in B cell development. Given the structural and composition similarity between the pre-BCR and the BCR complexes, our findings raise an intriguing question regarding substrate maturation in the ER and selection by ERAD. The binding of SLC to Igμ, unlike that of LC to Igμ, is likely to be much less efficient, associated more intensively with BiP and/or may expose folding intermediates of pre-BCR to the ERAD machinery. This model is consistent with earlier reports demonstrating that the pre-BCR complex is prone to misfolding and requires the activity of ER folding machinery (Hendershot, L. M., 1990; Minegishi, Coustan-Smith, et al., 1999), and that the majority of pre-BCR is retained in the ER, with less than 2 % reaching to the cell surface compared to 90 % of BCR reaching the cell surface in mature B cells (Brouns et al., 1996; Fagioli et al., 2001; Fang et al., 2001;

Hendershot, L. et al., 1987; Pillai et al., 1987). Moreover, the intrinsic ability of assembled pre-BCR complexes to exit the ER is limited by the unique region of λ5 (Fang et al., 2001; Minegishi, Hendershot, et al., 1999; Ohnishi et al., 2003). We show in this study that, while the unique region of λ5 controls the ER exit of the pre-BCR complex, it is dispensable for pre-BCR ERAD.

Our study demonstrates that the function of ERAD could not be accurately predicted solely on the basis of the abundance of secreted protein production. We speculate that this selectivity may derive from a particularly complex requirement for the folding of specific substrates in a cell type-specific manner, (in this case, the pre-BCR), rather than from the extent and the consequence of the UPR. Indeed, there was only very mild, if any, ER stress and cell death associated with Sel1L/ERAD deficiency in differentiating B cells. Moreover, *Sel1L*^{CD19} mice exhibited phenotypes largely distinct from those with B cell-specific UPR deficiency (Hetz, 2012; Sha et al., 2011). For example, *Xbp1* deficiency blocks plasma cell differentiation from B cells, whereas it has no effect on B cell development (Reimold et al., 2001; Todd et al., 2009). IRE1α deficiency causes a severe impairment of VDJ recombination of Ig genes and hence there is no expression of IgH and IgL chains of the BCR (Zhang, K. et al., 2005). This defect is associated with reduced expression of RAG1/2 and terminal deoxynucleotidyl transferase. On the other hand, the PERK-eIF2α branch of the UPR is not required for B cell differentiation (Zhang, K. et al., 2005). Therefore, Sel1L-Hrd1 ERAD manages B cell development in an UPR-independent manner.

Polypeptides that do not meet the quality-control standards are retained within the ER and are recognized and retrotranslocated by the ERAD machinery for proteasomal degradation in the cytosol. Mammals possess a number of ERAD complexes, with the Sel1L-Hrd1 being the most conserved and characterized biochemically. A limited number of endogenous substrates of

Sel1L-Hrd1 ERAD complex have been identified in vitro, including ER luminal hedgehog, ATF6 and CD147 (Chen, X. et al., 2011; Horimoto et al., 2013; Tyler et al., 2012). More recently, nuclear proteins such as NRF2, Blimp1 and PGC1β have been identified as cell type-specific Hrd1 substrates in vivo (Fujita et al., 2015; Wu, T. et al., 2014; Yang, H. et al., 2014). We recently reported IRE1α as an endogenous substrate for the Sel1L-Hrd1 ERAD in various cell types (Sun et al., 2015). Here we show that the pre-BCR complex is an endogenous ERAD substrate in developing B cells. Sel1L is required in this process likely as a key adaptor protein to stabilize Hrd1 protein as shown previously (Sun et al., 2014). Additionally, the physical interaction between the pre-BCR and Sel1L suggests that Sel1L may be intimately involved in pre-BCR turnover by recruiting the pre-BCR complex to the ligase Hrd1. As the pre-BCR is the first protein complex identified as an endogenous ERAD substrate to date, further investigation into its degradation mechanism may shed important mechanistic insights into substrate selection and protein degradation in vivo.

3.6. ACKNOWLEDGEMENT

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CHAPTER FOUR: B CELL SPECIFIC SEL1L ABLATION PROMOTES PROPAGATION OF COLITOGENIC MICROBIOTA

4.1 INTRODUCTION

Inflammatory bowel disease (IBD) is characterized by uncontrolled inflammatory responses in the gastrointestinal tract, mounted against antigens displayed by commensal bacteria. Although the combinations of different factors have been reported to initiate this disease, the exact etiology and mechanistic basis remain unclear. Thus a comprehensive understanding of the mechanisms underlying the regulation of innate immunity and inflammation is of both fundamental and clinical relevance.

The epithelial surface of the gastrointestinal tract serves as a physical barrier against gut microbiota, thereby providing a first layer of defense against infection and inflammation. At the luminal surface of the intestinal epithelial cells is layers of mucosa, which forms second layer of protection. Final layer of protection is mounted by numerous immune cells which resides in the gut, either in gut associated lymphoid tissues (GALT) such as Peyer's patches and mesenteric lymph nodes, or scattered throughout the intestinal epithelium and lamina propria.

Amongst different immunoglobulin isotypes, immunoglobulin A (IgA) is the main antibody isotype which is secreted into the intestinal lumen. This secretory IgA (sIgA) serves as the first line of defense in protecting the intestinal epithelium cells from enteric toxins and microbiota : 1) by antigen (Ag) neutralization; 2) prevention of microbial attachment to the epithelium; 3) elimination of excessive Ag load, and; 4) the overall maintenance of mucosal homeostasis (Johansen et al., 1999; Lamm, 1997; Langford et al., 2002; Underdown et al., 1986; Uren et al., 2003). The importance of IgA production and the quality of IgA affinity in maintaining gut homeostasis has been demonstrated by several groups (Cao et al., 2012; Cataldo et al., 1998; Friman et al., 2002; Malik et al., 2016; Meini et al., 1996; Murthy et al., 2006; Okai et al., 2016). Our previous study has demonstrated that Sel1L-Hrd1 ERAD manages a key

checkpoint in B cell development by selectively targeting the pre-BCR complex for proteasomal degradation, but has no apparent effect on plasma cell differentiation (Ji et al., 2016). However, the role of Sel1L and Sel1L-Hrd1 ERAD in the B cell function in mucosal immunity remains unknown.

Here, we tested sensitivity of wildtype (WT) and B cell specific Sel1L deficient mice to dextran sodium sulfate (DSS)-induced colitis. Our data show that *Sel1L*^{CD19} mice are hypersensitive to DSS-induced experimental colitis and strikingly, the susceptibility to colitis is transmissible to co-housed WT littermates. These data point to the presence of colitogenic microbiota in the intestinal tract of *Sel1L*^{CD19} mice. Mechanistically, we observed that *Sel1L*^{CD19} mice have reduced mature B cells in the gut-associated lymphoid tissues (GALT), reduced levels of IgA in lamina propria and feces. While more mechanistic studies are under way, our data show that B-cell-specific Sel1L-Hrd1 ERAD pathway is indispensable for B cell development and that ablation of this pathway leads to IgA deficiency and may create an intestinal niche that favors the outgrowth of colitogenic microbe(s).

4.2 MATERIALS AND METHODS

Mice

Sel1L$^{f/f}$ mice on the C57B/L background have been described (Sun et al., 2014) and crossed with CD19 promoter-driven Cre (*CD-19Cre*) mice from Jackson Laboratory (B6.129P2(C)-Cd19tm1(cre)Cgn/J, #006785) to generate *Sel1L*$^{f/f}$*; CD19-Cre+* (*Sel1L*^{CD19}) and control *Sel1L*$^{f/f}$*; CD19-Cre-*(*Sel1L*$^{f/f}$) littermates at a 1:1 ratio. Mice were housed under specific pathogen-free conditions and fed on a low-fat diet consisting of 13 % fat, 6 7% carbohydrate, and 20 % protein (Harlan Teklad 2914, Madison, WI). For cohousing experiments, age- and gender-matched *Sel1L*$^{f/f}$, *Sel1L*^{CD19} and C57BL/6J mice were co-housed at the age of 4 weeks, in

new cages at 1:1:1 ratio for 4 weeks. For cross-fostering experiments, newborn mice were exchanged between *Sel1L*^{CD19} and C57BL/6J mothers within 24 hr of birth. Mice were weaned between postnatal days 21-28 and used at the age of 6-12 weeks in all in vivo experiments.

In vivo experiments

Mice were fed ad libitum with 3 % (wt/vol) DSS (molecular weight 40-50 kDa; Affymetrix, Santa Clara, CA) dissolved in drinking water for 5 days and then fresh water thereafter. Body weights were monitored every other day until the end of experiments. Mice were sacrificed by cervical dislocation. Intestinal tissues were immediately harvested and fixed in 10% neutralized Formalin for histology or flushed with phosphate-buffered saline (PBS) for immune cell isolation from lamina propria. All animal procedures were approved by the Institutional Animal Care and Use Committee at Cornell University.

Isolation of lamina propria (LP) immune cells from colonic tissues

In brief, the intestine was removed from mesenteric fat tissues and Peyer's patches were excised. The feces were flushed with PBS and the tissues were cut into pieces in ice-cold PBS. Tissues are incubated with pre-digestion solution containing EDTA and dithiothreitol (DTT) in Hank's balance salt solution (HBSS) for 20 minutes at 37 ºC under slow rotation (40 g). The suspensions were vortexed and passed through a 100 μm cell strainer to remove epithelial cells, villus cells, subepithelial cells and intraepithelial lymphocytes (IELs). The remaining LPs and muscle layers were resuspended in digestion buffer containing collagenase D, DNase I, and dispase II and incubated at 37 ºC under slow rotation (40 g) for 20 minutes. After incubation, the suspensions were vortexed and passed through a 40 μm cell strainer. The supernatants were centrifuged for 10 min at 500 g at 20 ºC. The supernatants were discarded and the remaining pellets were resuspended in cold FACS buffer and centrifuged at 500 g for 10 minutes at 20 ºC.

The pellets were resuspended in 10 ml of the 40 % fraction of the Percoll solution and carefully overlayed on top of 5 ml of the 80 % fraction of the Percoll solution. The 40/80 Percoll gradient was centrifuged for 20 minutes at 1000 g at 20 ºC without brakes. The cells were resuspended in cold PBS (5 % FBS, 1 % Penicillin/Streptomycin) for flow cytometry analysis (Weigmann et al., 2007)

Flow Cytometry

Flow cytometric analysis of immune cells was performed as we previously described (Ji et al., 2014; Ji et al., 2012). Lymphocytes isolated from colonic lamina propria were washed and resuspended in cold PBS (5 % FBS, 1 % Penicillin/Streptomycin). The cell suspension was stained with either 1:100 or 1:200 fluorochrome- or biotin conjugated antibodies against B220 (RA3-6B2), CD4 (GK1.5), CD8 (YTS169), Gr-1 (RB6-8C5), CD11b (M1/70), CD69 (H1.2F3), IgA (RMA-1), avidin-FITC and isotype control antibodies were purchased from BioLegend or BD Biosciences. The samples were collected by BD LSR cell analyzer at the Flow Cytometry Core Facility at Cornell University and the data were analyzed using the CellQuest software (BD Biosciences) and Flowjo (Flowjo.com)

Histological analysis, Immunofluorescent staining and Immunohistochemistry

For the immunofluorescent staining, tissues were fixed in 10 % neutralized Formalin for 24 hours at 4 ºC, immersed in 30 % sucrose solution for 24 hours at 4 ºC and embedded Tissue-TeK OCT (optimum cutting temperature) solution and stored at -80 ºC until sectioning. The frozen intestinal sections were washed with PBS and blocked with 5 % normal donkey serums/ 1 % Triton X-100 in PBS for 45 minutes at room temperature. The sections were incubated with Biotinylated-IgA (1:100, BioLegend) overnight at 4 ºC. Next day, the sections were washed with PBS and incubated with Strepavidin-FITC (1:200) for 1 hour at room temperature. The sections

were washed at the end of the incubation and mounted with Prolong Gold Anti-fade Reagent with DAPI (Invitrogen). Fluorescent microscopic images were taken under a Nikon A1 confocal microscope at the Imaging Laboratory of the Brehm Center for Diabetes Research in University of Michigan.

For the immunohistochemistry (IHC) staining, tissues were fixed in 10 % neutralized Formalin and embedded in paraffin by the Cornell Histology Core Facility on a fee-for-service basis. The paraffin-embedded intestinal sections were rehydrated, boiled in 1 mM EDTA for antigen retrieval and stained with Histostain kit and DAB substrate from Invitrogen. Antibody used for IHCs is Ki-67 (1:50, Abcam). Sections stained with hematoxylin and eosin (H&E) and IHC were scanned using a brightfield ScanScope high-resolution digital image capture system (Aperio Technologies, Inc., Vista, CA) and photomicrographs were taken using Aperio ImageScope v.10.0 software.

For the assessment of the severity of experimental colitis, histological scores of the distal colon were blindly determined based on goblet cell loss, crypt abscesses, immune cell infiltration and epithelial erosion (0, none; 1, low; 2, moderate; 3, high; 4, maximal) as previously described (Wirtz et al., 2017).

Statistical analysis

Results are expressed as mean ± SEM. Comparisons between groups were made by unpaired two-tailed Student's t-test unless otherwise indicated. Survival curves were compared by the log-rank (Mantel–Cox) test. $p < 0.05$ was considered statistically significant. All experiments were repeated at least two to three times, and representative data are shown.

4.3 RESULTS

4.3.1. Defect in Gut Associated Lymphoid Tissue (GALT) development and reduced IgA production in in *Sel1L*^{CD19} mice.

The initial analysis revealed an aberrant reduction in the total number of Peyer's patches on the small intestine of *Sel1L*^{CD19} mice (Figure 4.1A). Moreover, the IHC analysis showed a dramatic reduction in the number of proliferating cells (Ki67+ cells) in the Peyer's patches of *Sel1L*^{CD19} mice, suggesting a reduction in germinal center B cells (Figure 4.1B). Flow cytometry analysis of Peyer's patches and mesenteric lymph node (mLN) also revealed a significant decrease in B220^{+}IgA^{+} cells (Figure 4.1C) in *Sel1L*^{CD19} mice. Consistent with the reduced IgA^{+} plasma cells, the total secreted IgA level in fecal samples of *Sel1L*^{CD19} mice was also reduced (Figure 4.1D). Of note, despite the reduction in IgA producing B cells, the circulating IgG and IgM concentrations remain comparable under steady state and after nitrophenylated-chicken gamma globulin (NP-CGG) antigen immunization (data not shown). Finally, immunofluorescent analysis of the large intestine revealed, that in comparison to the *Sel1L*$^{f/f}$ mice, IgA-expressing plasma cells in the lamina propria were also substantially reduced in *Sel1L*^{CD19} mice (Figure 4.1E).

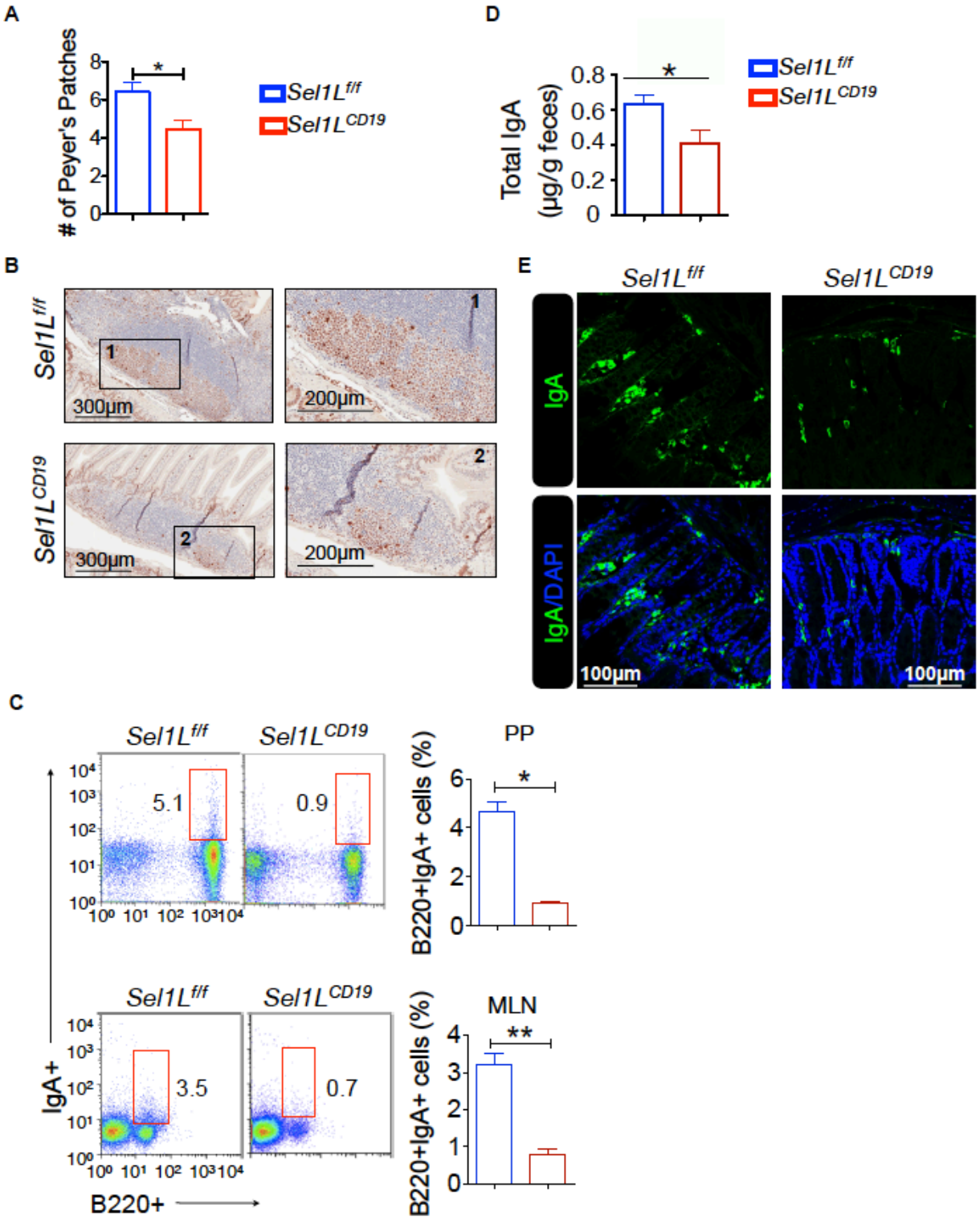

Figure 4. 1 Defect in Gut Associated Lymphoid Tissue (GALT) development and reduced IgA production in in *Sel1L*^{CD19} mice.

(A) The number of Peyer's patches (PP) from *Sel1L*$^{f/f}$ mice and *Sel1L*^{CD19} mice. (B) Immunohistochemistry of Ki67 of PP. Note decreased germinal center size. (C) Flow cytometric analysis of IgA^{+} B cells from PP and mesenteric lymph nodes (MLN) of *Sel1L*$^{f/f}$ mice and *Sel1L*^{CD19} mice with quantitation shown on the right (D) Total IgA levels in feces of *Sel1L*$^{f/f}$ mice and *Sel1L*^{CD19} mice (E) Representative confocal microscopic images of IgA+ B cells (green) in lamina propria of colon. Data are representative of two independent experiments in (A-E), n= 5 *Sel1L*$^{f/f}$ and 5 *Sel1L*^{CD19} Values shown as mean ± s.e.m.; **P< 0.01, ***P<0.001 by two-tailed Student's t-test.

4.3.2. *Sel1L*^{CD19} mice are more susceptible to DSS induced colitis and the phenotype is transmissible

Human and mice with IgA deficiency or mice with lower number of high-affinity IgA have been shown to be more susceptible to colitis (Cao et al., 2012; Cataldo et al., 1998; Friman et al., 2002; Malik et al., 2016; Meini et al., 1996; Murthy et al., 2006; Okai et al., 2016). To address whether reduced IgA producing plasma cells in GALT leads to dysregulated inflammatory response, mice were treated with 3 % DSS in water for 5 days. As we have speculated, *Sel1L*^{CD19} mice developed more severe colitis compared to singled-housed JAX B6 (SH) control mice (Figure 4.2A). The knockout mice displayed colon shortening, a typical clinical features of colonic inflammation (Figure 4.2B). Histological analysis of distal colon revealed almost complete loss of crypts and goblet cells, massive immune cell infiltration, transmural edema and increased colonic mucosal erosion/ulceration in DSS-treated *Sel1L*^{CD19} mice at day 10 (Figure 4.2C-D). Unexpectedly, co-housed wild type littermate *Sel1L*$^{f/f}$ mice also became more susceptible to colitis when compared to JAX B6 (SH) control (Figure 4.2A-D). The flow cytometry analysis of the mLN of these 3 cohorts, under experimental colitis revealed that, while B cell population was dramatically decreased only in *Sel1L*^{CD19} mice (Figure 4.3A), the neutrophil infiltration was significantly increased in both *Sel1L*^{CD19} and *Sel1L*$^{f/f}$ mice. (Figure 4.3B) when compared to JAX B6 (SH) mice. The activated T cell populations ($CD69^+CD4^+$ and $CD69^+CD8^+$ T) in *Sel1L*^{CD19} and *Sel1L*$^{f/f}$ mice were also higher that the JAX B6 (SH) mice, which is consistent with the histological analysis of the distal colon of these mice (Figure 4.3C-D).

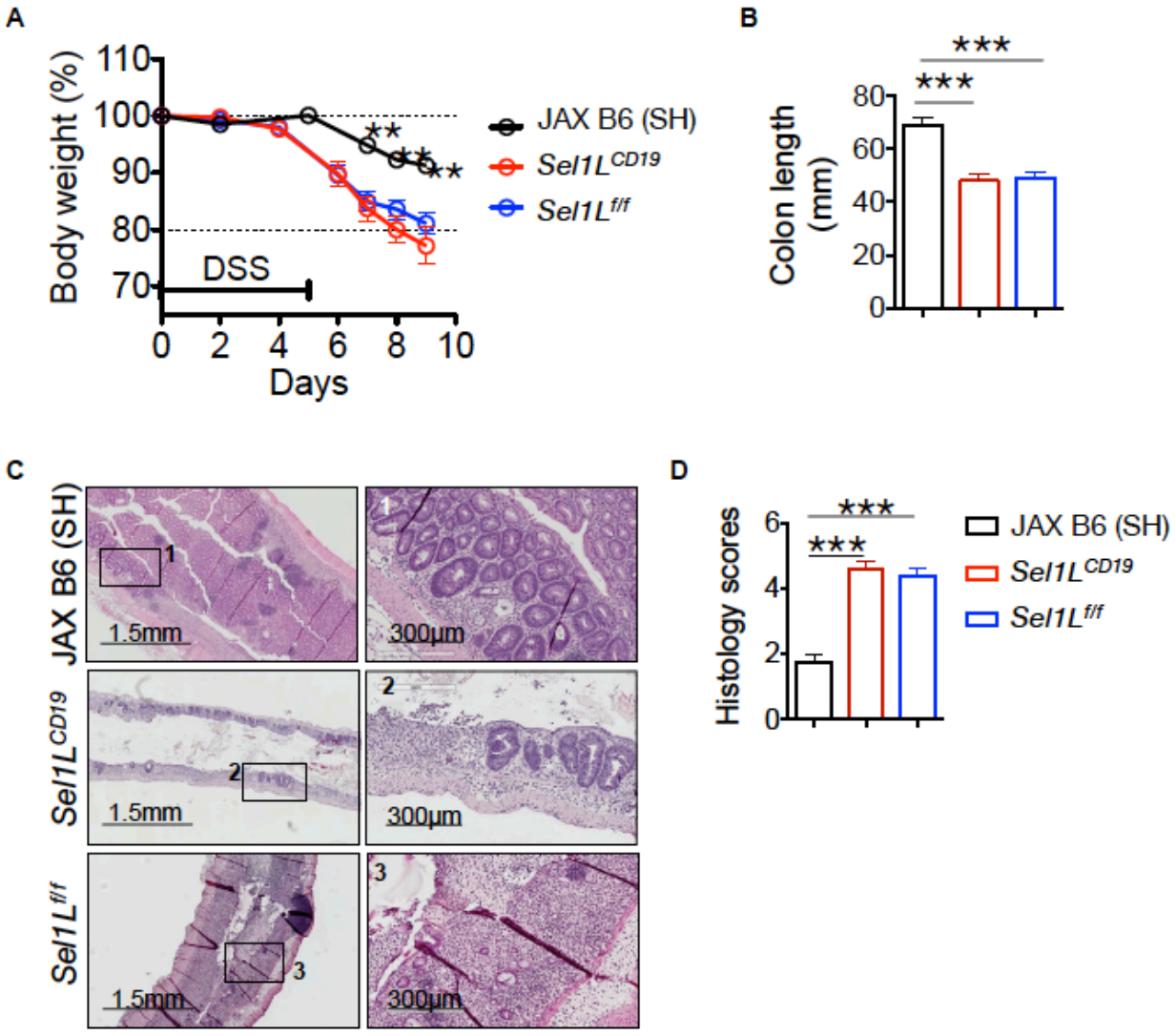

Figure 4. 2 *Sel1L*^{CD19} and *Sel1L*$^{f/f}$ mice are highly susceptible to DSS induced colitis.

(A-D) 8-week-old littermates and JAX B6 single-housed (SH) mice were treated with 3 % DSS for 5 days followed by fresh water. (A) Body weight change. (B) Colon length on day 9. (C) Haematoxylin and eosin (H&E) images of colon on day 9 showing epithelial cell ulceration, severed edema and regenerative crypts. (D) Inflammatory score of colon on day 9 as a blind study. 0, none; 1, low; 2, moderate; 3, high; 4, maximal. Data are representative of two independent experiments in (A-D), n= 5 each Values shown as mean ± s.e.m.; **P< 0.01, ***P<0.001 by two-tailed Student's t-test.

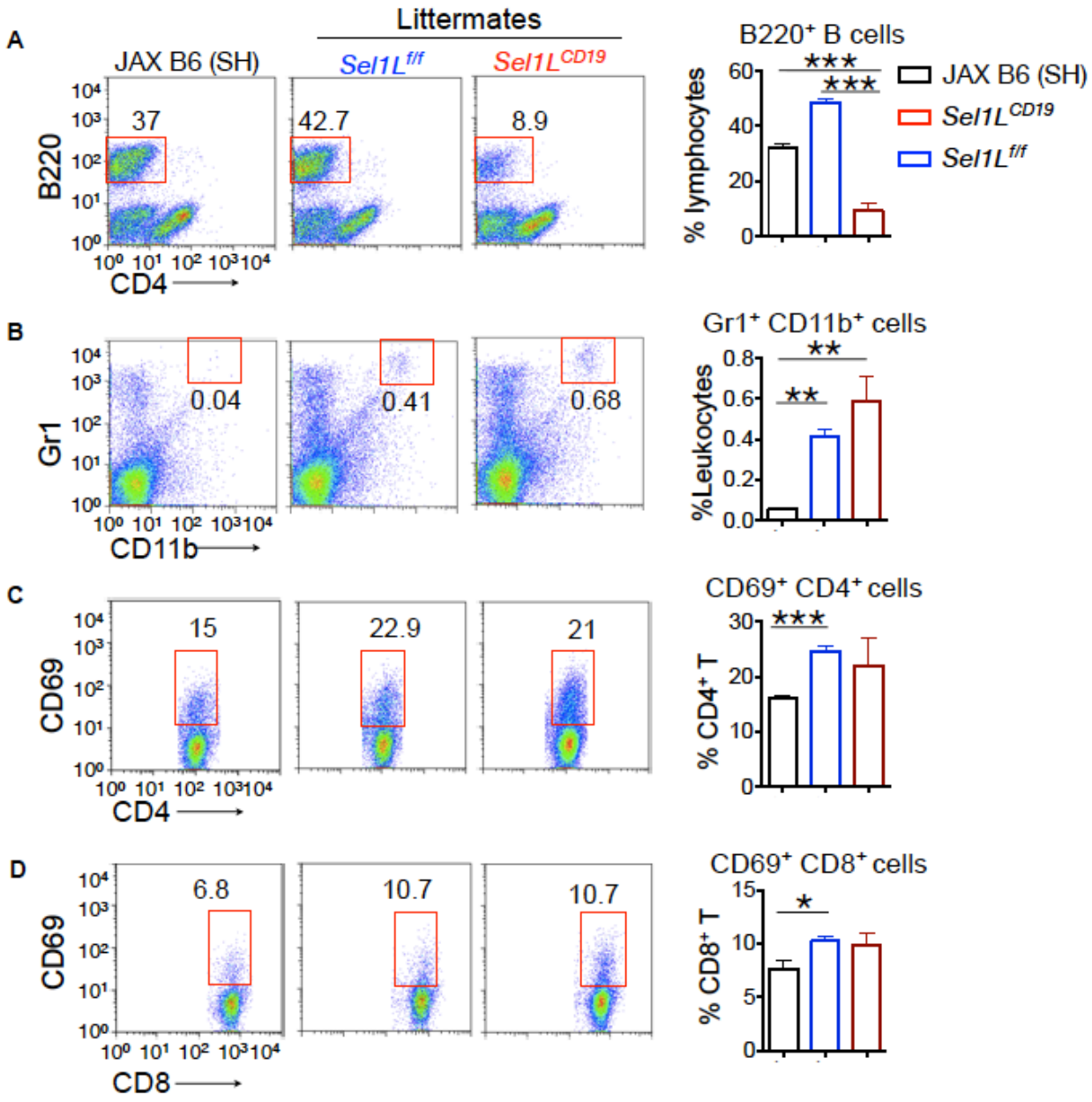

Figure 4. 3 Elevated immune cell activation in DSS treated *Sel1L^{CD19}* and *Sel1L$^{f/f}$* mice.

(A-D) Flow cytometric analysis of MLN cell isolated from *Sel1L$^{f/f}$* mice, Sel1LCD19and JAX B6 single-housed (SH) on day 9 post-DSS treatment: (A) B cells, (B) neutrophils, (C)activated CD4+ T cells, and (D) activated CD8+ T cells. Quantification in percentage shown on the right. Data are representative of two independent experiments in (A-D), n= 5 *Sel1L$^{f/f}$* and 5 *Sel1L^{CD19}*

and 5 JAX B6(SH). Values shown as mean ± s.e.m.; **P< 0.01, ***P<0.001 by two-tailed Student's t-test.

4.3.3. Colitogenic microbiota not transmissible to non-littermate wildtype mice.

To assess whether the differences in colitis severity observed between groups of littermates and JAX B6 (SH) mice were driven by differences in their intestinal microbiota, *Sel1L*^{CD19} mice were co-housed with JAX B6 mice at 1:1 ratio, at 3 weeks of age, for 4 weeks before chemically inducing colitis. To our surprise, the co-housed JAX B6 mice did not develop severe colitis as we had speculated, but remained comparable to JAX B6 (SH) mice (Figure 4.4A). Consistently, the histological analysis revealed similar levels of immune cell infiltration between two cohorts of JAX B6 mice (Figure 4.4B-C) and the structure of the crypt remained intact (Figure 4.4D)

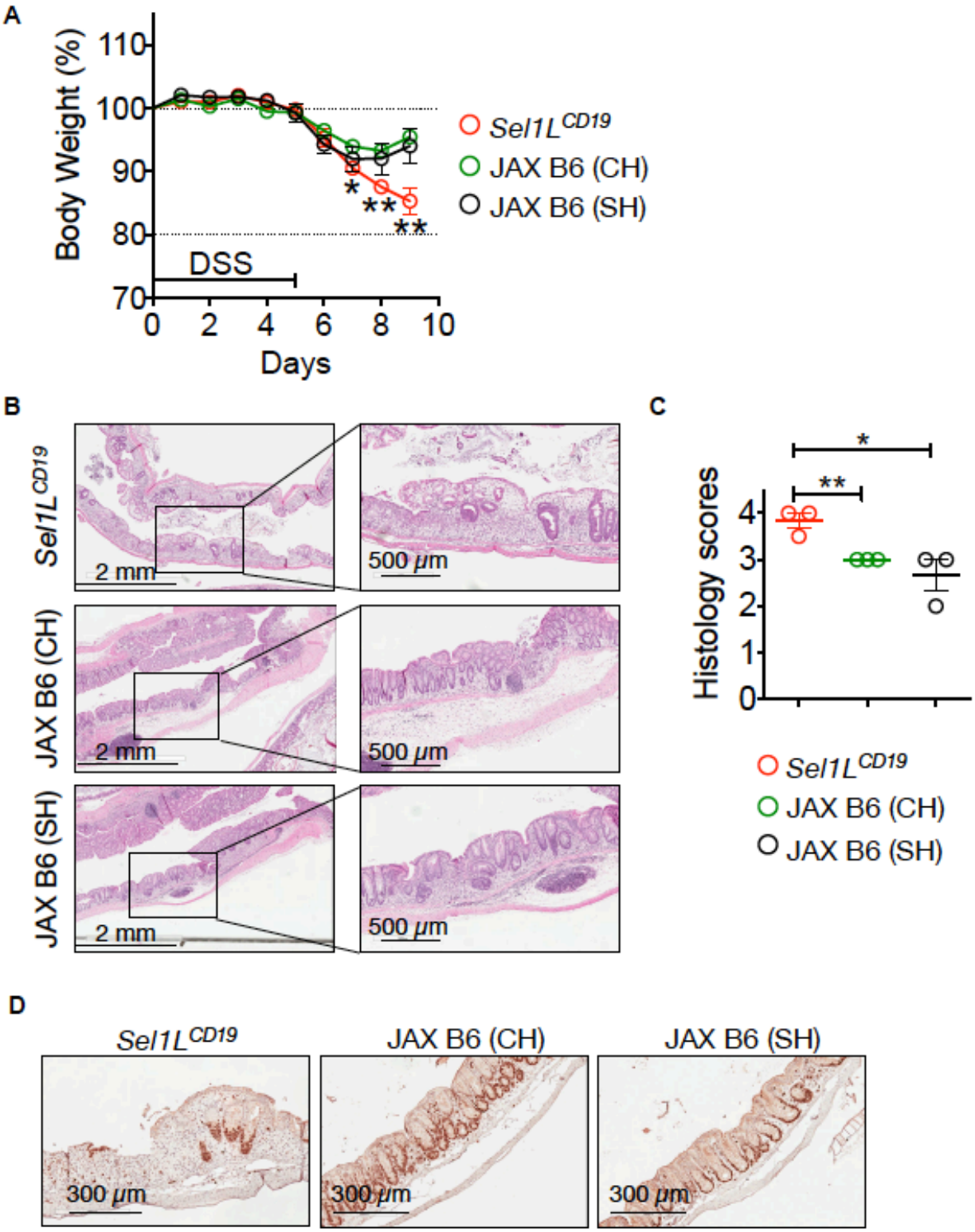

Figure 4. 4 Colitogenic microbiota are not transmissible to non-littermate wildtype mice.

(A-D) 3-week-old *Sel1L*^{CD19} mice and JAX B6 mice were co-housed for 4 weeks, followed by 3% DSS treatment for 5 days. (A) Body weight change. (B) H&E images of colon on day 9 showing epithelial cell ulceration, severed edema and regenerative crypts. (C) Inflammatory score of colon on day 9 as a blind study. 0, none; 1, low; 2, moderate; 3, high; 4, maximal. (D) Immunohistochemistry of Ki67 of colon collected on day 9 post-DSS treatment. Data are representative of two independent experiments in (A-D), n= 5 *Sel1L*$^{f/f}$ and 5 *Sel1L*^{CD19} Values shown as mean ± s.e.m.; **P< 0.01, ***P<0.001 by two-tailed Student's t-test

4.3.4. Colitogenic phenotype is vertically transmissible, only from *Sel1L*^{CD19} female mice.

Another route by which mice share intestinal microbiota is through mother to pups., as pups receive their first microbial exposure during vaginal delivery, from the vaginal and fecal microbiota of the mother. To further assess whether the intestinal microbiota, transmitted from mother to pups, contributes to differential inflammatory response, we performed cross-fostering experiments. Newborn JAX B6 mice cross-fostered (CF) at birth with *Sel1L*^{CD19} mother exhibited more severe colitis compared to non-cross-fostered JAX B6 mice (Figure 4.5A-D). Interestingly, newborn *Sel1L*^{CD19} mice exhibited milder body weight loss and milder colon shortening (Figure 4.5E-F) compared to non-cross-fostered *Sel1L*^{CD19} mice, while histological analysis revealed similar immune cell infiltration between two cohorts (Figure 4.5G-H).

Studies have shown that newborn relies on passive secretory IgA (sIgA) in breast milk to shape the composition of microbiota, which persist into adulthood (Brandtzaeg, 2010; Janoff et al., 2012; Macpherson et al., 2012; Rogier et al., 2014). Furthermore, epidemiological studies suggest that early exposure to breast milk reduces the risk of developing inflammatory bowel disease (IBD) in children (Barclay et al., 2009). To assess whethere the sIgA in breastmilk from *Sel1L*^{CD19} mother is the underlying factor which induces the propagation of colitogenic microbiota in offsprings, generated *Sel1L*^{CD19} offspring that would receive different amount of passive sIgA in breast milk. By breeding *Sel1L*$^{f/f}$ dams, with *Sel1L*^{CD19} males, we generated *Sel1L*^{CD19} offsprings that received breast milk abundant of passive sIgA, compared to offsprings from *Sel1L*^{CD19} dams and *Sel1L*$^{f/f}$ male (Figure 4.6A). The *Sel1L*^{CD19}♂ offsprings which received breastmilk with abundant IgA, exhibited much milder colitis when compared to *Sel1L*^{CD19}♀ offsprings, which received breastmilk with less abundant IgA (Figure 4.6B). The colon shortening and immune cell infiltration in *Sel1L*^{CD19}♂ offsprings were also less severe than that of *Sel1L*^{CD19}♀ offsprings (Figure 4.6C-D)

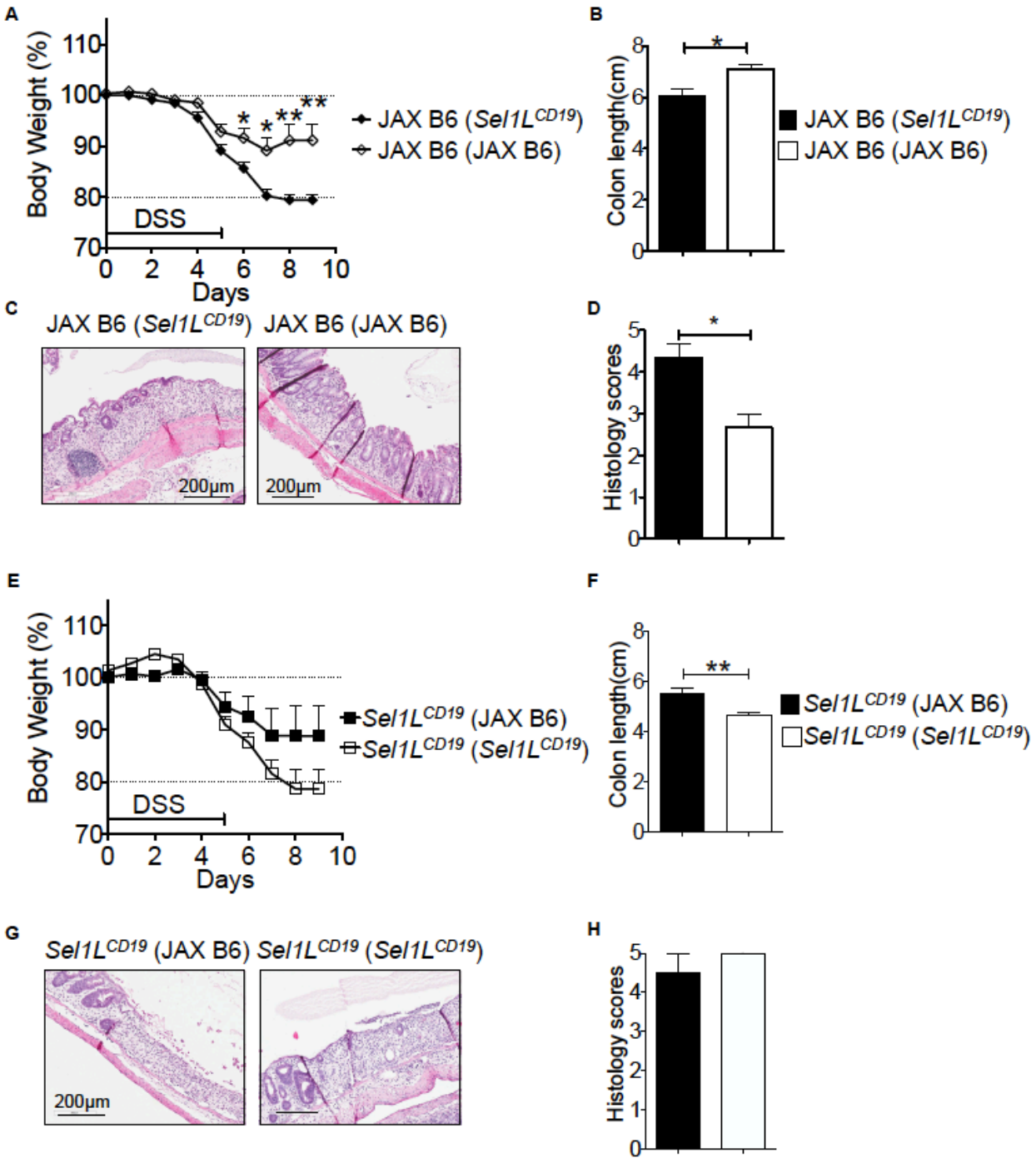

Figure 4. 5 Colitogenic phenotype is vertically transmissible, only from *Sel1L*^{CD19} female mice.

(A-H) Newborn *Sel1L*^{CD19} and JAX B6 mice were swapped between their respective mothers (cross-fostered), followed by induction of acute DSS colitis at 8 weeks of age.

(A, E) Body weight change and (B, F) colon length were measured in *Sel1L*^{CD19} and JAX B6 mice cross-fostered with either *Sel1L*^{CD19} or JAX B6 mothers. (C, G) H&E images of colon and (D, H) Inflammatory score of colon were analyzed on day 9 post 3 % DSS treatment. Data are representative of two independent experiments in (A-H), n= 5 mice each Values shown as mean ± s.e.m.; **P< 0.01, ***P<0.001 by two-tailed Student's t-test.

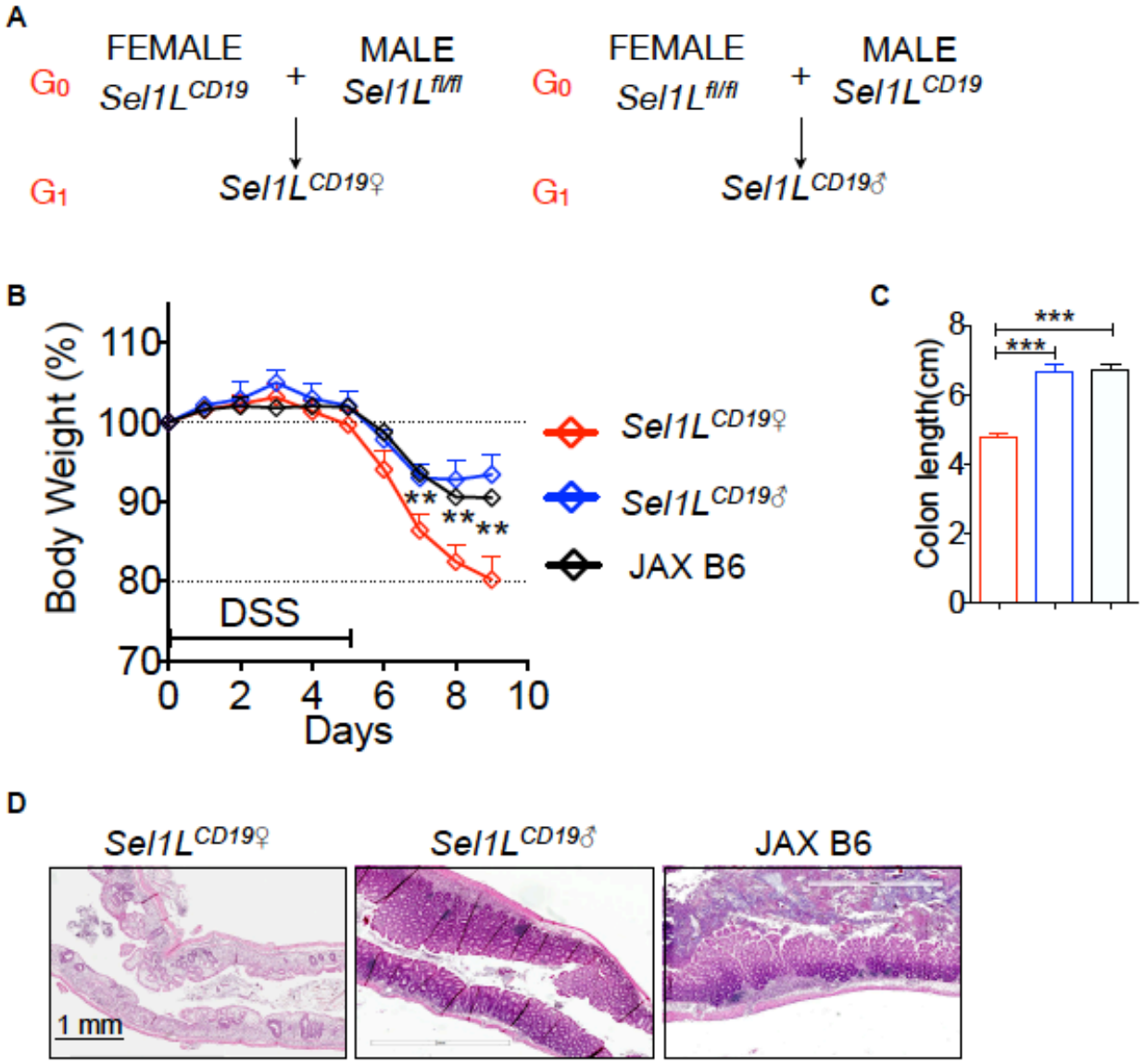

Figure 4. 6 Early exposure to reduced passive secretory IgA promotes susceptibility to colitis.

(A) Schematic diagram of *Sel1L*$^{CD19\,♀}$ and *Sel1L*$^{CD19\,♂}$ mice generation. (B-C) Body weight change and colon length were measured 9-days post 3 % DSS treatment. (D) H&E images of colon on 9-days post 3 % DSS treatment. Data are representative of two independent experiments in (B-D), n= 5 *Sel1L*$^{CD19\,♀}$ and 5 *Sel1L*$^{CD19\,♂}$ Values shown as mean ± s.e.m.; **P< 0.01, ***P<0.001 by two-tailed Student's t-test.

4.4 DISCUSSION

Previously, we have demonstrated Sel1L-Hrd1 ERAD as a key regulator of B cell development by targeting pre-BCR for proteasomal degradation in a BiP-dependent manner (Ji et al., 2016). Here, we demonstrated that loss of Sel1L-Hrd1 ERAD in B cells compromises mucosal innate immune response, which promote propagation of colitogenic bacteria.

In mice intestine, approximately 50 % of IgA secreting plasma cells derive from IgM expressing cells in the peritoneal cavity, known as B1 cells (Kroese et al., 1989) . While these precursors response rapidly against potential pathogen, the end product has relatively low affinity. The remaining 50 % of IgA secreting plasma cells derived from B2 cells in Peyer's patches and the response, although slower, is of high affinity and specificity (Tissot et al., 1995). Using flow cytometry analysis, we have previously shown decreased number of circulating $B220^{+}IgM^{+}$ B cells and peritoneal B1 cells in $Sel1L^{CD19}$ mice when compared to $Sel1L^{f/f}$ mice. In the current study, we further observed a significant decrease in size and number of Peyer's patches and MLN, as well as decrease in $B220^{+}IgA^{+}$ cells from these tissues. The number of plasma cells secreting IgA from colon lamina propria was also significantly reduced. Consequently, total secretory IgA(sIgA) measured in the fecal sample from $Sel1L^{CD19}$ mice was significantly lower than that of in $Sel1L^{f/f}$ mice.

The mammalian intestine provides a niche to an estimated 100 trillion microorganisms, which majority of them maintain a symbiotic relationship with the host. While epithelial surfaces provide a physical barrier to prevent invasion by non-commensal microbes, antibody secretion by B cells, especially secretory sIgA, acts as the first line of antigen-specific immunity at the interface between the gut microbiota and the intestinal epithelium. IgA is the predominant antibody isotype in the mucosal tissue and comprises approximately 75 % of the total

immunoglobulin production in the mammalian body (Hurley et al., 2011). However, in serum, IgA antibodies make up a minor population among all isotypes, underscoring the importance of sIgA in mucosal immunity. In the present study, the effect of reduced sIgA was reflected in increased susceptibility to chemically induced colitis in *Sel1L*^{CD19} mice. Interestingly, circulating IgG and IgM level in serum after immunization with NP-CGG antigen was comparable between two cohorts. These findings suggest that ERAD deficiency in B cells has drastic consequences on mucosal immunity, presumably via sIgA.

There are different ways in which the composition of the microbial communities is shaped. First, during vaginal delivery, the new born mammal receives its first microbial exposure from the vaginal and fecal microbiota of the mother as it leaves the womb (Dominguez-Bello et al., 2010; Hansen et al., 2012; Palmer et al., 2007) . Second, during postnatal life and during adulthood, the microbiota composition can change through cohabitation and coprophagy in mice (Elinav et al., 2011; Hildebrand et al., 2013). In line with these findings, *Sel1L*$^{f/f}$ littermates, which have comparable level of IgA production as the irrelevant wildtype mice JAX B6, had succumbed to DSS induced colitis as its littermate *Sel1L*^{CD19}mice. However, JAX B6 mice co-housed with *Sel1L*^{CD19} mice for 4 weeks, right after weaning, exhibited resistance to colitis. The transfer of colitogenic microbiota to JAX B6 pups was observed only after the pups had been cross-fostered by *Sel1L*^{CD19}dam. Based on this data, we speculate that the aberrant microbiota, which promote exaggerated inflammatory response in *Sel1L*^{CD19}mice and *Sel1L*$^{f/f}$ mice is only transmissible from dam to pups during birth (also known as vertical transmission) and is responsible for permanently shaping the life-long microbiota ecology.

As newborns are unable to produce its own sIgA (Rogier et al., 2014), the initial immune protection, which the newborn relies on, is provided through breast milk-derived sIgA antibodies;

also known as passive immunity. The initial passive immunity in suckling infants shape the composition of the gut microbiota and promote a mutualistic relationship with the host (Macpherson et al., 2012; Rogier et al., 2014). After weaning, plasma cells in the intestinal lamina propria produces IgA and provide lifelong antibody-mediated immune protection. Studies have demonstrated dysfunctional sIgA as a root of pathogenesis in IBD (Murthy et al., 2006). It has been demonstrated that cross-fostering, within 48 hours of birth, induces a permanent microbiota shift which is shaped by the nursing mother (Daft et al., 2015). Interestingly, in our cross-fostering experiment, we found that *Sel1L*^{CD19} pups fostered by JAX B6 dam exhibited milder inflammatory response under DSS treatment. Furthermore, *Sel1L*^{CD19}offspring from *Sel1L*$^{f/f}$ dam and *Sel1L*^{CD19}male breeding pairs were also protected from exacerbated inflammatory response. It has also been reported that a defect in passive sIgA of mothers can induce alteration in gut microbiota composition, subsequently leading to propagation of opportunistic and pathogenic microbes, contributing to the etiology of IBD (Rogier et al., 2014). In line with these findings, it can be speculated that early exposure of passive sIgA from *Sel1L*^{CD19} dam may be the primary contributing factor in development, propagation and transfer of colitogenic microbiota in pups.

Taken together, our findings suggest that Sel1L-Hd1 ERAD pathway in B cells is indispensable for innate mucosal immunity and subsequently impacts gut microbiota ecology through passive sIgA. Nevertheless, the mechanism by which Sel1L-Hrd1 ERAD regulates IgA production and the affinity of IgA remains unknown and needs further investigation.

CHAPTER FIVE: SUMMARY AND FUTURE DIRECTIONS

This thesis focused on delineating the physiological importance of ER-associated degradation (ERAD) in B cell development and its downstream impact on mucosal immunology. Humoral immunity involves a stepwise differentiation process and checkpoints during B-cell development in bone marrow, before reaching the antibody-producing mature stage. This complicated, yet refined development pathway requires the tightly orchestrated expression of many growth factors and cell surface-associated proteins. Hence, the magnitude of functional B cells reaching maturity depends on upregulation and downregulation of these proteins in a timely manner.

Along with UPR, ERAD is one of the quality control pathways responsible for maintaining ER homeostasis. Among several known mammalian ERAD complexes, Hrd E3 ligase and its adaptor protein, the Hrd1-Sel1L complex, targets the misfolded proteins in the ER for cytosolic proteasomal degradation. Various studies using tissue specific knockout animal models have revealed the physiological and pathological significance of ERAD and unveiled its tissue-specific endogenous protein substrates. In these studies, defective ERAD machinery resulted in accumulation of misfolded proteins and subsequently leading to either cell death or disease pathogenesis.

To this end, we generated B cell specific Sel1L knockout mice, under the assumption that ERAD deficiency would lead to: 1) impaired B cell development due to accumulation of misfolded proteins and; 2) defective B cell function. Indeed, we observed development block from large pre-B cell to small pre-B cell stage in B cells with ERAD deficiency (Chapter 3). While we hypothesized that impairment of an ERAD could induce ER stress response, Sel1L/ERAD deficiency in B cell resulted in a development block in a Chop-independent manner. Rather, ERAD regulates the turnover and signaling of pre-BCR. In the absence of

Sel1L/ERAD, the pre-BCR protein complex is stabilized and accumulated in large pre-B cells, leading to enhanced pre-B cell proliferation and a block of further B cell differentiation. Moreover, only pre-BCR, not BCR, complex is recognized as an endogenous substrate of Sel1L-Hrd1 ERAD. To our surprise, Sel1L/ERAD deficiency does not seem to affect either plasma cell differentiation or antibody production (IgG/IgM) of B cells in response to antigen immunization.

Further study in Appendix A revealed that ERAD deficiency in B cells compromises mucosal immune response and leads to propagation of transmissible colitogenic microbiota. While B cell development by Sel1L-Hrd1 ERAD deficiency did not interfere with IgG and IgM antibody production in response to antigen immunization, the total amount of IgA secreted into gut lumen was decreased. Under chemically induced colitis, the $Sel1L^{CD19}$ mice exhibited exaggerated inflammatory response. Interestingly, the wildtype littermate, which produces comparable levels of IgA, also exhibited exaggerated inflammatory response under DSS treatment. The current progress demonstrates that the colitogenic phenotype is only transmissible from Sel1L-Hrd1 ERAD deficient dam to pups. Moreover, Sel1L Hrd1 ERAD knockout pups bred from SeL1L-deficient male and Sel1L-sufficient female also exhibit very mild inflammatory response to DSS-induced colitis. Taken together, this study shows that Sel1L-Hrd1 ERAD is critical for IgA secretion which is responsible for maintaining gut homeostasis. Moreover, the transmissible colitogenic phenotype, between littermates, suggests that secretory IgA transferred from $Sel1L^{CD19}$ female to pups may shape the lifelong intestinal hoemostasis.

Many interesting questions are raised from this dissertation. First, using non-reducing sucrose gradient fractionation analysis, we observed accumulation of high molecular weight complex in the absence of Sel1L-Hrd1 ERAD. Hence, to confirm whether this high molecular weight complex is fully assembled whole pre-BCR complex or aggregates of individual pre-BCR

compartments (Igμ, λ5 or VpreB), further immunoprecipitation and western blot assays are required. Secondly, how ERAD distinguishes the pre-BCR complex in developing B cells versus the BCR complex in mature B cells, which share the common heavy chain Igμ, remains to be elucidated. Consequently, it would be interesting to compare the interaction between ERAD versus the individual pre-BCR components or different combination of the pre-BCR components through in vitro mechanistic studies. Although the role of autophagy in B cell development has not been addressed in the current study, it is worth investigating whether and how autophagy affects ER homeostasis and pre-BCR expression. Hence, dissecting the crosstalk between two protein-quality control pathways, ERAD and autophagy, in B cell biology may shed further mechanistic understanding in ER homeostasis and protein quality control during cell differentiation and development. Last but not the least, more in depth studies are necessary to dissect the detailed mechanisms by which the ERAD modulates secretory IgA production and affinity in B cells, but not IgG or IgM. In addition, considering the link between passive secretory IgA (sIgA) found in breast milk and the lifelong intestinal homeostasis (Rogier et al., 2014), it is also worthwhile to investigate the microbiota composition in suckling neonates to further identify the colitogenic microbiota via bacterial 16S rRNA gene sequencing. We propose that monoassociation studies, following the identification of the colitogenic microbiota in *Sel1L*^{CD19} mice will shed more insight into understanding pathogenesis of inflammatory bowel disease.

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Zhou, M., & Schekman, R. (1999). The engagement of Sec61p in the ER dislocation process. *Mol Cell, 4*(6), 925-934.

Curriculum Vitae

BIOSKETCH

NAME Hana Kim	POSITION TITLE Ph.D. candidate
EMAIL hk739@cornell.edu	DISSERTATION ADVISOR Dr. Ling Qi, Professor of Molecular & Integrative Physiology

A. EDUCATION/TRAINING

9/2011 – 8/2017 Ph.D., Immunology and Infectious Disease
Cornell University, Ithaca, NY (Advisor: Dr. Ling Qi, Professor)
GPA: 3.63

9/2008 - 7/2011 M.S. Biological Sciences California State Polytechnic University, Pomona, CA (Advisor: Dr. Jill Adler-Moore, Professor)
GPA: 3.51

6/2005 - 6/2008 B.S. Microbiology California State Polytechnic University, Pomona, CA (Advisor: Dr. Jill Adler-Moore, Professor)
GPA: 3.48

B. RESEARCH POSITIONS

8/2016 - present Research Intern. *University of Michigan, Ann Arbor, MI* (Advisor: Dr. Ling Qi, Professor)

9/2011 - present PhD Candidate. *Cornell University, Ithaca, NY* (Advisor: Dr. Ling Qi, Associate Professor)

9/2008 - 7/2011 Graduate research student. *California State Polytechnic University, Pomona, CA* (Advisor: Dr. Jill Adler-Moore, Professor)

6/2008 - 8/2010 Research Assistant. *California State Polytechnic University*, *Pomona, CA* (Advisor: Dr. Jill Adler-Moore, Professor)

9/2007 - 6/2008 Undergraduate research student. *California State Polytechnic University*, *Pomona, CA* (Advisor: Dr. Jill Adler-Moore, Professor)

C. OTHER WORK EXPERIENCES

8/2016 - present Laboratory Manager, *University of Michigan, Ann Arbor, MI*

1/2016 - 7/2016 Laboratory Manager, *Cornell University, Ithaca, NY*

9/2013 - 5/2016 Teaching Assistant, *Cornell University, Ithaca, NY*

9/2008 - 5/2011 Teaching Assistant, *California State Polytechnic, Pomona, CA*

D. PUBLICATIONS

1) Ji Y*, Sun S*, Shrestha N, Darragh L, Shirakawa J, Xing Y, He Y, Carboneau B, Kim G, **Kim H**, Shu X, An D, Ma M, Oberhozler J, Soleimanpour S, Gannon M, Liu C, Naji A, Kulkami R, Wang Y, Kersten S, Qi L. Toll-like receptors 2 and 4 act as brakes on adaptive β-cell mass expansion in diet-induced obesity. Nature immunology. *Under revision*

2) Ji Y*, **Kim H*,** Yang L, Sha H, Roman C, Long Q, Qi L. The Sel1L-Hrd1 endoplasmic reticulum-associated degradation complex manages sa key checkpoint in B cell development. Cell Report. 2016 September. PMID 27568564 (*Contributed equally)

3) Sun S*, Shi G*, Sha H, Ji Y, Han X, Shu X, Ma H, Inoue T, Gao B, **Kim H,** Bu P, Guber RD, Shen X, Lee AH, Iwawaki T, Paton AW, Paton JC, Fang D, Tsai B, Yates JR 3rd, Wu H, Kersten S, Long Q, Duhamel GE, Simpson KW, Qi L. IRE1α is an endogenous substrate of endoplasmic reticulum associated degradation. Nature cell biology. 2015 December. PMID 26551274

4) **Kim H*,** Bhattacharya A*, Qi L. Endoplasmic reticulum quality control in cancer: Friend or foe. Semin Cancer Biol. 2015 Mar 18. PMID 25794824 (*Contributed equally)

5) Ji Y*, Sun S*, Goodrich JK, **Kim H**, Poole AC, Duhamel GE, Ley RE, Qi L. Diet-indued alterations in gut microflora contribute to lethal pulmonary damage in TLR2/TLR4-deficient mice. Cell Rep. 2014 Jul 10. PMID 24953658

6) Iwata TN, Cowley TJ, Sloma M, Ji Y, **Kim H**, Qi L, Lee SS. The transcriptional co-regulator HCF-1 is required for INS-1 β-cell glucose-stimulated insulin secretion. PLoS One. 2013 Nov 8. PMID 24250814

7) Adler-Moore, J., M. Munoz, **H. Kim**, J. Romero, T. Tumpey, H. Zeng, C. Petro, W. Ernst, S. Kosina, G. Jimenez, and G. Fujii. 2011. Characterization of the murine Th2 response to immunization with liposomal M2e influenza vaccine. Vaccine. 2011 May 3. PMID 21545821

E. ABSTRACT PRESENTATIONS:

- *The Sel1L-Hrd1 Endoplasmic Reticulum-Associated Degradation Complex Manages a Key checkpoint in B cell development*
  Hana Kim, Yewei Ji, Liu Yang, Ling Qi
  October 13th, 2016
  3rd Annual Protein Folding Diseases Initiative Symposium, Ann Arbor, Michigan
- *The Sel1L-Hrd1 Endoplasmic Reticulum-Associated Degradation Complex Manages a Key checkpoint in B cell development*
  Yewei Ji, Hana Kim, Liu Yang, Ling Qi
  June 18th, 2015
  FASEB Science Research Conferences – From Unfolded Proteins in the ER to Disease, Saxtons Rivers, Vermont
- *B cell Specific Sel1L Ablation Promotes Propagation of Colitogenic Microbiota*
  Hana Kim, Yewei Ji, Iris Sun, Liu Yang, Ling Qi
  February 4th, 2014
  Biological and Biomedical Sciences Recruitment 2014 Poster Session, Ithaca, NY
- *Broad Neutralization of Type A Influenza Virus Strains by Anti-M2 Ectodomain Antibodies*
  Hana Kim[1], Juan Romero[1], Chris Petro[1], William Ernst[2], Gary Fujii[2], Hui Zeng[3], Terrence Tumpey[3], Jill Adler-Moore[1]
  October 28th, 2010
  10th Agricultural Research Institute Showcase Pomona, CA
- *Broad Neutralization of Type A Influenza Virus Strains by Anti-M2 Ectodomain Antibodies*
  Hana Kim[1], Juan Romero[1], Chris Petro[1], William Ernst[2], Gary Fujii[2], Hui Zeng[3], Terrence Tumpey[3], Jill Adler-Moore[1]
  July 17-21, 2010
  American Society for Virology 29th Annual Meeting

F. Research Skills/ Competencies

1) Research skills:

- Extensively trained in primary B cell isolation, activation, proliferation and differentiation.
- Highly skilled in animal handling, which includes, but not limited to intraperitoneal injection, tail vein injection, bone marrow transplantation, glucose tolerance test/ Insulin sensitivity test
- Trained in cryosectioning, immunohistochemistry (IHC) and immunofluorescence (IF) staining of mouse tissues such as spleen, pancreas, liver and intestine
- Trained in confocal microscopy and image analysis
- Highly skilled in designing and performing multicolor flow cytometry and analyze the data
- Extensively trained in immunoassays such as ELISA, ELISPOT, Multiplex
- Skilled in biochemistry techniques with experiences in protein isolation, immunoblot analysis and co-immunoprecipitation

2) Software:

- Knowledge on Microsoft Office, GraphPad Prism, FlowJo, Image J Analysis and Adobe Photoshop

3) Language proficiency:

- English and Korean (Native)

G. HONORS

2006-08 Golden Key International Honor Society, California State Polytechnic University, Pomona, CA
2006-08 Dean's List, California State Polytechnic University, Pomona, CA
2006-07 International Student Scholarships, California State Polytechnic University, Pomona, CA
2005-06 President's Honors List, California State Polytechnic University, Pomona, CA
2004-05 President's List, Mount San Antonio College, Walnut, CA
2002-04 Dean's List, Mount San Antonio College, Walnut, CA