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2 ***E3 Ligase UBR5 HECT domain mutations in lymphoma control maturation of B cells via***
3 ***alternative splicing***

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17 **RUNNING TITLE** – UBR5 HECT domain mutations in B cell development

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30 **KEY POINTS**

- 31
- 32 • Utilizing a novel mouse model mimicking MCL patient mutations, the loss of UBR5's HECT
33 domain causes alterations in B cell development.
 - 34 • UBR5 mutations lead to stabilization of UBR5 and aberrant splicing.
35

36 **ABSTRACT**

37 Coordination of a number of molecular mechanisms including transcription, alternative splicing,
38 and class switch recombination are required to facilitate development, activation, and survival of B
39 cells. Disruption of these pathways can result in malignant transformation. Recently, next generation
40 sequencing has identified a number of novel mutations in mantle cell lymphoma (MCL) patients
41 including the ubiquitin E3 ligase UBR5. Approximately 18% of MCL patients were found to have
42 mutations in UBR5 with the majority of mutations within the HECT domain of the protein which can
43 accept and transfer ubiquitin molecules to the substrate. Determining if UBR5 controls the maturation
44 of B cells is important to fully understand malignant transformation to MCL. To elucidate the role of
45 UBR5 in B cell maturation and activation we generated a conditional mutant disrupting UBR5's C-
46 terminal HECT domain. Loss of the UBR5 HECT domain leads to a block in maturation of B cells in
47 the spleen and up-regulation of proteins associated with mRNA splicing via the spliceosome. Our
48 studies reveal a novel role of UBR5 in B cell maturation by regulating alternative splicing of key
49 transcripts during B cell development and suggests UBR5 mutations may promote mantle cell
50 lymphoma initiation.

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55 INTRODUCTION

56 Mantle Cell Lymphoma (MCL) is a rare, aggressive form of Non-Hodgkin's Lymphoma
57 (NHL).¹ Although MCL represents only ~6% of NHL lymphoma cases, it has one of the highest
58 mortality rates of all lymphomas with only a 50% five year survival.² Given the high mortality
59 rate and propensity for recurrence, having better comprehension of mutations found in MCL
60 and how disease develops in B cells will open avenues for identifying new therapies. Recently,
61 the ubiquitin protein ligase E3 component n-recognin 5 (UBR5) was found mutated in ~18% of
62 patients with MCL.³ The majority of mutations identified in *UBR5* were frame shift mutations
63 found within its HECT domain, which can accept and transfer ubiquitin molecules to the
64 substrate, leading to a premature stop codon prior to the cysteine residue associated with
65 ubiquitin transfer.

66

67 UBR5 is a large ~300kDa protein HECT E3 ligase with a conserved carboxyl-terminal
68 HECT domain. In HECT E3 ligases, the N-terminal portion (N-lobe) of the enzyme interacts
69 with E2 ubiquitin-conjugating enzymes and determines substrate specificity while the C-
70 terminal HECT domain (C-lobe) contains a catalytic cysteine residue that binds ubiquitin.⁴ The
71 two lobes are connected by a flexible linker that allows for shifting orientation between N- and
72 C-lobes during ubiquitin transfer to allow for efficient movement of ubiquitin from the E3 ligase
73 to the substrate protein. UBR5 regulates a number of cellular processes including metabolism,
74 apoptosis, angiogenesis, gene expression, and genome integrity.⁵⁻¹⁰ Overexpression of UBR5
75 has been found in a number of cancers including ovarian, breast, hepatocellular, squamous
76 cell carcinoma, and melanoma.¹¹⁻¹⁴

77 Determining if, and at what stage – transcriptional, translational, or proteomic – UBR5
78 controls maturation of B cells is important for fully understanding B cell development and
79 lymphoma transformation. In order to elucidate the role of UBR5 in B cell maturation and
80 activation, we generated a conditional mutant disrupting the C-terminal HECT domain. Loss of
81 the HECT domain leads to a block in maturation of B cells, and follicular B cells are
82 phenotypically abnormal with low expression of IgD and high expression of IgM. Upon immune
83 stimulation, B cells lacking the HECT domain show decreased germinal center formation and
84 reduced antibody producing plasma cells suggesting both defects in phenotype and function
85 of mature B cells. Proteomic studies reveal up-regulation of proteins associated with mRNA
86 splicing via the spliceosome and indicates that UBR5 interacts with splicing factors (SF3B3,
87 SMC2, PRPF8, DHX15, SNRNP200, and EFTUD2). Our studies reveal a novel role of UBR5
88 in B cell maturation by regulating alternative splicing of key transcripts during B cell
89 development and suggests *UBR5* mutations in MCL lead to disease initiation.

90

91 **METHODS**

92 **Mice**

93 *Ubr5* HECT mutant mice were developed using Easi-CRISPR as previously published¹⁵
94 and crossed with *Mb1^{CRE}* mice (kind gift of Michael Reth). Floxed *Ubr5* alleles were validated
95 by PCR using primers designed to amplify targeted alleles (supplemental materials and
96 methods). For immune stimulation, mice were immunized by intraperitoneal injection with
97 1×10^8 sheep's red blood cells (SRBC) (Innovative Research). All mice were housed in a
98 pathogen-free facility at University of Nebraska Medical Center. Procedures performed were

99 approved by Institutional Animal Care and Use Committee of University of Nebraska Medical
100 Center in accordance with NIH guidelines.

101

102 **B cell isolation and culture**

103 Total bone marrow (BM) and splenocytes were isolated from 6-week-old mice. B220⁺ cells
104 were isolated using MojoSort Streptavidin Nanobeads (Biolegend, San Diego, CA) following
105 manufacturer's protocol. B cells were cultured in RPMI 1640 Base Media (Hyclone), 10% fetal
106 bovine serum (Atlanta Biologicals), 2mM L-glutamine (Corning), 50 μ M 2-mercaptoethanol
107 (Corning), 20mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (Hyclone), and 1X
108 penicillin/streptomycin. Cycloheximide (Millipore) was used at a final concentration of 10 μ g/mL.

109

110 **FlowCytometry analysis**

111 For flowcytometry analysis cells were stained for 1 hour in 3% FBS in PBS. Antibodies found
112 in supplemental materials and methods. For cell cycle analysis, cells were fixed and permeabilized
113 following Biolegend intracellular staining protocol and stained with Ki67 and DAPI (4',6-diamidino-
114 2-phenylindole).

115

116 **ELISA**

117 Mice were bled on Day 0 and Day 8 following immune stimulation. Serum was collected
118 following Abcam ELISA sample preparation guide, diluted 1:10 with PBS. SRBC, diluted 1:10 in
119 PBS, was used as a control for cross reactivity of antibodies due to presence of SRBC. ELISA was
120 performed with positive reference antigen mixture, and PBS as a negative control according to
121 manufactures directions (BD Pharmingen).

122

123 **Histological Staining**

124 Mouse spleens were fixed in 10% (vol/vol) buffered formalin phosphate for 24 hours then
125 placed in 70% ethanol. Sections were stained with H&E using standard protocols. Spleen sections
126 were stained in a 1:500 dilution of UBR5 antibody ab70311 (Abcam) and Ki67. For germinal center
127 analysis, mice were immunized with SRBC and sacrificed eight days later. Spleen sections were
128 stained in a 1:500 dilution of biotinylated peanut agglutinin (PNA) antibody B-1075 (Vector Labs).
129 Images were captured with a Zeiss Observer.Z1 microscope (Zeiss International, Germany) and
130 Zen Pro software (Zeiss International, Germany) was used to process the images.

131

132 **Nuclear Fractionation**

133 Nuclei from 293Ts (ATCC) were collected as described previously.¹⁶ Nuclei were lysed in low
134 salt buffer (10mM Tris-HCl pH7.4, 0.2mM MgCl₂, 1% Triton-X 100) containing protease and
135 phosphatase inhibitors for 15 minutes on a rocker at 4°C. 10-30% glycerol gradients were prepared
136 as previously described.¹⁷ Samples were spun at 28,000rpm for 13 hours at 4°C. 200uL fractions
137 were collected for a total of 25 fractions.

138

139 **Western Blot and Immunoprecipitation Analysis**

140 For western blot analysis, samples were lysed in Pierce RIPA buffer containing
141 protease and phosphatase inhibitor cocktail (ThermoFisher). Samples were separated by
142 SDS-PAGE, transferred onto PVDF (Millipore), and blocked in 5% milk. Antibodies were
143 prepared in 5% BSA. Horse Radish Peroxidase conjugated secondary antibodies (Jackson
144 laboratories) were prepared in 5% milk.

145

146 **RNA extraction and quantitative real-time PCR**

147 Total RNA was harvested using QIAGEN RNeasy Kit (QIAGEN, Hilden, Germany). Following
148 RNA extraction, cDNA synthesis was performed using High Capacity RNA-to-cDNA Kit
149 (ThermoFisher). qRT-PCR was carried out on equal concentrations of cDNA for each sample using
150 iTaq Universal SYBR Green Supermix. All primers can be found in supplemental materials and
151 methods.

152

153 **Mass Spectrometry**

154 For global proteome quantification, B220⁺ splenocytes were isolated from 3 mice per
155 genotype. Samples were prepared for and TMT labeled per manufacturer's protocol
156 (ThermoScientific TMT10plex Mass Tag Labeling Kits). Following TMT labeling, acetonitrile
157 was removed by speedvac and samples were resuspended in 0.1% trifluoroacetic acid (TFA).
158 Sample cleanup with C18 tips was performed per manufacturer's protocol (Pierce). Sample
159 concentrations were re-quantified (Pierce Quantitative Colorimetric Peptide Assay kit) and then
160 combined in equal concentration. Following combination, samples were dried by speedvac and
161 fractionated by ThermoScientific high pH reverse phase fractionation kit following
162 manufacturer's protocol for TMT. Resulting fractions were run in a speedvac to dryness and
163 resuspended in 0.1% Formic Acid for mass spectrometry (MS) analysis (see supplemental
164 methods). Data are available via ProteomeXchange with identifier PXD014307.

165

166 For immunoprecipitation, cells were lysed in 20mM Tris pH 7.5, 150mM NaCl, 1mM
167 EDTA, and protease and phosphatase inhibitors. Immunoprecipitation was performed

168 overnight at 4°C using anti-UBR5 antibody from Cell Signaling (8755) or rabbit IgG control.
169 Protein A agarose beads (Cell signaling) were used for IgG pulldown. Samples were washed
170 5X with lysis buffer prior to analysis by MS.

171

172 **Statistical analysis**

173 All experiments were performed in triplicate unless noted and statistical analyses were performed
174 using paired two-tailed Student's t-test assuming experimental samples of equal variance. * P<0.05,
175 ** P<0.01, *** P<0.001, **** P<0.0001

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179 **RESULTS**

180

181 ***Ubr5* mutations are specific to MCL.**

182 Meissner, B. et al originally identified UBR5 mutations in ~18% of MCL patients.³ In recent
183 cross-sectional genomic profiling of multiple lymphoma subtypes, we identified UBR5 as one of 8
184 genes that had a significantly higher frequency of mutation in MCL compared to other lymphoma
185 subtypes. These mutations were observed in 21 out of 196 MCL tumors (10.7%) and 15 out of 559
186 tumors (2.7%) of other histologic subtypes. However, mutations within the HECT domain were found
187 only in MCL tumors (Figure 1B).¹⁸ Mutations within the HECT domain of UBR5 are therefore a
188 disease-specific genetic feature of MCL.

189

190 **Generation of a conditional UBR5 HECT domain mutant.**

191 Since the role of *Ubr5* in lymphopoiesis is unknown, we first evaluated the expression of *Ubr5*
192 in B cell sub-groups during development by purifying pro, pre, and immature B cells from BM of 6-

193 week-old C57/BL6 wild-type (WT) mice. Additionally, transitional, follicular, and marginal zone B cell
194 populations were purified from spleens. The pro B cell population showed lowest expression of *Ubr5*
195 at both the RNA and protein level, whereas the highest expression of *Ubr5* was found in mature
196 splenic populations (follicular and marginal B cells) (Figure 1B-C). These studies suggest a role for
197 UBR5 at different stages of B cell development.

198

199 Saunders *et al.* generated a *Ubr5*-null mouse; unfortunately, these mice die during
200 embryogenesis prior to onset of definitive hematopoiesis at E10.5.¹⁹ In order to understand the role
201 of UBR5 mutations in B cell development, we generated a conditional allele targeting exon 58 that
202 would lead to a truncated protein lacking a critical cysteine required for ubiquitin conjugation in the
203 HECT domain and mimicking mutations found in MCL patients (Figure 1D).²⁰ We first crossed our
204 *Ubr5*^{ΔHECT/+} mice to *E2A*^{CRE};*Ubr5*^{ΔHECT/+} mice that delete in early embryogenesis.^{19, 21} As with null
205 mice, *E2A*^{CRE};*Ubr5*^{ΔHECT/ΔHECT} were not viable. In contrast to *Ubr5*-null mice, 3 of 48 pups found dead
206 at birth were *E2A*^{CRE};*Ubr5*^{ΔHECT/ΔHECT}, suggesting the HECT domain is not required for embryogenesis
207 and early stages of hematopoiesis (Figure 1E-F).

208

209 Since *E2A*^{CRE};*Ubr5*^{ΔHECT/ΔHECT} mice die prior to or at birth, to study B cell development we
210 crossed *Ubr5*^{ΔHECT/ΔHECT} mice to *Mb1*^{WT/CRE}; *Ubr5*^{ΔHECT/ΔHECT}, which deletes in early B-lymphocytes
211 (Figure 1G).²² To determine the specific loss of the HECT domain, we performed qRT-PCR with
212 primers flanking the HECT domain and primers amplifying a region on the N-terminus in B220⁺
213 splenocytes. qRT-PCR showed no decrease in *Ubr5* expression, but significant decrease in *Ubr5*
214 HECT domain expression (Figure 1H). These studies demonstrate the loss of HECT domain of UBR5
215 targeted mice.

216

217 **Impaired B cell maturation following deletion of UBR5 HECT domain..**

218 Early B cell development occurs in the BM compartment so we examined BM B cells in 6-
219 week-old *Mb1^{WT/CRE};Ubr5^{ΔHECT/ΔHECT}* mice. In *Ubr5^{ΔHECT/ΔHECT}* and *Ubr5^{ΔHECT/WT}* mice, the number of
220 total BM cells and frequency of B220⁺ B cells showed no significant difference compared to their WT
221 littermates (Figure 2A-C; Supplemental Figure 1A-B). Further analysis of specific subtypes of B cells
222 revealed a striking decrease in IgD⁺ mature B cell populations (Figure 2D-E; Supplemental Figure
223 1C-D). Decreases in mature B cells were compensated in BM by a slight increase in pro/pre B cell
224 population (Figure 2F-G). Further breakdown of the population revealed an increase in pro B, but not
225 pre B cell population (Figure 2F-I). These studies demonstrate an impact to mature cells within BM,
226 as well as changes to composition of pro and pre B cells following loss of HECT domain.

227

228 Following development of B cells in BM, cells migrate to the spleen where they undergo
229 maturation and activation.²³ *Mb1^{CRE/WT};Ubr5^{ΔHECT/ΔHECT}* mice have smaller spleens as well as a
230 reduction in number of total splenocytes (Figure 3A-B). Although the mice had a total reduction of
231 splenocytes, frequency of B220⁺ cells in both WT littermates and *Ubr5^{ΔHECT/ΔHECT}* mice was ~45%
232 (Figure 3C), and splenic architecture was unaltered following loss of the HECT domain of *Ubr5* (Figure
233 3D). In addition, the transitional B cell population stages T1, T2, and T3 in the spleen had no
234 significant differences in *Ubr5^{ΔHECT/ΔHECT}* mice versus WT littermates (Figure 3 E-F). However, there
235 was significant impact on mature B1 and B2 subsets within the spleen. We found that in the B2 subset,
236 marginal zone B cells were significantly reduced from approximately 10% of B220⁺ splenocytes to
237 2% in *Ubr5^{ΔHECT/ΔHECT}* mice whereas the follicular B cell compartment frequency was slightly

238 increased, despite a reduction in absolute number of follicular B cells in *Ubr5*^{ΔHECT/ΔHECT} mice (Figure
239 3H-I; Supplemental Figure 1E-F).

240

241 The B1 population is responsible for innate immunity and is the first line of defense for infection.
242 In spleen, the B1 population was reduced by almost 2-fold in *Ubr5*^{ΔHECT/ΔHECT} mice and when further
243 separated into B1a and B1b, the reduction was exclusively found in the B1a subpopulation (Figure
244 3K-M). We further evaluated B1 populations in the peritoneal cavity and found a ~75% reduction in
245 B1 cells, mainly from a reduction of B1a cells (Supplemental Figure 2). These findings demonstrate
246 a significant loss of populations required for innate immunity with loss of B1 and marginal zone B cells
247 following deletion of the HECT domain of *Ubr5*.

248

249 **Alterations in follicular B cell subsets and activation of B cells.**

250 Evaluating cell surface markers on the follicular B cell population, we found that
251 *Ubr5*^{ΔHECT/ΔHECT} follicular B cells had abnormal protein expression with low IgD and high IgM
252 compared to their WT littermates (Figure 4A-B). The follicular population in *Ubr5*^{ΔHECT/ΔHECT} mice also
253 had high CD23 protein expression, but normal expression of CD5 and CD1d on follicular B cells
254 (Figure 4A-B). To further define alterations in the B cell compartment, we analyzed cell cycle status.
255 While follicular B cells are typically in the resting state, *Ubr5*^{ΔHECT/ΔHECT} cells are more quiescent and
256 had increased cells in G0 for both transitional and mature B cells (Supplemental Figure 3A).
257 Additionally, staining with cellular proliferation marker Ki67 on spleen sections showed a reduction of
258 Ki67 staining specifically in white pulp of spleens from *Ubr5*^{ΔHECT/ΔHECT} mice (Supplemental Figure
259 3B). These studies demonstrated alterations in mature spleen cells with both phenotypic changes
260 and cell cycle alterations.

261

262 Activation of follicular B cells by T dependent antigens leads to formation of germinal centers
263 (GC) in secondary lymphoid tissues and generation of antibody producing plasma cells. In order to
264 determine if *Ubr5*^{ΔHECT/ΔHECT} follicular B cells have normal function and whether germinal center
265 formation can be induced, we immunized 6-week-old mice with SRBC. Eight days following
266 immunization, we performed immunohistochemical (IHC) analysis on spleens with peanut agglutinin,
267 a GC marker. Staining revealed a significant decrease in overall number and size of GCs in
268 *Ubr5*^{ΔHECT/ΔHECT} mice, but in white pulp with GCs, there were multiple small GCs (Figure 4C). Only
269 ~65% of white pulp in spleens of *Ubr5*^{ΔHECT/ΔHECT} mice contained GCs compared to ~83% in WT
270 littermate controls. We evaluated the ability of follicular B cells to terminally differentiate into plasma
271 cells within PB and found a ~50% reduction of B cells and plasma cells (Figure 4D-E; Supplemental
272 Figure 1G-H). Although we had a significant decrease in CD138⁺ antibody producing cells, levels of
273 IgG1, IgG3, and IgA were not changed in unstimulated *Ubr5*^{ΔHECT/ΔHECT} mice (Figure 4F), and
274 measurement of antibodies IgG, IgM, and IgA within sera were unaltered suggesting that *Ubr5* HECT
275 domain deletion does not affect basal antibody levels (Figure 4G). These studies indicate an important
276 role of Ubr5 in the function and activation of mature B cells.

277

278 **Loss of UBR5 HECT domain leads to increased expression of spliceosome components found** 279 **to interact with UBR5**

280 The HECT domain of UBR5 is thought to be required for its ubiquitination activity implicating
281 deletion could lead to increased accumulation of UBR5 substrates targeted for degradation by the
282 proteasome. To quantitatively determine protein differences in *Ubr5*^{ΔHECT/ΔHECT} versus WT littermates,
283 we labeled splenic B220⁺ cells with tandem mass tags (TMT), combined samples in equal

284 concentrations, and analyzed by MS (Figure 5A). Proteomic analysis identified 15,584 unique
285 peptides, 2,797 quantifiable proteins, and 1,675 proteins with greater than or equal to three unique
286 peptides. We identified 143 proteins that were either significantly decreased or enriched in
287 *Ubr5*^{ΔHECT/ΔHECT} B220⁺ splenocytes (Figure 5B and Supplementary Table 2). Principle component
288 analysis (PCA) plot showed protein isolated from 3 independent mice from either genotype clustered
289 together, but that the two independent genotypes clustered separately (Figure 5C). In addition,
290 identified proteins were distributed in different cellular compartments (Figure 5D). Of the differentially
291 expressed proteins, 104 ≥1.3 fold significantly overexpressed proteins were enriched for proteins
292 associated with mRNA processing, RNA splicing, and mRNA splicing via the spliceosome, whereas
293 the 39 ≤0.70 fold significantly decreased proteins in spleen B220⁺ cells from *Ubr5*^{ΔHECT/ΔHECT} were
294 proteins associated with immune system process and protein transport (Figure 5E-G). As suggested
295 by flowcytometry, IgD protein was the highest down-regulated protein following deletion of *Ubr5* HECT
296 domain (Figure 5E). CD22, which is associated with B cell activation, was also down-regulated in
297 correlation with the follicular B cell phenotype in *Ubr5*^{ΔHECT/ΔHECT} mice (Figure 5E). Intriguingly, the
298 second highest expressed protein in splenic B cells of *Ubr5*^{ΔHECT/ΔHECT} was UBR5 (Figure 5E-F).
299 Proteomic profiling revealed an increase in proteins associated with mRNA splicing in B cells lacking
300 HECT domain of UBR5 suggesting a novel role of UBR5 in mRNA splicing.

301

302 To identify putative substrates and interacting partners of UBR5 in MCL, we performed
303 immunoprecipitation followed by mass spectrometry (IP-MS) utilizing human MCL patient derived cell
304 lines, Jeko1 and Mino (Figure 6A).^{24, 25} We identified 115 proteins with ≥3 unique peptides and, similar
305 to our TMT analysis, gene ontology analysis showed proteins enriched in mRNA splicing via the
306 spliceosome (Figure 6B and Supplementary Table 3). Comparing MS datasets from TMT labeled

307 proteins and endogenous IP-MS revealed 89 proteins overlapping and seven proteins were up-
308 regulated in the TMT study including UBR5 (Figure 7 D-E). Intriguingly, all six of the other identified
309 proteins are associated with mRNA splicing (SF3B3, SMC2, PRPF8, DHX15, SNRNP200, and
310 EFTUD2). These proteins are classified as core spliceosome components including U2 (SF3B3) and
311 associated U5 small nuclear ribonucleoprotein (snRNP) complex (EFTUD2, SNRNP200, and PRPF8)
312 (Figure 6E). Of the identified proteins, none have previously been characterized as UBR5 interactors
313 or substrates, suggesting a novel pathway of UBR5 regulation and the identification of potential novel
314 UBR5 substrates.

315

316 **Alterations in splicing factors and splicing in B cells.**

317 UBR5 has been implicated in a number of cellular processes including cell cycle (CDK9), DNA
318 damage (RNF168), and cell division (BUB3).^{5-7, 26} BUB3 was found in the quantitative MS analysis;
319 however, accumulation was not seen in cells with deletion of *Ubr5* HECT domain (Supplementary
320 Table 1). In addition, western blot confirmed that BUB3, CDK9, and RNF-168 did not accumulate in
321 *Ubr5*^{ΔHECT/ΔHECT} B220⁺ splenocytes (Figure 7A). Flowcytometry validated cell surface markers
322 associated with activation and maturation, CD22 and IgD, expression was significantly decreased in
323 total B220⁺ splenocytes (Figure 7B-C). UBR5 was the second highest expressed protein in the MS;
324 however, none of the identified peptides had coverage within the HECT domain (data not shown). To
325 confirm overexpression of UBR5 protein, we performed IHC and western blot analysis and found
326 higher protein expression of UBR5 in *Ubr5*^{ΔHECT/ΔHECT} spleens (Figure 7A and D). In order to determine
327 if loss of HECT domain leads to stabilization of the protein, we performed half-life analysis with
328 cycloheximide treatment. *Ubr5*^{ΔHECT/ΔHECT} B220⁺ splenocytes had higher protein expression due to

329 stabilization of the protein (Figure 7E). These studies validate the MS findings that known substrates
330 do not accumulate and loss of the HECT domain leads to overexpression and stabilization of UBR5.

331

332 To evaluate protein overexpression of RNA splicing components, we performed western blot
333 analysis on B220⁺ splenocytes. *Ubr5*^{ΔHECT/ΔHECT} B220⁺ splenocytes had increased expression of
334 EFTUD2, SMC2, and PRPF8 (Figure 7F). Because three of the identified proteins EFTUD2,
335 SNRNP200, and PRPF8 are part of the U5 snRNP complex, we wanted to determine whether UBR5
336 elutes with the U5 complex. We performed glycerol density gradient and found that EFTUD2,
337 SNRNP200, PRPF8, and UBR5 eluted in fractions 15-19 suggesting they are found in the same
338 complex (Figure 7G).

339

340 Expression of *IgM* and *IgD* is regulated by alternative splicing of the Ig heavy chain locus. As
341 shown in the follicular B cell population, *IgM* and *IgD* have aberrant expression suggesting defects in
342 splicing. To confirm altered splicing, we isolated follicular B cells from *Ubr5*^{ΔHECT/ΔHECT} and WT
343 littermates for analysis of transcript levels. PCR confirmed increased *IgM* transcripts and decreased
344 *IgD* transcripts in *Ubr5*^{ΔHECT/ΔHECT} follicular B cells (Figure 7H). It was previously shown that mRNA of
345 Zinc Finger Protein 318 (*Zfp318*) has two known splice variants.²⁷ It is thought that the longer
346 transcript form containing exon 1-10 plays a key role in alternative splicing of immunoglobulin heavy
347 chain locus. We found decreased expression of transcript spliced from exon 7 to exon 11 and aberrant
348 variant transcripts in *Ubr5*^{ΔHECT/ΔHECT} follicular B cells, and decreased transcript containing exon 7 to
349 exon 9 suggesting expression of *Zfp318* splice variants may also be contributing to B cell defects
350 (Figure 7H). These studies demonstrate that loss of HECT domain leads to increased expression of
351 spliceosome components and aberrant splicing.

352

353 **DISCUSSION**

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In this report, we demonstrate that ubiquitin E3 ligase UBR5 plays a key role in B cell maturation and activation via regulation of alternative splicing. While UBR5 is present in all B cell populations, deletion of *Ubr5* HECT domain had limited identifiable impact on development of BM B cell populations. This suggests a crucial role for UBR5 in maturation of B cells. With the loss of the UBR5 HECT domain, there is normal frequency of transitional B cells that migrate to the spleen; although there is a block in differentiation of naïve B cells which corresponds to the MCL tumor population. The greatest impact is on B1a and marginal zone B cells that are significantly reduced within the spleen and peritoneal cavity suggesting impaired innate immunity. Although follicular B cell population frequency is only slightly impacted, these cells are phenotypically abnormal and have a reduced capacity to generate plasma cells.

Interrogation of global proteomic changes in the splenic B cell population demonstrates a novel role of UBR5 in mRNA splicing via the spliceosome. The spliceosome plays an important role in providing genetic diversity. In immune cells, alternative splicing is suggested to play a key role in B cell differentiation, activation, and survival.²⁸ A number of important pathways and genes in the immune system require alternative splicing. In human B cells, ~90% of genes with multiple exons undergo alternative splicing.²⁹ The spliceosome involves multicomponent complexes including five snRNPs that coordinate mRNA splicing.³⁰ Our finding that components of the spliceosome are both up-regulated and found interacting with UBR5 in MCL cell lines identifies a new pathway of UBR5 involvement. The striking phenotype of IgD in HECT domain mutants supports defects in splicing

376 since IgM and IgD are generated from alternative splicing of the heavy chain gene.³¹ It is well
377 established that IgM and IgD play a key role in immunity and are required for B cell receptor
378 signaling and immune response, but little is known regarding how alternative splicing of *IgD* and
379 *IgM* occur. In addition, markers of mature naïve B cells, *CD21* and *CD23*, have multiple splice
380 variants regulated by alternative splicing.^{32, 33} Our studies find that in transitional and follicular B
381 cell populations, *CD23* has altered expression, which may be due to splicing. Importantly,
382 expression of SF3B3 identified in our MS has been suggested to lead to alternative splicing of *Ezh2*
383 and promote tumorigenesis.³⁴ In addition, EZH2 has been previously shown to play a key role in
384 germinal center formation and EZH2 mutations promote lymphoid transformation.³⁵ Furthermore,
385 SF3B1, another component of the U2 snRNP complex that interacts with SF3B3 is frequently
386 mutated in chronic lymphoblastic leukemia and a variant of Myelodysplastic Syndrome, suggesting
387 that dysregulation of this complex may be important for development of hematopoietic
388 malignancies.^{36, 37} Although these studies suggest a role of UBR5 in the spliceosome, further
389 investigation is required to determine if ubiquitination activity is required or whether UBR5 acts as
390 a scaffolding protein during splicing.

391

392 Loss of the HECT domain of UBR5 leads to stabilization and overexpression which could be
393 due to UBR5 potentially undergoing self-ubiquitination.³⁸ Also, UBR5 is predicted to bind RNA
394 further supporting its role in RNA splicing.³⁸ More importantly these studies identifying the
395 consequences of UBR5 HECT domain loss on alternative splicing in the immune system not only
396 reveal mechanisms regulating B cells development, but since ~18% of MCL patient genomes
397 contain UBR5 mutations, it may lead to further understanding of lymphoma transformation and
398 provide a potential therapeutic target. Overall, our findings reveal a novel mechanism of regulation

399 by UBR5 via alternative mRNA splicing, provides evidence that the ubiquitin E3 ligase is a novel
400 regulator of B cell maturation, and suggests a role of UBR5 in lymphoma transformation.

401

402 **AUTHOR CONTRIBUTIONS**

403 S.S., T.G, and S.M.B. conceived and designed the experiments. S.S., T.G., H.V., J.H.G, M.R.G.
404 and S.M.B performed experiments and analysis. S.S., T.G., R.W.H., and S.M.B. wrote the
405 manuscript. H.C.H.L. and N.T.W. provided technical and material support. All authors reviewed the
406 manuscript before submission.

407

408

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422

423 **DISCLOSURES OF CONFLICTS OF INTEREST**

424 The authors have no conflicts of interest related to this work.

425

426

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521

522

523 **FIGURE LEGENDS**

524

525 **Figure 1. Expression of *Ubr5* in B cells and generation of conditional *Ubr5* HECT domain**
526 **knockout model.** (A) Frequency of UBR5 mutations in lymphoma patients. orange =
527 nonsense/frameshift; green = missense. (B) Relative qRT-PCR and (C) Western blot
528 expression of UBR5 within different B cell populations: pro B cells (B220⁺IgM⁻ckit⁺), pre B cells
529 (B220⁺IgM⁻CD25⁺), immature B cells (B220⁺IgM^{lo}IgD⁻) from the BM, and transitional
530 (B220⁺CD93⁺), marginal zone B cells (B220⁺CD21⁺CD23⁻), and follicular B cells
531 (B220⁺CD21⁺CD23⁺) isolated from the spleen of 6 weeks old WT C57Bl6 mice. (D) Schematic
532 of targeting strategy used to insert loxP sites flanking exon 58 of *Ubr5*. (E) Distribution of pups
533 born from crossing *Ubr5*^{ΔHECT/ΔHECT} with E2A^{CRE} (F) Pups alive at birth per genotype. (G)
534 Genotyping PCR of targeted alleles and deletion in six-week old mice bred to B lymphocyte
535 specific *Mb1*^{Cre} mouse model. PCR was performed on BM. (H) Relative expression of *Ubr5*
536 and *Ubr5* HECT domain by qRT-PCR in spleen B220⁺ cells.

537

538 **Figure 2. The loss of the HECT domain of *Ubr5* leads to decreased numbers of mature**
539 **B cells within the BM.**

540 (A) Bar graph of the total number of cells per femur. (B) Representative flowcytometry plot of
541 total B220⁺ in the BM. (C) Bar graph of the frequency of B220⁺ cells per femur. (D)
542 Representative flowcytometry plots gated on B220⁺ cells for pro and pre B cells (B220⁺IgM⁻
543 IgD⁻), immature B cells (B220⁺IgM^{lo}IgD⁻), transitional B cells (B220⁺IgM⁺IgD⁻), early mature B
544 cells (B220⁺IgM⁺IgD⁺), and late mature B cells (B220⁺IgM⁻IgD⁺). (E) Bar graphs of B cell
545 populations shown in D. (F) Representative flow cytometry plots gated for pro and pre B cells

546 (B220⁺IgM⁻), immature B cells (B220⁺IgM^{lo}), and recirculating B cells (B220⁺IgM⁺). (G) Bar
547 graph of the population breakdown shown in F. (H) A representative flowcytometry plot gated
548 on B220⁺ cells gating for pro B cells (B220⁺IgM⁻c-kit⁺) and pre B cells (B220⁺IgM⁻CD25⁺) (I)
549 Bar graph representing the population breakdown shown in H.
550 (N=10, * P<0.05, ** P<0.01, *** P<0.001)

551

552 **Figure 3. The loss of the HECT domain of *Ubr5* leads to reduction in marginal and B1a**
553 **splenic cells, but no effects on transitional B cells.**

554 (A) Bar graph of spleen weight. (N=19) (B) Bar graph of the total number of splenocytes. (N=4)
555 (C) Bar graph of frequency of B220⁺ cells per spleen. (N=4) (D) An example hematoxylin and
556 eosin stain of spleens. (N=4) (E) Representative flow cytometry plot gated on B220⁺ for
557 transitional cells: T1 cells (B220⁺CD93⁺IgM⁺CD23⁻), T2 cells (B220⁺CD93⁺IgM⁺CD23⁺), and
558 T3 cells (B220⁺CD93⁺IgM⁻CD23⁺). (N=5) (F-G) Bar graphs of percentages and absolute
559 number of transitional cells within the spleen. (H) Representative flowcytometry plots gated on
560 B220⁺ for follicular B cells (B220⁺CD21⁺CD23⁺) and marginal zone B cells (B220⁺CD21⁺CD23⁻
561). (N=7) (I-J) Bar graphs of percentages and absolute number of follicular and marginal zone
562 B cells within the spleen. (K) Representative flowcytometry plots gated for B1a
563 (B220⁺CD19^{lo}CD5⁺) and B1b cells (B220⁺CD19^{lo}CD5⁻). (N=8) (L & M) Bar graph of
564 percentages and absolute number of B1a and B1b cells. (* P<0.05, ** P<0.01, *** P<0.001,
565 **** P<0.0001)

566

567 **Figure 4. The follicular B cells in *Ubr5*^{ΔHECT} mice have altered phenotype and diminished**
568 **differentiation capabilities.**

569 (A) Representative histogram of cell surface markers of *Ubr5^{WT}* and *Ubr5^{ΔHECT}* follicular B cells
570 (B220⁺CD21⁺CD23⁺). (B) Bar graphs of relative MFI of follicular B cells depicted in A. (C)
571 Representative PNA IHC staining of spleen 7 days post stimulation with SRBC. (D)
572 Representative flowcytometry plots of B220⁺ and CD138⁺ plasma cells in the peripheral blood.
573 (E) Bar graphs representing percentages of B220⁺ and CD138⁺ cells in the peripheral blood
574 depicted in D. (F) Bar graphs representing percentages of IgG1⁺, IgG3⁺, and IgA⁺ B220⁺ cells
575 in the peripheral blood. (G) Bar graphs of ELISA indicating relative expression for different
576 immunoglobulin types before (Day 0) and after (Day 8) immune system stimulation with SRBC.
577 (N=4) (* P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001)

578

579 **Figure 5. Loss of HECT domain results in a reduction in B cell development proteins**
580 **and an enrichment in proteins regulating mRNA splicing.** (A) Schematic of sample
581 preparation of *UBR5^{WT}* and *Ubr5^{ΔHECT}* spleens for TMT MS analysis. (N=3). (B) Quantification
582 of identified peptides and proteins. (C) PCA using components 1 and 2 showing clustering of
583 *WT* and *ΔHECT* samples for significantly up-regulated proteins. (D) Pie chart of localizations
584 for all 2,786 proteins identified by mass spec. (E) Volcano plot of the Log₂ fold change
585 *WT/ΔHECT* showing proteins significantly up-regulated (p<0.05) 1.3-fold or more and
586 significantly (p<0.05) down-regulated 0.7-fold or more. (F) Heatmap of the significantly up-
587 regulated and significantly down-regulated proteins showing the top 10 up-regulated and top
588 10 down-regulated proteins. (G) Gene ontology analysis showing pathways known to be
589 associated with significantly up-regulated (≥1.3, p≤0.05) and significantly down-regulated
590 (≤0.7, p≤0.05) proteins using DAVID 6.8 software. For up-regulated proteins, includes

591 pathways with ≥ 10 proteins associated and a p-value ≤ 0.01 . Down-regulated proteins
592 pathways with p-value ≤ 0.05 .

593

594 **Figure 6. UBR5 interacts with spliceosome components.** (A) Western blot of
595 immunoprecipitation of UBR5 from JEKO1 MCL cell line used for MS analysis. MS experiment
596 was performed in duplicate (B) Gene ontology analysis of immunoprecipitated proteins. (C)
597 Venn diagram showing the significant overlap of proteins identified in the TMT labelled *UBR5^{WT}*
598 and *Ubr5^{ΔHECT}* MS and those identified in the UBR5 endogenous IP MS. (D) Venn diagram
599 showing overlap of the proteins significantly up-regulated in the *Ubr5^{ΔHECT}* samples and those
600 identified in the UBR5 endogenous IP. (E) Spliceosome associated proteins found in
601 endogenous immunoprecipitation and/or up-regulated in spleen B220⁺ cells.

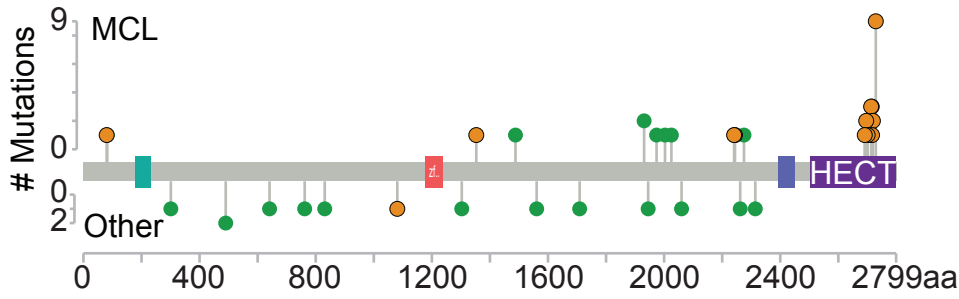
602

603 **Figure 7. Loss of HECT domain leads to aberrant splicing.**

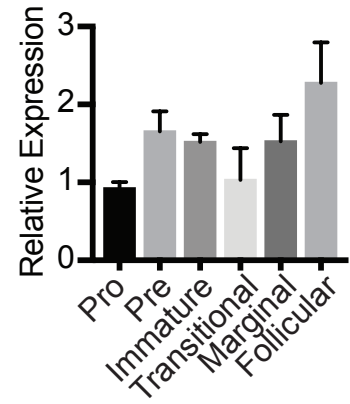
604 (A) Western blot of known UBR5 substrates in spleen B cells (B220⁺) *Ubr5^{WT}* and *Ubr5^{ΔHECT}*
605 mice. (B) Representative histogram of cell surface markers of spleen cells (B220⁺). (C) Bar
606 graphs of relative MFI of the markers in B. (D) Representative immunohistochemistry staining
607 of UBR5 protein in spleen. (E) Western blot of UBR5 in follicular B cells (B220⁺CD21⁺CD23⁺)
608 *Ubr5^{WT}* and *Ubr5^{ΔHECT}* after treatment with 10μg/mL cycloheximide. (F) Western blot of proteins
609 identified in MS in splenic B cells (B220⁺) *Ubr5^{WT}* versus *Ubr5^{ΔHECT}* mice. (G) 10-30% glycerol
610 fractionation of nuclear lysate from HEK293T cells followed by western blot for UBR5 and
611 spliceosome components. (H) RT-PCR of mRNA in *Ubr5^{WT}* and *Ubr5^{ΔHECT}* follicular B cells
612 (B220⁺CD21⁺CD23⁺). Arrows point to the bands of interest. (N=3) (** P<0.01, **** P<0.0001).

613

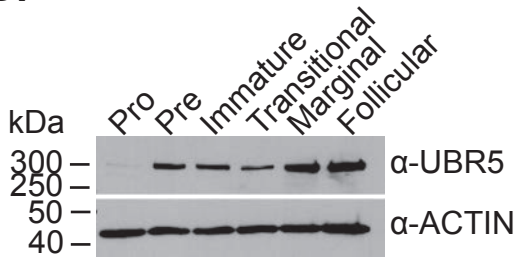
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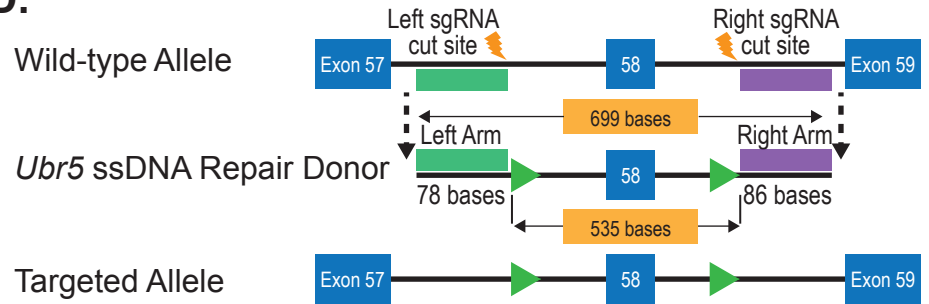
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C.

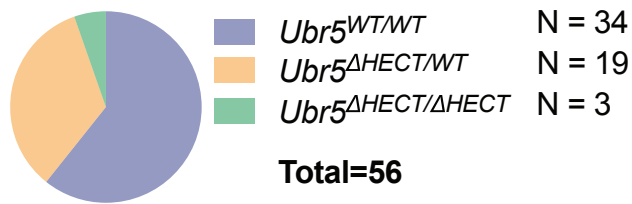


D.



E.

At Birth

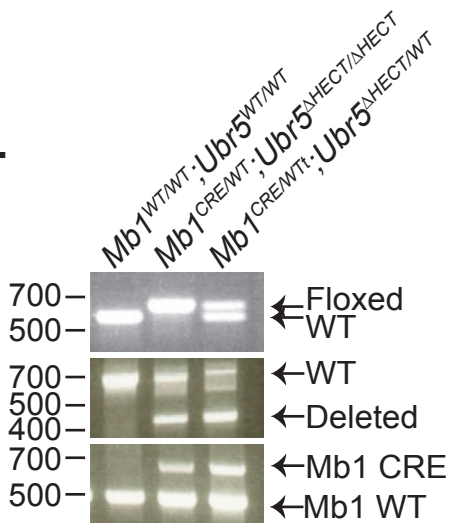


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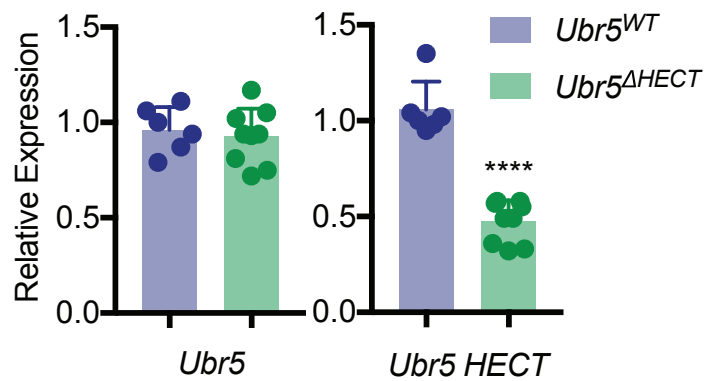
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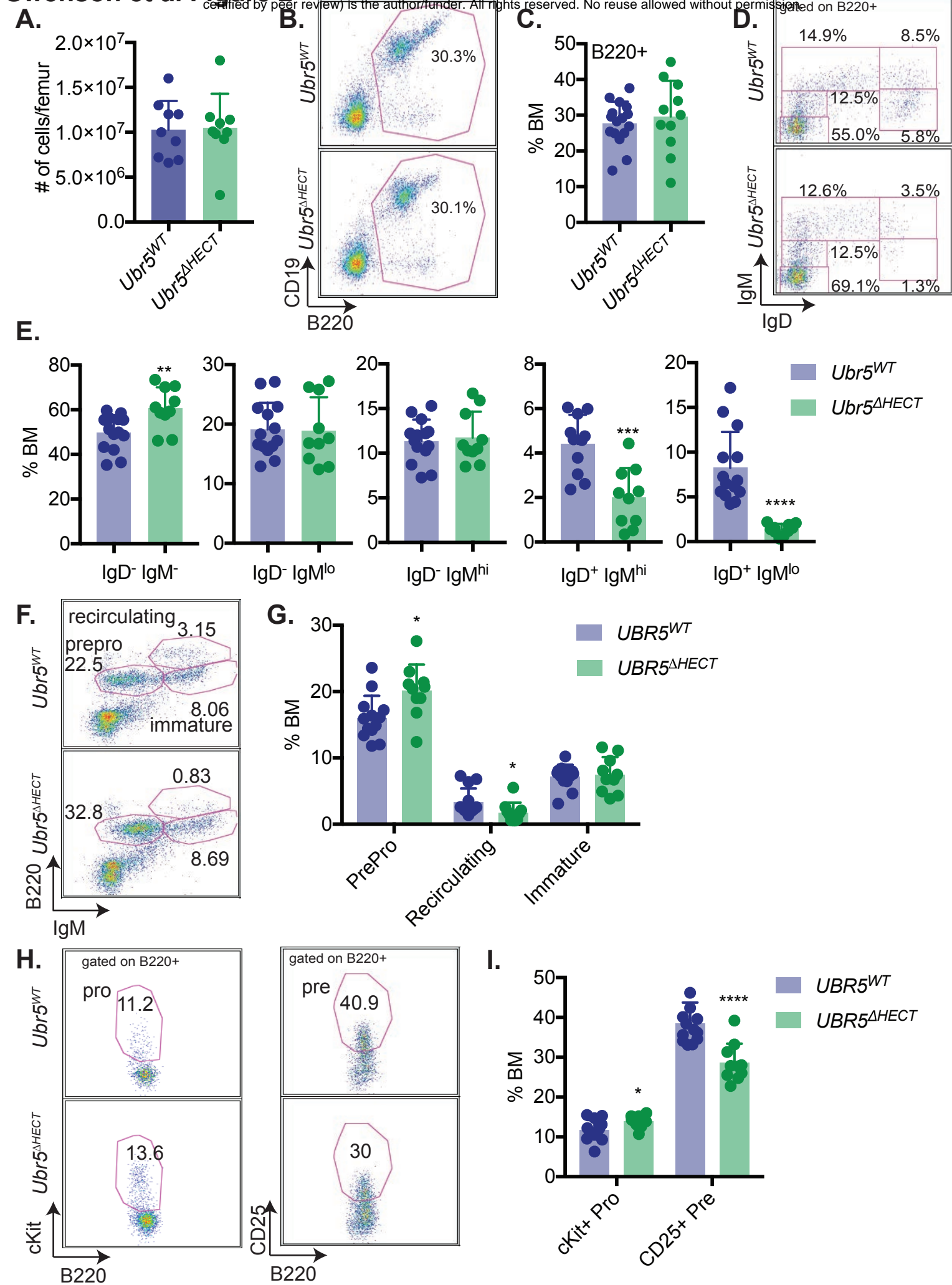


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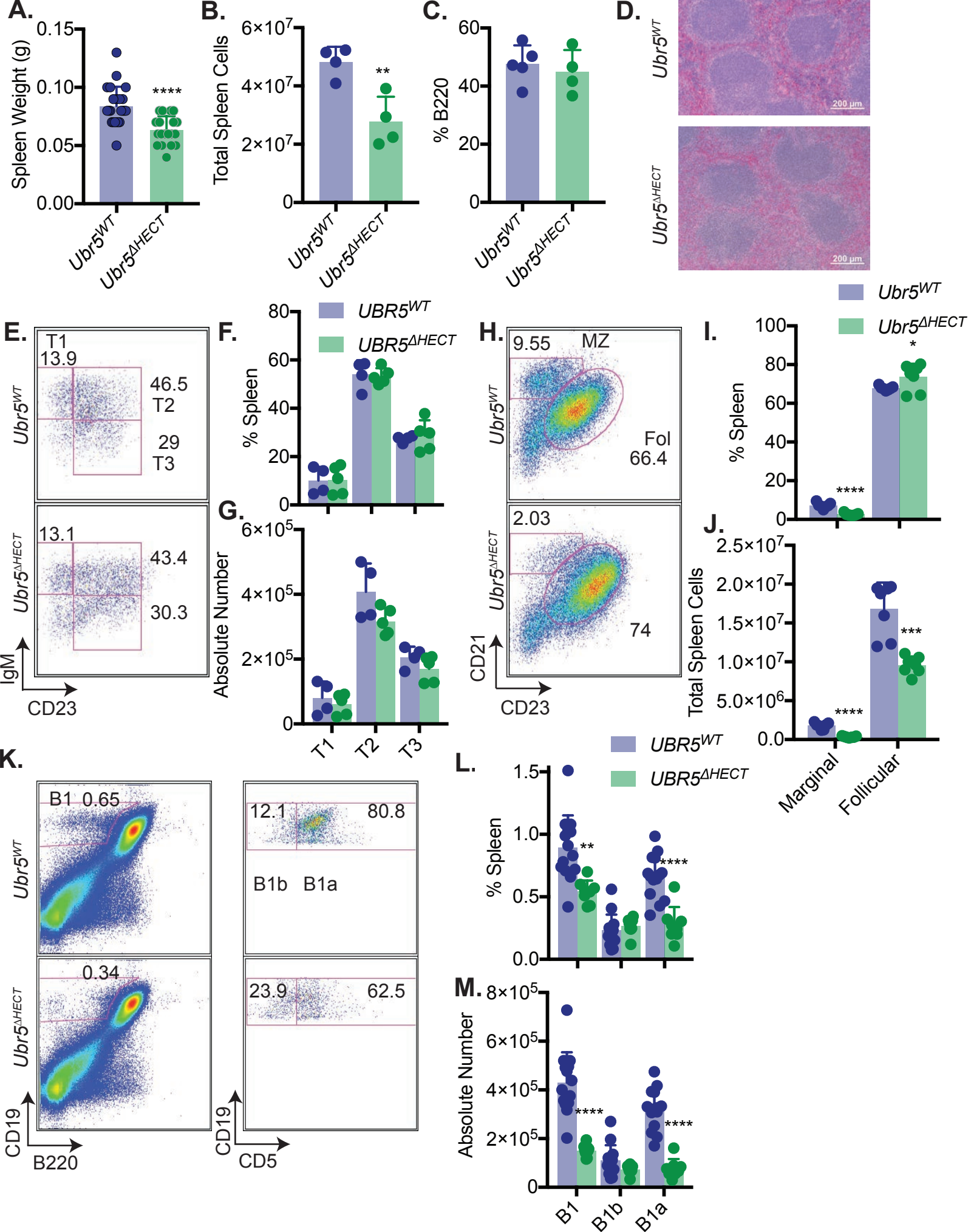


H.

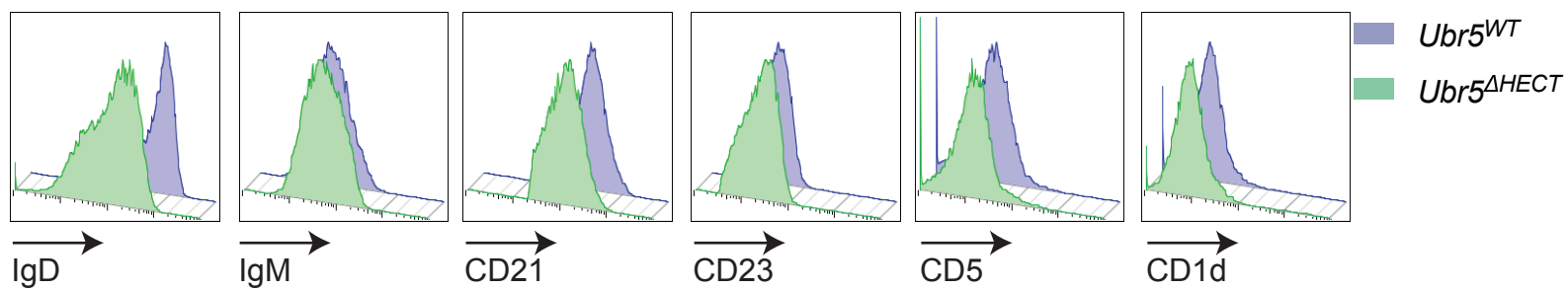




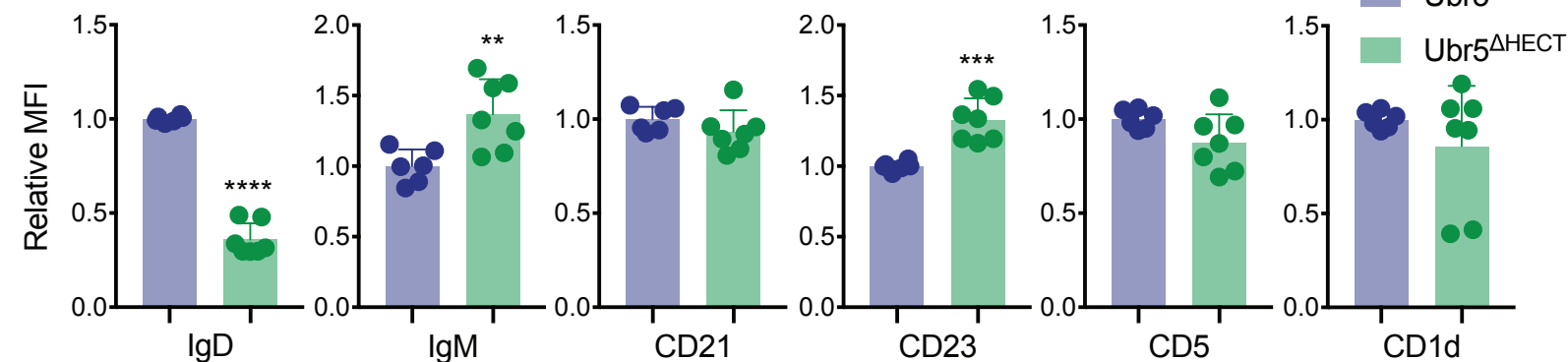
Swenson et al Figure 3



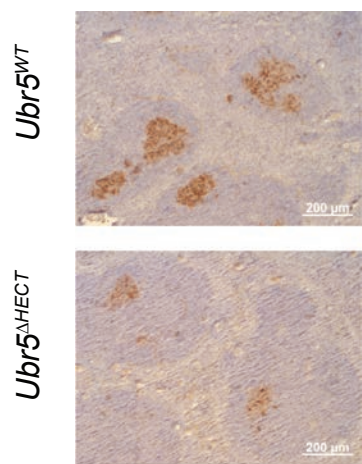
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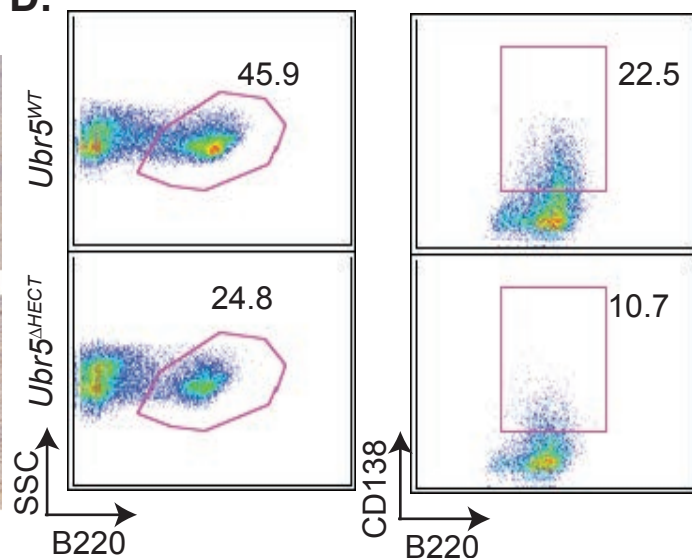
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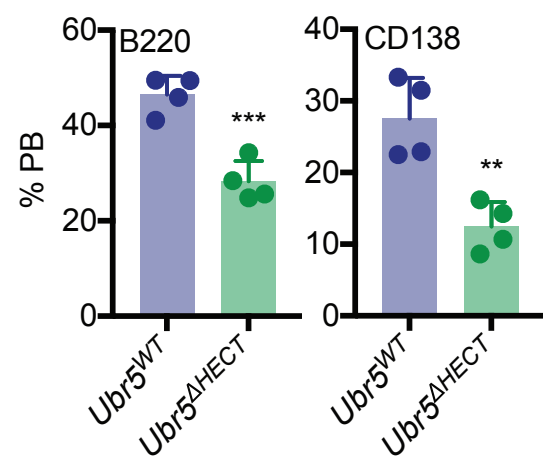
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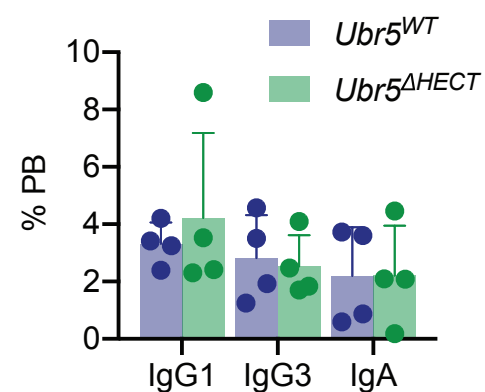
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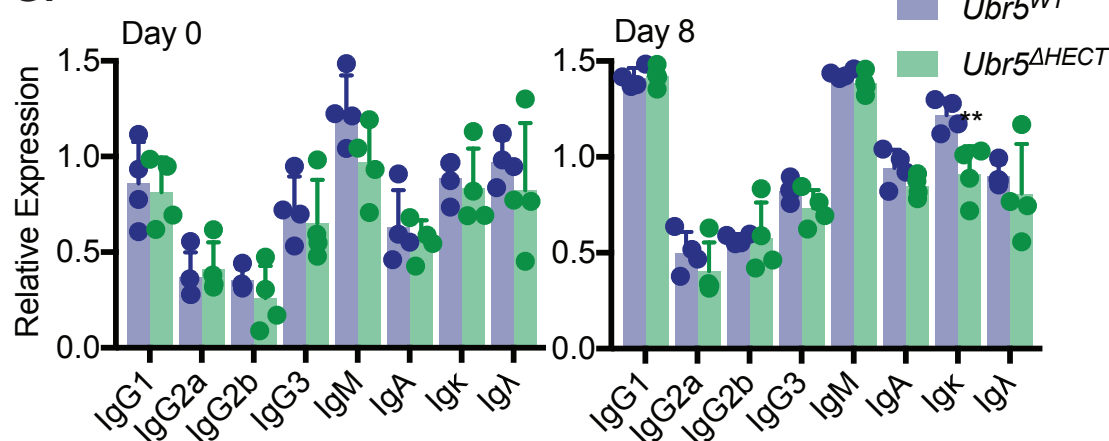
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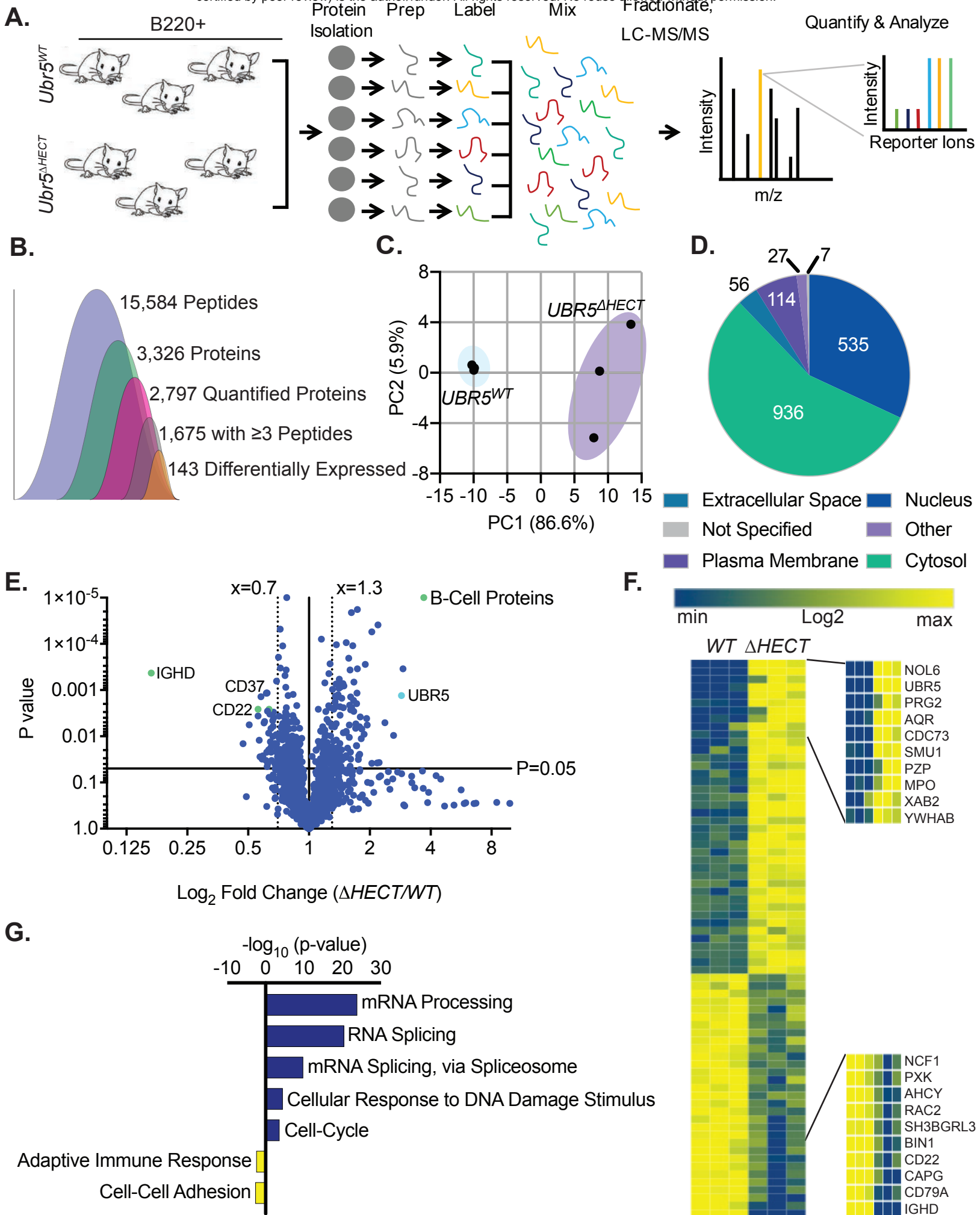


F.



G.





Swenson et al Figure 8

