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1 **_KLF2_ mutation is the most frequent somatic change in splenic marginal zone lymphoma and**

2 **identifies a subset with distinct genotype**

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27

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52

53 **ABSTRACT**

54

55 To characterise the genetics of splenic marginal zone lymphoma (SMZL), we performed whole
56 exome sequencing of 16 cases and identified novel recurrent inactivating mutations in *KLF2*, a gene
57 whose deficiency was previously shown to cause splenic marginal zone hyperplasia in mice. *KLF2*
58 mutation was found in 40 (42%) of 96 SMZLs, but rarely in other B-cell lymphomas. The majority of
59 *KLF2* mutations were frameshift indels or nonsense changes, with missense mutations clustered in
60 the C-terminal zinc finger domains. Functional assays showed that these mutations inactivated the
61 ability of KLF2 to suppress NF-κB activation by TLR, BCR, BAFFR and TNFR signalling. Further
62 extensive investigations revealed common and distinct genetic changes between SMZL with and
63 without *KLF2* mutation. *IGHV1-2* rearrangement and 7q deletion were primarily seen in SMZL with
64 *KLF2* mutation, while *MYD88* and *TP53* mutations were nearly exclusively found in those without
65 *KLF2* mutation. *NOTCH2*, *TRAF3*, *TNFAIP3* and *CARD11* mutations were observed in SMZL both with
66 and without *KLF2* mutation. Taken together, *KLF2* mutation is the most common genetic change in
67 SMZL and identifies a subset with a distinct genotype characterised by multi-genetic changes. These
68 different genetic changes may deregulate various signalling pathways and generate cooperative
69 oncogenic properties, thereby contributing to lymphomagenesis.

70

71

72

73 **INTRODUCTION**

74

75 Splenic marginal zone lymphoma (SMZL), a low grade B-cell lymphoma, is difficult to diagnose
76 accurately due to a lack of specific histological, immunophenotypic and genetic markers.[1] Patients
77 with SMZL present with a highly variable clinical course with the majority showing a median survival
78 of 10 years, ~25% of cases die of the disease within 5 years and a further ~5% of cases display high-
79 grade transformation.[2,3] Despite the advances in treatment of other lymphomas, the survival of
80 patients with SMZL has not been improved over the last decade.[4] These dilemmas in diagnosis and
81 clinical management are largely due to poor understanding of its genetics and molecular mechanism.
82
83 There is mounting evidence suggesting a role for antigenic stimulation in the pathogenesis of SMZL.
84 Approximately 20% of patients with SMZL present with autoimmune phenomena. A small
85 proportion of cases are associated with HCV infection and can be effectively treated by antiviral
86 therapy.[5] Importantly, >30% of SMZL has biased usage of *IG* heavy chain variable gene, *IGHV1-2.*[6,7]
87 Most of the *IGHV1-2* rearrangements are characterised by minimal somatic mutations and longer
88 complementarity determining region-3 (CDR3) sequence with common motifs, suggesting a possible
89 selection by superantigens.[7] Together, these findings indicate a critical role of active BCR signalling
90 in the pathogenesis of SMZL.
91
92 SMZL lacks recurrent chromosome translocations. Approximately 30% of SMZLs show hemizygous
93 7q deletion, which is also seen frequently in splenic B-cell lymphoma/leukaemia unclassifiable
94 (SBCLU), but rarely in other lymphoma subtypes.[8-10] The gene(s) targeted by the 7q deletion remain
95 obscure despite the combined investigation of genomic and transcriptomic profiles and mutation
96 analysis of a number of candidate genes.[11,12]
97

Recent studies by whole exome sequencing (WES) identified a plethora of somatic mutations in SMZL.[13-16] These studies together with candidate gene sequencing showed a diverse spectrum of mutations in the NOTCH, NF-κB, BCR and TLR pathways, and in histone modifiers and transcriptional regulators.[13-18] Most of these mutations were found in <10% of cases, with only *NOTCH2* mutations occurring more frequently, but variably among different studies (6.5-25%).[13-16] Importantly, a significant proportion of SMZL lack any of these candidate pathogenic mutations. As the number of cases investigated by WES in each of the above studies was small (6-15 cases),[13-16] the mutation landscape in SMZL is likely not yet fully characterised. In this study, we identified further novel recurrent mutations in SMZL by WES and showed that *KLF2* (Kruppel-like factor 2) was inactivated by mutations in 42% of SMZL. Mechanistically, *KLF2* mutations abrogated KLF2-mediated suppression of NF-κB activation by TLR, BCR, BAFFR and TNFR signaling. *KLF2* mutation is significantly associated with 7q deletion, *IGHV1-2* usage, *NOTCH2*, *TNFAIP3* and *TRAF3* mutation, and may potentially cooperate with these genetic changes in oncogenesis.

MATERIALS AND METHODS

Patients samples: Tumour DNA was extracted from 105 cases of SMZL (77 from fresh frozen [FF] lymphoma tissues, 3 from leukemic peripheral blood samples, 25 from formalin-fixed paraffin-embedded [FFPE] lymphoma tissue), SBCLU (n=3), chronic lymphocytic leukaemia (CLL, n=39, all from bone marrow aspirate), hairy cell leukaemia (HCL, n=30, all from bone marrow aspirate), extranodal marginal zone lymphoma of mucosa-associated lymphoma tissue (MALT lymphoma, n=47, all from FFPE diagnostic tissue biopsies), mantle cell lymphoma (MCL, 7 from FF and 4 from FFPE diagnostic tissue biopsies), follicular lymphoma (FL, 5 from FF and 6 from FFPE diagnostic tissue biopsies), and diffuse large B-cell lymphoma (DLBCL, n=28, all FFPE diagnostic tissue biopsies). The lymphoma diagnosis was made according to the 2008 WHO classification of tumours of haematopoietic and lymphoid tissues. Where indicated, germline DNA was prepared from non-

124 neoplastic cells of tissues. The DNA quality was assessed by PCR of variably sized genomic
125 fragments,[19] and samples with successful amplifications of >300bp were used for genetic analyses.
126 Partial data on 7q deletion and *IGHV* usage in SMZL were available from previous studies,[10-12] with all
127 other genetic data collected in this study. Local ethical guidelines were followed for the use of
128 archival tissues for research with the approval of the ethics committees of the involved institutions.
129
130 **Exome sequencing and somatic variant calling:** These were carried out by the Wellcome Trust
131 Sanger Institute. High molecular weight (HMW) tumour DNA samples from 16 cases of SMZL and
132 matched germline DNA samples from 3 of these cases (non-neoplastic FFPE tissues in 1, buccal swap
133 or non-involved peripheral blood sample in 2) were used to generate genomic libraries with the
134 Illumina Paired End Sample Prep Kit (Table S1). Enrichment was performed using the Agilent
135 SureSelect Human All Exon 50Mb kit.[20-22] Each exome was sequenced using a 75bp paired-end
136 protocol on an Illumina HiSeq platform. Sequencing reads were aligned to the hg19 reference
137 genome using the BWA algorithm on default settings.
138
139 Novel variants were called by comparison of tumour and germline sequence reads. CaVEMan
140 (Cancer Variants through Expectation Maximisation) was used to call single nucleotide substitution,[21]
141 while Pindel was used to call insertions and deletions.[23] Post processing filters were applied to
142 increase the specificity of the output, remove variants reported in poor quality sequences and
143 remove known SNPs in databases and unmatched normals from this study and the 10,000 genomes
144 project.
145
146 **Somatic variant validation by PCR and Sanger sequencing:** Where indicated, novel variants
147 identified by WES and their potential somatic origin were first confirmed by PCR and Sanger
148 sequencing. Depending on the nature of gene sequences, different approaches were employed for
149 mutation screening. Mutations in *KLF2* were screened by PCR and Sanger sequencing as the gene

150 has a high GC content (Table S2). In each case, sequence change was confirmed by at least two
151 independent PCR and sequencing experiments. The somatic mutation was ascertained by excluding
152 germline changes through SNP database search and analysis of germline DNA samples where
153 possible.
154
155 **Somatic variant validation by Fluidigm Access Array PCR and Illumina MiSeq sequencing:**
156 Mutations in *NOTCH2*, *TNFAIP3, TRAF3*, *MYD88, IKBKB, CARD11, BCL10, CD79A, CD79B* and *TP53*
157 were screened by massive parallel Fluidigm Access Array PCR and Illumina MiSeq sequencing using
158 our established protocol from a parallel investigation (manuscript in preparation). The in house
159 variant calling algorithm was developed and optimised against a large number of various known
160 somatic mutations by Sanger sequencing. Please refer to Supplementary Methods for experimental
161 details and variant calling algorithms (Table S3). Each sample was investigated in duplicate to
162 eliminate any potential false positives. Any novel variants seen in both replicates of the same
163 sample were further ascertained by an independent Fluidigm PCR and MiSeq sequencing or Sanger
164 sequencing. Where indicated, their somatic nature was confirmed by PCR and Sanger sequencing of
165 the paired non-tumour DNA sample, or by search of COSMIC somatic mutation database.

166 **NF-κB reporter assay:** The full-length coding sequence and various truncated forms of *KLF2* were
167 amplified from pCMV6-AC-GFP (OriGene, USA) by PCR and cloned into the pIRES-puro2-HA vector at
168 the *EcoRI* and *BamHI* sites. The *KLF2* mutant containing a single point mutation was generated from
169 the wild type using the QuickChange Site-directed mutagenesis kit (Stratagene, USA). PCR and
170 sequencing were performed to verify the *KLF2* sequence and reading frame. The effect of KLF2 and
171 its mutants on suppression of NF-κB activation by various stimuli (TNFα, BAFF, mutant MYD88 or
172 mutant CARD11) was investigated where appropriate in both HEK293T and OCI-LY19 human B-cell
173 lymphoma cell lines using a Dual-Luciferase reporter assay (Promega, UK).[24,25] Please refer to
174 Supplementary Methods for experimental details.
175

176

177 **Analysis of rearranged *IGH* genes by PCR and Sanger sequencing:** The rearranged *IGH* genes were
178 amplified using BIOMED-2 FR1 and consensus JH primer sets.[26] The PCR product was purified and
179 sequenced as previously described.[10] The VH sequence was identified using the IMGT/V-QUEST
180 database (http://www.imgt.org). Cases harbouring < 97% homology were considered significantly
181 mutated, while those with 97-99.9% identity were regarded minimally mutated.[7]

182

183 **Statistical analyses:** The student's t-test was performed using GraphPad Prism version 5.00
184 software (GraphPad Software, San Diego, USA). The correlation among categorical variables was
185 evaluated by Fisher's exact probability test. Overall survival (OS) was measured from the date of
186 diagnosis to death from any cause. Probabilities of OS were calculated by the Kaplan–Meier method,
187 and the comparison between subgroups was performed via the log-rank test. Kaplan-Meier analysis,
188 log-rank test and Fisher's exact test were carried out using SPSS, version 13.

189

190 **RESULTS**

191

192 **Identification of *KLF2* mutation by WES**

193

194 WES was successful for all 16 tumour and 3 matched germline DNA samples (Table S4). Based on
195 the 3 cases with matched germline DNA, a total of 174 variants in 163 genes (average 58/case; range
196 45-82/case) were seen, with variants in 135 genes being novel, not reported previously in SMZL
197 (Table S5). The number of variants in the remaining cases was much higher due to a lack of WES data
198 from matched germline DNA, thus preventing the filter of all SNPs. Nonetheless, a total of 223
199 variants were observed in 159 genes known to be mutated in SMZL by previous studies, including
200 those described in the NOTCH2 signalling pathway (*NOTCH2*, *NOTCH4*, *SPEN*), NF-κB pathway
201 (*TNFAIP3*, *TRAF3*, *BIRC3*), BCR pathway (*CARD11*) and TLR pathway (*MYD88*) (Figure S1).[13-16]

202 Comparative analyses of the exome sequencing data from the 4 published WES studies also revealed
203 little overlap among the variants identified in these studies (Figure S2).[13-16] Together, these findings
204 suggest the presence of a remarkable heterogeneity or incomplete discovery of the somatic
205 mutation profile in SMZL, or both.
206
207 Among the variants not reported previously, there were several recurrent changes not seen in the
208 matched control DNA. Based on the frequency, possible functional impact and a comprehensive
209 literature search, we identified *KLF2* mutation as a potentially significant genetic abnormality in
210 SMZL (Figure S1). Among the 16 SMZLs investigated by WES in this study, *KLF2* mutation was seen
211 in 5 cases, all being deleterious changes (frameshift insertion/deletion in 2, nonsense mutation in 2,
212 and substitution change at an essential splice site in 1 case). Further PCR and Sanger sequencing
213 confirmed these mutations, and their somatic origin in all 5 cases. In support of the pathogenic
214 importance of these mutations in SMZL, Klf2 deficient mice were previously shown to have a marked
215 increase in marginal zone B-cells and splenic marginal zone hyperplasia.[27-29]
216
217 ***KLF2* is frequently targeted by mutation in SMZL.**
218
219 Next, we investigated *KLF2* mutation in 96 cases of SMZL including 13 of the 16 cases investigated by
220 WES, and 7 other B-cell lymphoma entities to determine its frequencies and mutation spectrum. As
221 the *KLF2* gene has a high GC content that may have accounted for the failure of detection of its
222 mutation by previous exome sequencing studies,[13-16] PCR and Sanger sequencing were used for
223 mutation screening. A total of 47 *KLF2* mutations were seen in 40 (42%) of the 96 cases of SMZL
224 with double mutations in 7 cases, and their somatic nature was confirmed in each of the 8 cases
225 (including 3 indels and 7 substitutions), for which non-tumour DNA was available (Figure 1A, Figure
226 S3, Table S6). Of note, Sanger sequencing identified 4 additional mutations in 3 cases missed by WES,
227 which included 1 frameshift deletion, 1 in-frame deletion and 2 missense substitutions. In contrast,

228 *KLF2* mutation was not or rarely seen in SBCLU (0/3), CLL (0/39), HCL (3/30), FL (1/11), MCL (1/11),
229 MALT lymphoma (2/47) and DLBCL (0/28) (Figure 1B).
230
231 Among the 47 *KLF2* mutations identified in SMZL, 27 were frameshift insertions / deletions,
232 nonsense mutations, or substitutions affecting the essential splice site, thus resulting in a potentially
233 truncated protein product (Figure 1A). Importantly, a high proportion of these deleterious
234 mutations were localised toward the N-terminal activation domain (AD) and middle inhibitory
235 domain (ID). The remaining 20 mutations seen in SMZL were 19 missense substitutions and 1 in-
236 frame deletion, with 12 clustered in the C-terminal zinc finger (ZF) 1, mainly at conserved amino acid
237 residues (Figure 1A). Fourteen missense mutations were predicted to be damaging by the
238 PolyPhen-2 program,[30] and a further 2 missense mutations abolished the stop codon with potential
239 extension of a further 62 amino acids. Among the 7 *KLF2* mutations found in other lymphoma
240 entities, 2 were frameshift deletions in the ID and ZF3 domains respectively, and 5 were missense
241 mutations with only 1 in the C-terminal ZF1 domain (Figure 1A).
242
243 **Functional characterisation of *KLF2* mutations**
244
245 The nature and distribution of the *KLF2* mutations suggest that these genetic changes are likely to
246 inactivate KLF2 function. We thus generated a series of *KLF2* expression constructs, representing
247 various C-terminal truncated products that were lacking 1 or more ZF domains, and recurrent
248 missense or in-frame deletion mutants that affected conserved amino acid residues in the ZF1
249 domain, and tested their impact on KLF2 function using *in vitro* reporter assays in both HEK293T and
250 OCI-LY19 B-lymphoma cells. Previous studies have shown that KLF2 inhibits the transcriptional
251 activity of NF-κB.[31] We therefore tested the ability of various KLF2 mutants to suppress NF-κB
252 activation by TNFα, MYD88(S219C), CARD11(F130V) and BAFF, which were used to activate TNFR,
253 TLR, canonical and non-canonical NF-κB signalling pathways respectively.

254

255

256 As expected, wild type KLF2 was highly potent in the suppression of NF-κB activation by different
257 signalling pathways including stimulation by TNFα, MYD88(S219C), CARD11(F130V) and BAFF
258 (Figure 2B). With the exception of KLF2-A291V mutant, all other 5 mutants including KLF2-C274Y
259 and KLF2-ΔTY mutants showed a total or major loss in NF-κB suppression, although to varying
260 extents depending on stimuli used to activate NF-κB. Interestingly, both cases with KLF2-A291V had
261 a second mutation, one with P70S in the activation domain, the other with C274S, at which a C274Y
262 change was shown to impair KLF2 function as described above (Table S6).
263
264 ***KLF2* mutation identifies a subset of SMZL with distinct genotypes**
265
266 To further characterise the genetics of SMZL and understand their potential cooperation in
267 lymphomagenesis, we comprehensively investigated somatic mutations in *NOTCH2*, *TNFAIP3*, *TRAF3*,
268 *MYD88*, *CD79A*, *CD79B*, *CARD11*, *BCL10*, *IKBKB* and *TP53* (Figure 3, Table S6), 7q deletion and *IGHV*
269 usage in the entire cohort of SMZL and correlated their changes with *KLF2* mutation using the
270 Fisher's exact probability test. The analyses revealed several significant associations.
271
272 First, *KLF2* mutation identified a subset of SMZL with distinct genetic changes. The mutation was
273 significantly associated with both 7q deletion (P=7.33×10^{-7}) and *IGHV1-2* usage (P=1.02×10^{-7}), seen
274 in 77% cases with 7q deletion and 83% of those with *IGHV1-2* (Figure 4). As with previous studies,[6,7]
275 the rearranged *IGHV1-2* was characterised by minimal somatic mutations (Figure 4A).
276
277 Second, mutations in the *NOTCH2*, *TRAF3*, *TNFAIP3* and *CARD11* genes were found in SMZL both
278 with and without *KLF2* mutation, with *NOTCH2*, *TRAF3* and *TNFAIP3* mutations being significantly
279 associated with *KLF2* mutations (Figure 4C, P=0.007, P=0.012 and P=0.015 respectively). In line with

280 the recent studies, [13,14,16] *NOTCH2* mutations were characterised by frameshift insertion/deletion,
281 and nonsense mutations, which were clustered at the C-terminus and predicted to eliminate the C-
282 terminal PEST, a domain critical for NOTCH2 proteasomal degradation (Figure 3). *TRAF3* and
283 *TNFAIP3* mutations were featured by frameshift insertions/deletion and nonsense mutations, while
284 *CARD11* mutations were typically activating changes reported elsewhere (Figure 3).[32,33]
285

286 Third, most SMZL without *KLF2* mutation showed heterogeneous usage of *IGHV* in their rearranged
287 *IGH* genes, and the majority of these rearranged *IGHV* harboured high loads of somatic mutations.
288 Interestingly, *MYD88* mutations were exclusively seen in cases without *KLF2* mutation (Figure 4A&C,
289 *P*=0.021). *MYD88* mutations were typically those of activating changes, while *TP53* mutations were
290 characteristic inactivating changes reported elsewhere.
291

292 Mutation in *CD79A* and *CD79B* (both seen in a single case), *BCL10* [2 cases, both mutations predicted
293 a C-terminal truncated BCL10 with a potential gain of function[34]], and *IKBKB* (1 case) was found to
294 be low in SMZL. Interestingly, these mutations were mutually exclusive from *CARD11* mutations.

295 **Correlation among genetic abnormalities and clinicopathological parameters**

296

297 Follow-up data were available for 60 cases of SMZL, ranging from 12 to 288 months (median = 55
298 months). Kaplan-Meier univariate analysis of the genetic and clinical variables showed that only
299 *TP53* mutation was significantly associated with poor 5-year overall survival (*P* =0.002) (Table S7).
300 However, the number of cases and death events were not sufficient for reliable multivariate analysis.

301

302 **DISCUSSION**

303

304 By WES and validation of the mutations identified, we have made several novel and significant
305 discoveries in the present study. First, *KLF2* is frequently mutated in SMZL (42%), but not or rarely in
306 other lymphomas; second, *KLF2* mutations are characterised by frameshift insertion/deletion,
307 nonsense mutations, and a cluster of missense mutations in the ZF1 domain, which impair KLF2
308 function; third, there are distinct genetic changes according to *KLF2* mutation status. *IGHV1-2*
309 rearrangement and 7q deletion are essentially seen in SMZL with *KLF2* mutation, while *MYD88* and
310 *TP53* mutations are nearly exclusively seen in those without *KLF2* mutation. Mutations in *NOTCH2*,
311 *TRAF3*, *TNFAIP3* and *CARD11* genes were found in SMZL both with and without *KLF2* mutation.
312 These distinct mutation patterns indicate overlapping molecular mechanisms between SMZL with
313 and without *KLF2* mutation, and also suggest the presence of different oncogenic cooperation
314 between the two subgroups.
315
316 ***KLF2* mutation and its distinctively associated genetic changes**
317
318 Among the diverse spectrum of mutations identified in SMZL, *KLF2* mutation (42%) is the most
319 frequent genetic change, much higher than the recently identified *NOTCH2* mutation (6.5-25%).[13-16]
320 The nature of *KLF2* mutations and our *in vitro* functional studies of *KLF2* mutants indicate that these
321 mutations inactivate KLF2 function.
322
323 A pathogenic role of *KLF2* inactivating mutations in SMZL is strongly supported by recent findings,
324 particularly those by studies of Klf2 knockout mice. Remarkably, B-cell specific Klf2-deficient mice
325 show a dramatic increase of marginal zone B-cells.[27-29] Klf2 deficiency appears to promote follicular
326 B-cells to gain a marginal zone like phenotype and migrate to the splenic marginal zone, but have
327 little impact on their proliferation.[27-29] The molecular mechanism underlying the altered B-cell
328 homeostasis and trafficking in Klf2-deficient mice is unclear although Klf2 most likely exerts such
329 effects through transcriptional regulation of its target genes.

330

331 KLF2 is a member of the KLF family of transcription factors, and has been recently shown to be a

332 negative regulator of inflammation and NF-κB activities.[35-38] KLF2 appears to regulate NF-κB

333 activities by modulating recruitment of critical NF-κB coactivators.[38] Using an *in vitro* reporter assay,

334 we showed that wild type KLF2 was a potent inhibitor of NF-κB activation by several signalling

335 pathways including BCR (CARD11 mutant), TLR (MYD88 mutant), TNFR (TNFα) and BAFFR (BAFF). In

336 contrast, KLF2 mutants had a total or major loss in suppression of NF-κB activation triggered by

337 these signals. Given the importance of TLR, canonical and non-canonical NF-κB pathways in the

338 development of marginal zone B-cells,[39,40] KLF2 inactivation by mutation may exert its oncogenic

339 activities at least in part by deregulation of NF-κB activities triggered by these signals, leading to

340 altered gene expression favouring B-cells homing to the marginal zone. However, *KLF2* inactivation

341 alone is insufficient for malignant transformation, and requires cooperating genetic and cellular

342 events in SMZL development.

343

344 The majority of SMZL with *KLF2* mutation have both 7q deletion and *IGHV1-2* rearrangement. The

345 genes targeted by 7q deletion are unclear.[11,12] *IGHV1-2* usage is over-represented in SMZL,

346 accounting for 30% of cases. Although the epitope recognised by *IGHV1-2* expressing BCR and its

347 potential impact on clinicopathological presentation are unknown, the features of *IGHV1-2*

348 rearrangements, including minimal somatic mutations and longer CDR3 sequence with common

349 motifs, suggest a possible selection of T-cell independent marginal zone B-cells by superantigens,[7]

350 thus a role of antigenic drive in the lymphomagenesis (Figure 5). Such active BCR signalling may

351 cooperate with *KLF2* inactivation in SMZL development. Apart from *IGHV1-2* BCR stereotype, there

352 are further genetic changes that potentially cooperate with KLF2 inactivation although these genetic

353 changes occur in SMZL both with and without *KLF2* mutation.

354

355 **Genetic changes common to SMZL both with and without *KLF2* mutation**

356

357 *NOTCH2*, *TRAF3*, *CARD11* and *TNFAIP3* mutations were found in SMZL both with and without *KLF2*
358 mutation. *NOTCH2*, *TRAF3* and *CARD11* mutations are most likely to enhance the NOTCH2, non-
359 canonical NF-κB and BCR signalling respectively,[13,14,16,41,42] while *TNFAIP3* mutation may augment
360 several molecular pathways including TNFR, TLR/IL1-R and BCR signalling.[43] All these innate signals
361 are critical for the development of marginal zone B-cells although their precise role remains to be
362 dissected. NOTCH2 signalling is critical for generation of marginal zone B-cells and their retention in
363 the splenic marginal zone.[44-47] Active NOTCH2 signalling alone appears to have little impact on cell
364 proliferation and survival, but sensitises B-cells to stimulation of surface TLR and CD40.[46,47] Non-
365 canonical NF-κB signalling, typically triggered by stimulation of surface BAFFR and CD40, is also
366 pivotal in development of marginal zone B-cells and formation of the splenic marginal zone.[48]
367 Chronic active BCR signalling promotes cellular proliferation and survival, and TLR signalling may
368 contribute to both the development and survival of marginal zone B-cells.[40] Marginal zone B cells
369 express NOTCH2, BAFFR, CD40, and high levels of TLRs, while the splenic innate lymphoid cells (ILCs)
370 express surface DLL1 (Notch ligand Delta-like 1), BAFF, CD40L and provide contact-dependent help
371 to marginal zone B-cells by stimulation of the respective receptors in a cooperative manner.[39,40,49]
372 Thus, *NOTCH2*, *TRAF3*, *CARD11* and *TNFAIP3* mutation may cooperate with the aforementioned
373 surface receptor stimulation and cause constitutive activation of the corresponding signalling
374 pathway.

375

376 The respective signalling enhanced by *NOTCH2*, *TRAF3*, *TNFAIP3* and *CARD11* mutation likely
377 complements the molecular mechanism deregulated by *KLF2* mutation, *IGHV1-2* expressing BCR and
378 genes targeted by 7q deletion, thus cooperating in SMZL development (Figure 5). However, the
379 genetic events that cooperate with these mutations in SMZL without *KLF2* mutation are unclear.

380

381 **Genetic changes preferentially associated with SMZL without *KLF2* mutation**

382

383 There are also several interesting features in SMZL without *KLF2* mutation, including infrequent 7q
384 deletion and *IGHV1-2* rearrangement, and nearly exclusive association with *MYD88* and *TP53*
385 mutations (Figure 5). Among SMZL without *KLF2* mutation, there is a heterogeneous usage of *IGHV*
386 and the majority of these rearranged *IGHV* genes show high levels of somatic mutations, suggesting
387 origin from T-cell dependent marginal zone B-cells.[50]

388

389 The *MYD88* mutations seen in SMZL are typically those of gain-of-function change, capable of
390 spontaneously assembling a signalling complex to activate NF-κB, STAT3 and AP1 transcription
391 factors.[51] *MYD88* activation by mutation may lead to biological consequences similar to that by TLR
392 activation implicated by KLF2 and/or TNFAIP3 inactivation.

393

394 The *TP53* mutations seen in SMZL were typically those reported elsewhere, and these mutations
395 likely inactivate TP53 function. *TP53* mutation in SMZL, like in other lymphomas, is likely to be a
396 secondary genetic event. In line with this, TP53 inactivation is associated with progression and poor
397 prognosis in SMZL.[12,52,53]

398

399 In summary, there are common and distinct genetic changes between SMZL with and without *KLF2*
400 mutation and these different genetic changes most likely deregulate several signalling pathways
401 important for the generation of marginal zone B-cells, their migration and retention in the splenic
402 marginal zone. Each of these genetic changes may have a predominant impact on a particular
403 biological process and contribute to the lymphoma development through oncogenic cooperation
404 with other concurrent changes (Figure 5). It is pertinent to tentatively speculate that 1) KLF2
405 inactivation may deregulate gene expression through the modulation of NF-κB activities and other
406 unknown mechanisms, thereby promoting B-cells homing to the splenic marginal zone; 2) NOTCH2
407 activation, TRAF3 and TNFAIP3 inactivation and MYD88 activation by mutations may contribute to

408 the generation of marginal zone B-cells and their retention in the splenic marginal zone by
409 augmenting the NOTCH2, non-canonical NF-κB pathway and TLR signalling respectively; 3) *IGHV1-2*
410 rearrangement and CARD11 activation by mutations may lead to chronic active BCR signalling,
411 consequently enhancing cell proliferation and survival. A simultaneous deregulation of the above
412 signalling pathways in SMZL with *KLF2* mutation may generate complementary properties in
413 oncogenic cooperation, leading to lymphoma development.
414
415
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423 DO; Manuscript writing and preparation: MQD, AC, MW. Study design and coordination: MQD, AJW.
424 All authors commented on the manuscript and approve its submission for publication. There is no
425 conflict of interest to declare.

426

427 Supplementary information is available at Leukemia's website.
428

429

430 **References:**

431 1 Isaacson PG, Piris MA, Berger F, Swerdlow SH, Thieblemont C, Pittaluga S, Harris NL. Splenic B-
432 cell marginal zone lymphoma. In: Swerdlow SH, Campo E, Harris NL, Jaffe ES, Pileri SA, Stein H,
433 Thiele J, Vardiman JW, editors. WHO Classification of Tumous of Haematopoietic and
434 Lymphoid Tissues. Lyon: International Agency for Research on Cancer, 2008: 185-187.

435 2 Arcaini L, Lazzarino M, Colombo N, Burcheri S, Boveri E, Paulli M et al. Splenic marginal zone
436 lymphoma: a prognostic model for clinical use. *Blood* 2006; **107**: 4643-4649.

437 3 Montalban C, Abraira V, Arcaini L, Domingo-Domenech E, Guisado-Vasco P, Iannito E et al. Risk
438 stratification for Splenic Marginal Zone Lymphoma based on haemoglobin concentration,
439 platelet count, high lactate dehydrogenase level and extrahilar lymphadenopathy:
440 development and validation on 593 cases. *Br J Haematol* 2012; **159**: 164-171.

441 4 Olszewski AJ, Castillo JJ. Survival of patients with marginal zone lymphoma: Analysis of the
442 Surveillance, Epidemiology, and End Results database. *Cancer* 2013; **119**: 629-638.

443 5 Arcaini L, Bruno R. Hepatitis C virus infection and antiviral treatment in marginal zone
444 lymphomas. *Curr Clin Pharmacol* 2010; **5**: 74-81.

445 6 Zibellini S, Capello D, Forconi F, Marcatili P, Rossi D, Rattotti S et al. Stereotyped patterns of B-
446 cell receptor in splenic marginal zone lymphoma. *Haematologica* 2010; **95**: 1792-1796.

447 7 Bikos V, Darzentas N, Hadzidimitriou A, Davis Z, Hockley S, Traverse-Glehen A et al. Over 30%
448 of patients with splenic marginal zone lymphoma express the same immunoglobulin heavy
449 variable gene: ontogenetic implications. *Leukemia* 2012; **26**: 1638-1646.

450 8 Mateo M, Mollejo M, Villuendas R, Algara P, Sanchez-Beato M, Martinez P et al. 7q31-32 allelic
451 loss is a frequent finding in splenic marginal zone lymphoma. *Am J Pathol* 1999; **154**: 1583-
452 1589.

453 9 Gruszka-Westwood AM, Hamoudi R, Osborne L, Matutes E, Catovsky D. Deletion mapping on
454 the long arm of chromosome 7 in splenic lymphoma with villous lymphocytes. *Genes*
455 *Chromosomes Cancer* 2003; **36**: 57-69.

456 10 Watkins AJ, Huang Y, Ye H, Chanudet E, Johnson N, Hamoudi R et al. Splenic marginal zone
457 lymphoma: characterization of 7q deletion and its value in diagnosis. *J Pathol* 2010; **220**: 461-
458 474.

459 11 Watkins AJ, Hamoudi RA, Zeng N, Yan Q, Huang Y, Liu H et al. An integrated genomic and
460 expression analysis of 7q deletion in splenic marginal zone lymphoma. *PLoS One* 2012; **7**:
461 e44997.

462 12 Fresquet V, Robles EF, Parker A, Martinez-Useros J, Mena M, Malumbres R et al. High-
463 throughput sequencing analysis of the chromosome 7q32 deletion reveals IRF5 as a potential
464 tumour suppressor in splenic marginal-zone lymphoma. *Br J Haematol* 2012; **158**: 712-726.

465 13 Rossi D, Trifonov V, Fangazio M, Bruscaggin A, Rasi S, Spina V et al. The coding genome of
466 splenic marginal zone lymphoma: activation of NOTCH2 and other pathways regulating
467 marginal zone development. *J Exp Med* 2012; **209**: 1537-1551.

468 14 Kiel MJ, Velusamy T, Betz BL, Zhao L, Weigelin HG, Chiang MY et al. Whole-genome sequencing
469 identifies recurrent somatic NOTCH2 mutations in splenic marginal zone lymphoma. *J Exp Med*
470 2012; **209**: 1553-1565.

471 15 Martinez N, Almaraz C, Vaque JP, Varela I, Derdak S, Beltran S et al. Whole-exome sequencing
472 in splenic marginal zone lymphoma reveals mutations in genes involved in marginal zone
473 differentiation. *Leukemia* 2014; **28**: 1334-1340.

474 16 Parry M, Rose-Zerilli MJ, Gibson J, Ennis S, Walewska R, Forster J et al. Whole exome
475 sequencing identifies novel recurrently mutated genes in patients with splenic marginal zone
476 lymphoma. *PLoS One* 2013; **8**: e83244.

477 17 Yan Q, Huang Y, Watkins AJ, Kocialkowski S, Zeng N, Hamoudi RA et al. BCR and TLR signaling
478 pathways are recurrently targeted by genetic changes in splenic marginal zone lymphomas.
479 *Haematologica* 2012; **97**: 595-598.

480 18 Rossi D, Deaglio S, Dominguez-Sola D, Rasi S, Vaisitti T, Agostinelli C et al. Alteration of BIRC3
481 and multiple other NF-{kappa}B pathway genes in splenic marginal zone lymphoma. *Blood*
482 2011.

483 19 Liu H, Bench AJ, Bacon CM, Payne K, Huang Y, Scott MA et al. A practical strategy for the
484 routine use of BIOMED-2 PCR assays for detection of B- and T-cell clonality in diagnostic
485 haematopathology. *Br J Haematol* 2007; **138**: 31-43.

486 20 Papaemmanuil E, Cazzola M, Boultwood J, Malcovati L, Vyas P, Bowen D et al. Somatic SF3B1
487 mutation in myelodysplasia with ring sideroblasts. *N Engl J Med* 2011; **365**: 1384-1395.

488 21 Varela I, Tarpey P, Raine K, Huang D, Ong CK, Stephens P et al. Exome sequencing identifies
489 frequent mutation of the SWI/SNF complex gene PBRM1 in renal carcinoma. *Nature* 2011; **469**:
490 539-542.

491 22 Stephens PJ, Tarpey PS, Davies H, Van Loo P, Greenman C, Wedge DC et al. The landscape of
492 cancer genes and mutational processes in breast cancer. *Nature* 2012; **486**: 400-404.

493 23 Ye K, Schulz MH, Long Q, Apweiler R, Ning Z. Pindel: a pattern growth approach to detect
494 break points of large deletions and medium sized insertions from paired-end short reads.
495 *Bioinformatics* 2009; **25**: 2865-2871.

496 24 Bi Y, Zeng N, Chanudet E, Huang Y, Hamoudi RA, Liu H et al. A20 inactivation in ocular adnexal
497 MALT lymphoma. *Haematologica* 2012; **97**: 926-930.

498 25 Yan Q, Wang M, Moody S, Xue X, Huang Y, Bi Y et al. Distinct involvement of NF-kappaB
499 regulators by somatic mutation in ocular adnexal malt lymphoma. *Br J Haematol* 2013; **160**:
500 851-854.

501 26 van Dongen JJ, Langerak AW, Bruggemann M, Evans PA, Hummel M, Lavender FL et al. Design
502 and standardization of PCR primers and protocols for detection of clonal immunoglobulin and
503 T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2
504 Concerted Action BMH4-CT98-3936. *Leukemia* 2003; **17**: 2257-2317.

505 27 Hoek KL, Gordy LE, Collins PL, Parekh VV, Aune TM, Joyce S et al. Follicular B cell trafficking
506 within the spleen actively restricts humoral immune responses. *Immunity* 2010; **33**: 254-265.

507 28 Winkelmann R, Sandrock L, Porstner M, Roth E, Mathews M, Hobeika E et al. B cell
508 homeostasis and plasma cell homing controlled by Kruppel-like factor 2. *Proc Natl Acad Sci U S*
509 *A* 2011; **108**: 710-715.

510 29 Hart GT, Wang X, Hogquist KA, Jameson SC. Kruppel-like factor 2 (KLF2) regulates B-cell
511 reactivity, subset differentiation, and trafficking molecule expression. *Proc Natl Acad Sci U S A*
512 2011; **108**: 716-721.

513 30 Adzhubei IA, Schmidt S, Peshkin L, Ramensky VE, Gerasimova A, Bork P et al. A method and
514 server for predicting damaging missense mutations. *Nat Methods* 2010; **7**: 248-249.

515 31 Das H, Kumar A, Lin Z, Patino WD, Hwang PM, Feinberg MW et al. Kruppel-like factor 2 (KLF2)
516 regulates proinflammatory activation of monocytes. *Proc Natl Acad Sci U S A* 2006; **103**: 6653-
517 6658.

518 32 Lenz G, Davis RE, Ngo VN, Lam L, George TC, Wright GW et al. Oncogenic CARD11 mutations in
519 human diffuse large B cell lymphoma. *Science* 2008; **319**: 1676-1679.

520 33 Dong G, Chanudet E, Zeng N, Appert A, Chen YW, Au WY et al. A20, ABIN-1/2 and CARD11
521 mutations and their prognostic value in gastrointestinal diffuse large B-cell lymphoma. *Clin*
522 *Cancer Res* 2011.

523 34 Du MQ, Peng H, Liu H, Hamoudi RA, Diss TC, Willis TG et al. BCL10 gene mutation in lymphoma.
524 *Blood* 2000; **95**: 3885-3890.

525 35 Mahabeleshwar GH, Kawanami D, Sharma N, Takami Y, Zhou G, Shi H et al. The myeloid
526 transcription factor KLF2 regulates the host response to polymicrobial infection and endotoxic
527 shock. *Immunity* 2011; **34**: 715-728.

528 36 Heo KS, Chang E, Takei Y, Le NT, Woo CH, Sullivan MA et al. Phosphorylation of protein
529 inhibitor of activated STAT1 (PIAS1) by MAPK-activated protein kinase-2 inhibits endothelial
530 inflammation via increasing both PIAS1 transrepression and SUMO E3 ligase activity.
531 *Arterioscler Thromb Vasc Biol* 2013; **33**: 321-329.

532 37 Zahlten J, Steinicke R, Opitz B, Eitel J, N'guessan PD, Vinzing M et al. TLR2- and nucleotide-
533 binding oligomerization domain 2-dependent Krüppel-like factor 2 expression down-regulates
534 NF-kappa B-related gene expression. *J Immunol* 2010; **185**: 597-604.

535 38 Nayak L, Goduni L, Takami Y, Sharma N, Kapil P, Jain MK et al. Kruppel-like factor 2 is a
536 transcriptional regulator of chronic and acute inflammation. *Am J Pathol* 2013; **182**: 1696-1704.

537 39 Pillai S, Cariappa A. The follicular versus marginal zone B lymphocyte cell fate decision. *Nat Rev*
538 *Immunol* 2009; **9**: 767-777.

539 40 Cerutti A, Cols M, Puga I. Marginal zone B cells: virtues of innate-like antibody-producing
540 lymphocytes. *Nat Rev Immunol* 2013; **13**: 118-132.

541 41 Sun SC. The noncanonical NF-kappaB pathway. *Immunol Rev* 2012; **246**: 125-140.

542 42 Niemann CU, Wiestner A. B-cell receptor signaling as a driver of lymphoma development and
543 evolution. *Semin Cancer Biol* 2013; **23**: 410-421.

544 43 Catrysse L, Vereecke L, Beyaert R, Van Loo G. A20 in inflammation and autoimmunity. *Trends*
545 *Immunol* 2014; **35**: 22-31.

546 44 Tanigaki K, Han H, Yamamoto N, Tashiro K, Ikegawa M, Kuroda K et al. Notch-RBP-J signaling is
547 involved in cell fate determination of marginal zone B cells. *Nat Immunol* 2002; **3**: 443-450.

548 45 Saito T, Chiba S, Ichikawa M, Kunisato A, Asai T, Shimizu K et al. Notch2 is preferentially
549 expressed in mature B cells and indispensable for marginal zone B lineage development.
550 *Immunity* 2003; **18**: 675-685.

551 46 Hampel F, Ehrenberg S, Hojer C, Draeseke A, Marschall-Schroter G, Kuhn R et al. CD19-
552 independent instruction of murine marginal zone B-cell development by constitutive Notch2
553 signaling. *Blood* 2011; **118**: 6321-6331.

554 47 Simonetti G, Carette A, Silva K, Wang H, De Silva NS, Heise N et al. IRF4 controls the positioning
555 of mature B cells in the lymphoid microenvironments by regulating NOTCH2 expression and
556 activity. *J Exp Med* 2013; **210**: 2887-2902.

557 48 Guo F, Weih D, Meier E, Weih F. Constitutive alternative NF-kappaB signaling promotes
558 marginal zone B-cell development but disrupts the marginal sinus and induces HEV-like
559 structures in the spleen. *Blood* 2007; **110**: 2381-2389.

560 49 Magri G, Miyajima M, Bascones S, Mortha A, Puga I, Cassis L et al. Innate lymphoid cells
561 integrate stromal and immunological signals to enhance antibody production by splenic
562 marginal zone B cells. *Nat Immunol* 2014; **15**: 354-364.

563 50 Pillai S, Cariappa A. The follicular versus marginal zone B lymphocyte cell fate decision. *Nat Rev*
564 *Immunol* 2009; **9**: 767-777.

565 51 Ngo VN, Young RM, Schmitz R, Jhavar S, Xiao W, Lim KH et al. Oncogenically active MYD88
566 mutations in human lymphoma. *Nature* 2011; **470**: 115-119.

567 52 Gruszka-Westwood AM, Hamoudi RA, Matutes E, Tuset E, Catovsky D. p53 abnormalities in
568 splenic lymphoma with villous lymphocytes. *Blood* 2001; **97**: 3552-3558.

569 53 Salido M, Baro C, Oscier D, Stamatopoulos K, Dierlamm J, Matutes E et al. Cytogenetic
570 aberrations and their prognostic value in a series of 330 splenic marginal zone B-cell
571 lymphomas: a multicenter study of the Splenic B-Cell Lymphoma Group. *Blood* 2010; **116**:
572 1479-1488.

573 **FIGURE LEGENDS:**

574 **Figure 1:** Nature and incidence of *KLF2* mutations in SMZL and other B-cell lymphomas.

575

576 A) Nature and distribution of *KLF2* mutations in lymphoma. The majority of *KLF2* mutations seen in
577 SMZL are frameshift deletion / insertion or nonsense mutations. Missense mutations are largely
578 clustered in ZF1 (zinc finger), particularly at conserved amino acid residues. Mutations were
579 confirmed by two independent PCR and sequencing experiments. Where possible the somatic
580 nature of the mutation identified was confirmed by PCR and sequencing analysis of the paired non-
581 tumour DNA and indicated by red symbols. Mutations identical to those confirmed to be somatic
582 are highlighted in blue. Concurrent mutations seen in the same cases are indicated by their case
583 number.

584

585 B) Frequencies of *KLF2* mutations in SMZL and various other B-cell lymphomas.

586

587 ZF: zinc finger; NLS: putative nuclear localisation signal.

588

589 **Figure 2:** Functional characterisation of *KLF2* mutations.

590

591 A) The representative *KLF2* mutants investigated by *in vitro* reporter assays, which include three
592 truncation, two recurrent missense and one in-frame deletion mutants. The missense change and
593 in-frame deletion affect the conserved amino acid residues.

594

595 B) NF-κB reporter assay shows that wild type KLF2 is a potent inhibitor of NF-κB activation by TNFα,
596 BAFF, MYD88 and CARD11 mutants in both HEK293T and OCI-LY19 B-lymphoma cells. With the
597 exception of KLF2-A291V mutant, all other 5 mutants including KLF2-C274Y and KLF2-ΔTY mutants
598 showed a total or major loss in NF-κB suppression. Interestingly, both the cases with KLF2-A291V

599 had a second mutation, one with P70S in the activation domain, the other with C274S. The data is
600 from at least three independent experiments and presented as a mean ± standard deviation, and the
601 difference between KLF2 and its mutants is analysed by the Student t-test. As indicated by an
602 arrowhead, the KLF2-AD-ZF1 mutant consistently shows an additional band, ~7 kDa larger than the
603 expected size (32 kDa). We have performed a series of experiments to confirm the correct sequence
604 and also rule out any cross-contamination of an additional clone. The band shift is most likely
605 caused by post-translational modifications, but its nature remains to be established. * P < 0.05, ** P
606 < 0.01, *** P < 0.001.
607
608
609 **Figure 3:** Nature and distribution of mutations in *NOTCH2*, *TNFAIP3*, *TRAF3*, *CARD11*, *MYD88* and
610 *TP53* in SMZL. *NOTCH2* mutations are characterised by frameshift insertion/deletion, and nonsense
611 mutations that are clustered at the C-terminus and predicted to eliminate the C-terminal PEST, a
612 domain critical for NOTCH2 proteasomal degradation. *TNFAIP3* and *TRAF3* mutations are featured
613 by frameshift insertion/deletion and nonsense mutations, which inactivate their protein functions.
614 *CARD11* and *MYD88* mutations are typically those of activating changes reported elsewhere. *TP53*
615 mutations are also characteristic inactivating changes extensively reported in the literature. The
616 mutations identified by Fluidigm PCR and MiSeq sequencing are confirmed either by an independent
617 Fluidigm PCR and MiSeq sequencing or Sanger sequencing. Where possible, the somatic nature of
618 mutation was determined: those confirmed by PCR and sequencing of the paired non-tumour DNA
619 are shown by red symbols, while those identified in a search of the COSMIC somatic mutation
620 database are shown by blue symbols. Concurrent mutations seen in the same cases are indicated by
621 their case number.
622
623 LNR: LIN-12/NOTCH repeats; HD: heterodimerisation; TM: transmembrane; RAM: regulation of
624 amino acid metabolism; TAD: transactivation domain; PEST: Proline, glutamic acid, serine and

625 threonine rich domain; ZF: zinc finger; CARD: caspase recruitment domain; PDZ: (PSD95, DLG and
626 ZO1 homology) domain; SH3: Src homology motif; GUK: guanylate kinase domain
627

628 **Figure 4:** Correlation of *KLF2* mutation with other genetic changes in SMZL.

629 A) Heatmap shows *KLF2* mutation and other genetic changes in 101 cases of SMZL. Rows
630 correspond to genetic change, while columns indicate individual cases. Positive genetic changes are
631 shown in green. Genes included in the BCR pathway mutations are *CARD11*, *BCL10*, *CD79A* and
632 *CD79B*.

633 B) Frequencies of *KLF2* mutation and other genetic changes in SMZL;

634 C) Correlation among *KLF2* mutation and other genetic changes in SMZL. *KLF2* mutation is
635 significantly and positively associated with 7q deletion, *IGHV1-2* usage, *NOTCH2*, *TRAF3* and *TNFAIP3*
636 mutations, but negatively correlated with *MYD88* mutation. BCR: B-cell receptor; Mut: mutation;
637 Del: deletion.

638

639 **Figure 5:** A summary of the proposed molecular mechanism of SMZL.

640 The majority of SMZL with *KLF2* mutation have the rearranged *IGHV1-2* that carries minimal levels of
641 somatic mutations, suggesting derivation of these lymphoma cells from T-cell independent marginal
642 zone B-cells. The biased usage of *IGHV1-2* indicates possible antigenic drive by superantigen, hence
643 chronic BCR signalling. *KLF2* inactivation by mutation may facilitate marginal zone B-cell
644 differentiation and their homing to the splenic marginal zone. 7q deletion is predominately seen in
645 cases with *KLF2* mutation and its role in the lymphoma pathogenesis is unknown.

646 *CARD11*, *NOTCH2*, *TRAF3* and *TNFAIP3* mutations are found in cases with and without *KLF2* mutation.
647 Mutations in *CARD11* and others (*CD79A/B*, *BCL10*) may lead to active BCR signalling, thereby

648 promoting cell proliferation and survival. Activation of NOTCH2, BAFFR/CD40 and TLR signalling by
649 *NOTCH2*, *TRAF3* and *TNFAIP3* mutation may primarily contribute to marginal zone B-cell generation
650 and their retention in the splenic marginal zone.

651 The majority of SMZL without *KLF2* mutation have heterogeneous usage of *IGHV* that carries high
652 loads of somatic mutation, suggesting origination of these lymphoma cells from T-cell dependent
653 marginal zone B-cells. *MYD88* and *TP53* mutations are nearly exclusively seen in cases without *KLF2*
654 mutation. *MYD88* mutation most likely causes constitutive TLR signalling, while *TP53* mutation
655 inactivates its tumour suppressor function and may promote disease progression and high-grade
656 transformation.

657 **SUPPLEMENTARY MATERIALS:**

658 **Supplementary methods**

659

660 **List of Supplementary Figures**

661 **Figure S1:** An overview of the variants in signalling pathways and molecular processes known to be

662 affected in SMZL.

663 **Figure S2:** Summary of a combined analysis of the novel variants identified by whole exome or

664 genome sequencing among the 4 published studies

665 **Figure S3:** Example of *KLF2* mutations detected by Sanger sequencing in SMZL.

666 **Figure S4:** Example of mutations detected by Fluidigm PCR and Illumina MiSeq sequencing.

667 **List of Supplementary tables**

668 **Table S1:** List of SMZL used for whole exome sequencing study.

669 **Table S2:** Primers used for PCR and sequencing of the KLF2 gene.

670 **Table S3:** Primers used for PCR with Fluidigm Access Array system.

671 **Table S4:** Performance data of whole exome sequencing.

672 **Table S5:** Sequence variants identified by whole exome sequencing in cases with matched non-

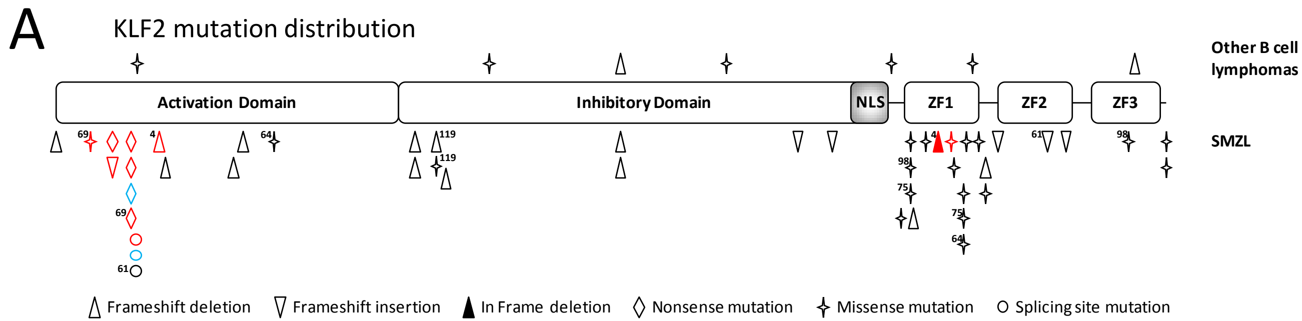
673 tumour DNA.

674 **Table S6:** List of mutations in the validation cohort of SMZL identified by Sanger sequencing, and

675 Fluidigm PCR / MiSeq sequencing.

676 **Table S7:** Impact of clinicopathological parameters and genetic changes on overall survival of

677 patients with SMZL.



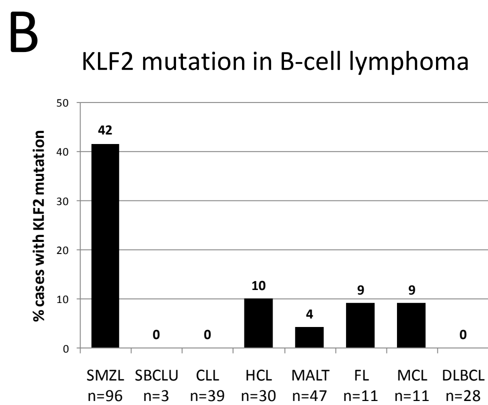
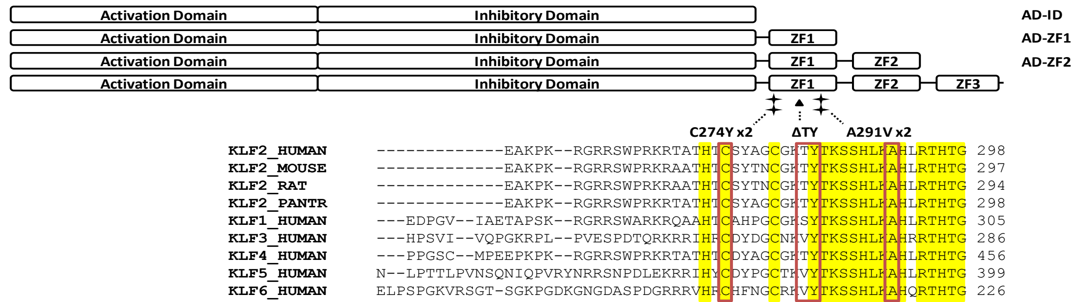


Figure 1: Nature and incidence of *KLF2* mutations in SMZL and other B-cell lymphomas.

A **Representative KLF2 mutants**



B **NF-κB reporter assay**

HEK293T

OCI-LY19 B-lymphoma cells

NFκB activity fold change

vector, vector, KLF2-WT, AD-ID, AD-ZF1, AD-ZF2, C274Y, A291V, ΔTY

KLF2 mutants

TNFα stimulation - + + + + + + + +

HA-KLF2 39 kDa 28 kDa

β-actin 42 kDa

BAFF stimulation - + + + + + + + +

MYD88-S219C - + + + + + + + +

Flag-MYD88 34 kDa

CARD11-F130V - + + + + + + + +

Flag-CARD11 134 kDa

Figure 2: *KLF2* mutations and their functional characterisation.

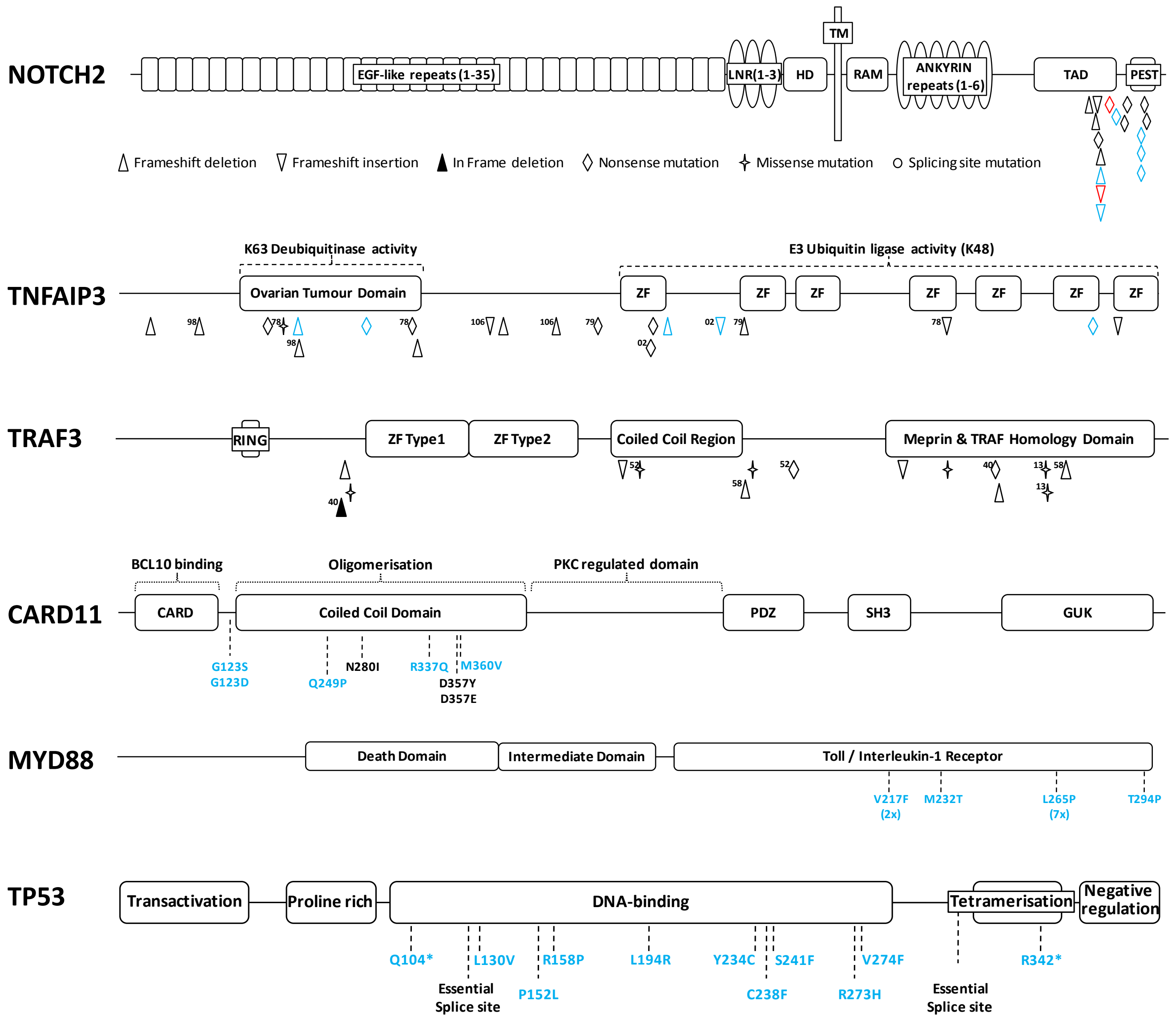
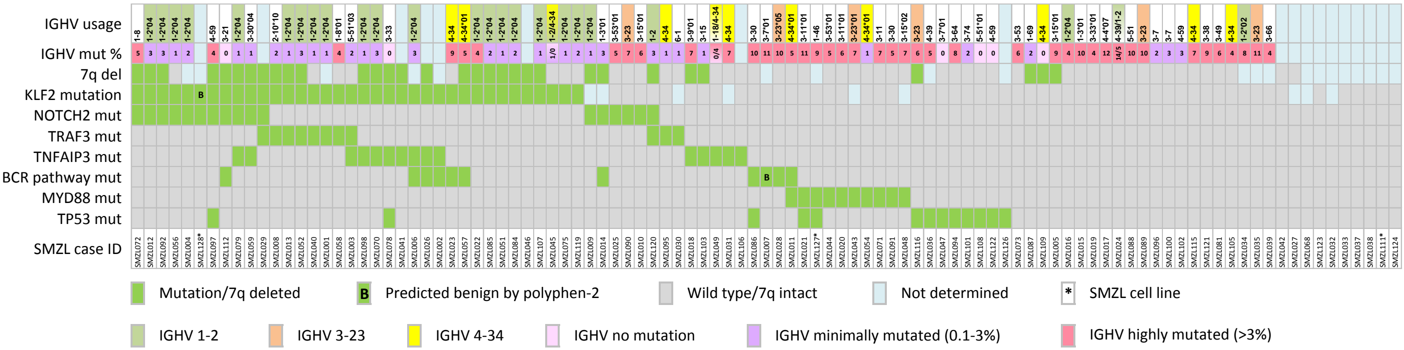
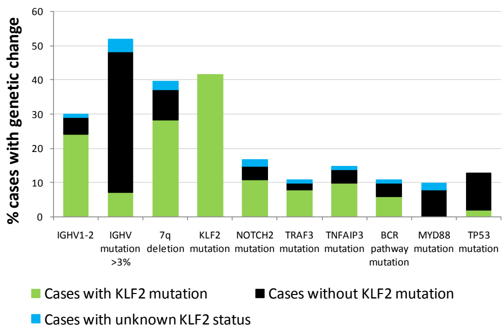


Figure 3: Nature and distribution of mutations in *NOTCH2*, *TNFAIP3*, *TRAF3*, *CARD11*, *MYD88* and *TP53* in SMZL.

A



B



C

	IGHV1-2	7q deletion	KLF2 mutation	NOTCH2 mutation	TRAF3 mutation	TNFAIP3 mutation	BCR pathway mutation	MYD88 mutation
7q del	P = 0.003							
KLF2 mut	P = 1.02×10^{-7}	P = 7.33×10^{-7}						
NOTCH2 mut	P = 0.134	P = 0.007	P = 0.007					
TRAF3 mut	P = 0.159	P = 0.505	P = 0.012	P = 1				
TNFAIP3 mut	P = 0.727	P = 0.028	P = 0.015	P = 1	P = 1.000			
BCR pathway mut	P = 0.266	P = 0.426	P = 0.147	P = 1	P = 0.596	P = 0.167		
MYD88 mut	P = 0.028	P = 0.010	P = 0.021	P = 0.205	P = 0.596	P = 0.351	P = 1	
TP53 mut	P = 0.015	P = 0.732	P = 0.071	P = 0.690	P = 0.350	P = 0.685	P = 0.352	P = 0.613

Red cell: positive correlation; black cell: negative correlation

Figure 4: Correlation of *KLF2* mutation with other genetic changes in SMZL.

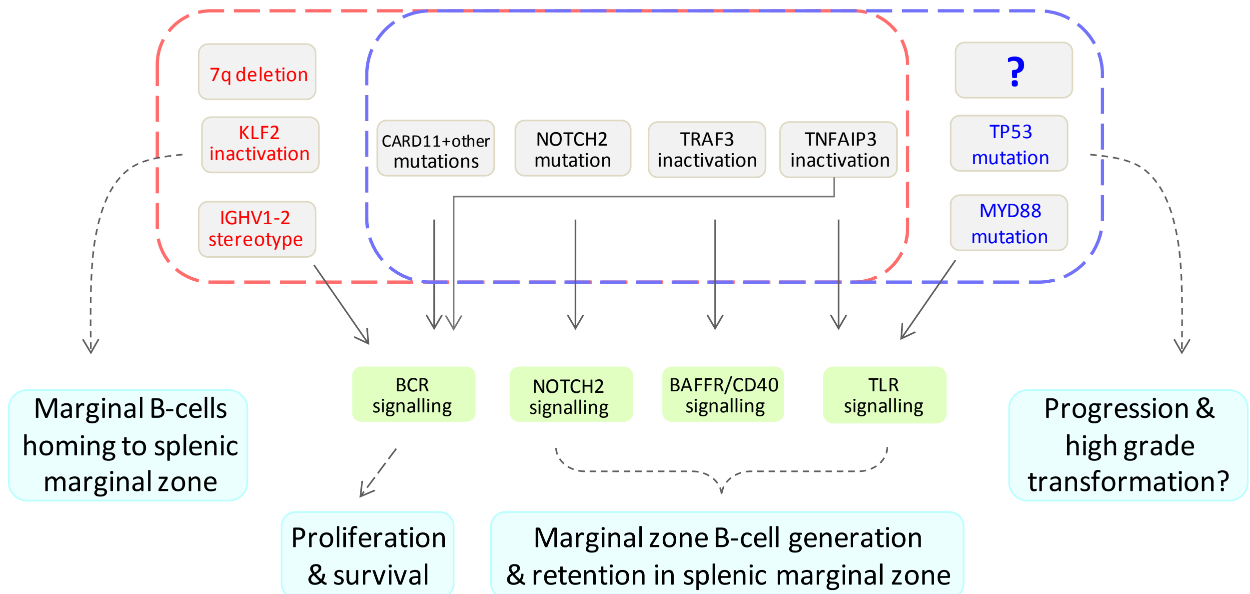


Figure 5: A summary of the proposed molecular mechanism of SMZL.