1 # Epigenomic evolution in chronic lymphocytic leukaemia
2 # cells from patients treated with ibrutinib and showing
3 # disease progression

4 Katie B. Holmes[1], Ildar I. Sadreev[1], Charlotte L. Evans[1], Fraser Thomas[1], Andy C. Rawstron[2],
5 Peter Hillmen[1], Darren J. Newton[1] and Pascal F. Lefevre[1]
6
7 *[1]Division of Haematology and Immunology, Leeds Institute of Medical Research at St.*
8 *James's, University of Leeds, Leeds, UK; [2]Haematological Malignancy Diagnostic Service*
9 *(HMDS), St. James's Institute of Oncology, Bexley Wing, Beckett Street, Leeds LS9 7TF, UK*
10
11 Correspondent author: Dr Darren J. Newton, Division of Haematology and Immunology,
12 Leeds Institute of Medical Research, St. James's University Hospital, Leeds LS9 7TF UK,
13 Tel.: +44-113-3437645, Fax: +44-113-3438502, email: D.J.Newton@leeds.ac.uk
14
15 **Running title:** Epigenomic changes in ibrutinib-treated CLL cells
16
17
18
19
20

Abstract

Background

The Bruton's tyrosine kinase (BTK) inhibitor ibrutinib provided a breakthrough in the treatment of chronic lymphocytic leukaemia (CLL), but cases of resistance are now emerging. Whilst resistance is commonly associated with mutations in BTK itself and the downstream signalling molecule PLCγ2, this is not always the case. In a recent study, we have detected some epigenomic plasticity correlated with the dynamics of CLL cell response to ibrutinib. To understand the mechanisms mediating resistance in CLL, it is important to be able to determine whether the observed phenotypic changes are driven solely by resistance cues (e.g. clonal evolution, activation of signalling bypassing BTK inhibition), or if they could also be the consequence of unrelated events.

Methods

To answer to this question, we have monitored chromatin changes happening in response to ibrutinib from the start of treatment until relapse in CLL cells from both a patient carrying a previously identified BTK C481S mutation and from a relapsing patient, for whom none of the classical genetic lesions associated with ibrutinib relapse was detected.

Results

We established that the epigenome and gene expression in CLL cells from patients on ibrutinib changes with time independently of disease progression and identified two patterns of chromatin alterations, which are independent of resistance mechanisms; one ibrutinib-dependent and the other related to quiescence. Furthermore, by defining the main characteristics of resistance-independent epigenomic plasticity and excluding them from the analysis, we identified candidate genes potentially mediating disease progression.

Conclusion

49 This two-step strategy could fundamentally alter the understanding of resistance to
50 treatment in CLL.

51

52 **Key words:** CLL, ibrutinib, relapse, epigenome, plasticity

53 **Background**

54 Chronic lymphocytic leukaemia (CLL), the most frequent leukaemia in western
55 countries, originates from clonal proliferating B-cells (1, 2). Treatment with the Bruton's
56 Tyrosine Kinase (BTK) inhibitor ibrutinib has shown extremely promising results in front-line
57 treatment as well as in relapsed/refractory CLL (3, 4) and is now being tested in combination
58 with other therapies (5-7). However, a significant number of cases of both primary and
59 secondary resistance have been reported (8, 9) and when treatment is discontinued, long-
60 term outcome is generally poor (10).

61 Intensive efforts are undertaken to better understand the mechanism of response to
62 ibrutinib and the development of drug resistance. In CLL cells, the initial response is a sharp
63 downregulation of the NF-κB and BCR signalling pathways (11-14) and a reduced activity of
64 lineage specific transcription factors (14). This is followed by ibrutinib-dependent
65 lymphocytosis (15), cells from lymph nodes being expelled into the peripheral blood (PB).
66 These cells are typically over-expressing genes related to proliferation pathways and cell cycle
67 (16). Accordingly, epigenomic analysis of these cells reveals transient increases in histone H3
68 lysine 4 trimethylation (H3K4me3), a histone post-translational modification (PTM) associated
69 with open chromatin at cis-elements targeted by lineage defining transcription factors
70 providing a signature of activation (17). Longer exposure to ibrutinib induces erosion of CLL
71 identity correlated with a quiescent-like gene expression signature (14), global loss of both
72 H3K27 acetylation (activating) and trimethylation (repressive) marks (17). Furthermore, while
73 H3K4me3 stays globally relatively stable with time on ibrutinib, this histone PTM is distinctly
74 reduced in regions identified as bivalent (H3K4me3+/H3K27me3+) before treatment (17). All
75 of these observations indicate an important epigenomic plasticity of CLL cells in response to
76 ibrutinib treatment.

77 Relapsed CLL, after ibrutinib therapy, is commonly associated with genomic
78 alterations in *BTK* and its downstream target *PLCγ2*. However, cases of progressive disease
79 in which targeted sequencing is unable to explain resistance have also been reported (12)

80 and activation of alternative signalling pathways that bypass BCR signalling might also play a
81 role in such process.

82 In the present analysis, we monitored chromatin structure evolution in CLL cells from
83 patients treated with ibrutinib before and after disease progression (Supplementary tables 1
84 and 2). In contrast with genetic abnormalities, the specificity of the chromatin landscape for a
85 given individual correlates with cell phenotype and is, consequently, relevant for this particular
86 individual when compared to clinical outcome. This study, combined with gene expression
87 analysis, suggests coordinated phenotypic variations of CLL cells independent of disease
88 evolution, characteristic of either cell activation or quiescence, consistent with our previous
89 observations (17), and not explained by technical variability. We also determined that WNT5A
90 might participate in the mechanism of relapse in a patient with no detectable mutation of either
91 the *BTK* or *PLCγ2* genes.

92

93 **Results**

94

95 **Chromatin structure varies with time in CLL cells from patients on ibrutinib**

96 We have previously analysed the evolution of the chromatin structure in CLL cells from
97 patients enrolled in the IcICLLe trial, a feasibility study investigating the mechanism of action
98 of ibrutinib. Patients received continuous oral therapy with ibrutinib (420 mg OD) and CLL cells
99 were collected from PB before and at regular time points during treatment (17). From this trial,
100 we identified a CLL patient (IbruR3) who progressed after 24 months of ibrutinib therapy
101 (Supplementary Fig. S1A). We screened for previously identified mutations associated with
102 relapse (18, 19). Interestingly, we detected the previously characterised BTK C481S mutation
103 after 32 months, but not at month 30 even though the percentage of CLL cells in the PB was
104 already notably increasing (Fig. 1A, Supplementary Fig. S1A). In addition, the level of
105 H3K27me3 and H3K27ac was low at 6 months post treatment initiation for this patient, in
106 agreement with our previous observation showing global disappearance of both histone marks

107 after months on ibrutinib (17) and was elevated at 33 months corresponding with progression.
108 The 9 months' time point showed a normal level of H3K27me3 as well as a slight and global
109 increase in H3K27ac compared with 3 months earlier (Fig. 1B and C, Supplementary Fig.
110 S1B). However, CLL cell count was decreasing at 9 months suggesting that the patient was
111 responding well to the treatment at that time (Supplementary Fig. S1A). Furthermore, we
112 compared this observation with the analysis of the chromatin structure of CLL cells obtained
113 from peripheral blood samples from a patient with acquired resistance to ibrutinib (IbruR1),
114 who was not enrolled in the trial. For this patient, we analysed chromatin changes in two
115 samples collected within an interval of 3 months, both after the diagnosis of relapse. PCR
116 screening for mutations including *BTK* C481S and the *PLCγ2* L845F, R665W and S707Y
117 mutations (18, 19) was negative for both time points. A genome-wide increase in the monitored
118 histone marks was observed in this patient for the first time point, however, a noticeable
119 reduction in both H3K27me3 and H3K27ac was observed 3 months later, suggesting that
120 disease progression might not be a continuous process (Fig. 1B and C, Supplementary Fig.
121 S1B and C). Altogether, these data suggested that some chromatin changes are dependent
122 and some independent of disease evolution.
123
124 **H3K27ac can vary independently of disease progression**
125 To further characterise the observed changes and determine if technical variability
126 could be ruled out, we performed a quantitative analysis of H3K27ac evolution compared to
127 before treatment. Loss or reduction in H3K27ac was global and partially restored at 33 months
128 compared to before treatment (Fig. 2A). Interestingly, the global change of H3K27ac peak
129 intensity was comparable between IbruR3 before treatment and in the progressing sample
130 (33M) and between the 3 months interval samples for IbruR1, suggesting a similar reduced
131 transcriptional activity in both later time points (Fig. 2A).
132 Moreover, when comparing this histone mark at 9M and 6M (IbruR3), we observed
133 that 56% and 11% of these peaks were higher at 9M and 6M respectively (Fig. 2B,
134 Supplementary Fig. S1D). Gene ontology (GO) analysis of the nearest genes associated with

135 the selected peaks, did not reveal any specific functional association for the 6M high peaks
136 but the 9M high fraction was enriched for genes of the canonical WNT and TGFβ-signalling
137 pathways as well as genes associated with regulation of cell growth (Supplementary Fig. S2A
138 Supplementary Table 4). Next, we analysed the intersection between our peak libraries and
139 merged transcription factor ChIP-seq data obtained from a publicly available database
140 (ReMap). This analysis, referred to as REMI, calculates the number of cis-regulatory elements
141 enriched for a specific histone mark and containing a validated consensus sequence
142 corresponding to a specific transcription factor (17). This allowed us to generate signatures
143 associated with determined peak populations and to assess the relative variation of these
144 signatures when comparing one peak population to another (17). Using REMI analysis, we
145 identified an enrichment for peaks containing validated binding-sites for the mediator complex
146 (CDK8, MED12) and the Polycomb Receptor Complex 2 (PRC2) (EZH2, SUZ12) in the 9M
147 high peak population and for SOX2 and PRDM1 in the 6M high one (Fig. 2C).
148 The same analysis performed to compare the H3K27ac peak population between
149 IbruR1 and IbruR3 BT suggested that histone acetylation was increased for genes of the
150 stress activated signalling cascade or cell-cell adhesion in the nearest genes associated with
151 peaks high in IbruR1 or IbruR3 cells respectively (Supplementary Fig. S2B, Supplementary
152 table 4). Interestingly, EZH2 and SUZ12 functional binding sites were found in the decreased
153 H3K27ac peaks fraction and SOX2 in peaks enhanced in IbruR1 compared to IbruR3,
154 respectively (Fig. 2D). Moreover KLF4, which was among the genes with higher level of
155 H3K27ac at associated cis-regulatory elements in IbruR3 compared to IbruR1, was also found
156 enriched in the IbruR3 high peak population (Fig. 2D, Supplementary table 4). Altogether
157 these data suggested that loss of H3K27ac at EZH2/SUZ12-associated peaks was correlated
158 with a gain in SOX2-associated peaks in these cells, such coordinated changes ruling out
159 technical artefacts.
160
161 **Changes in H3K4me3 peak number follow H3K27ac evolution**

162 We have shown previously that H3K4me3 was progressively disappearing at bivalent
163 cis-elements and transiently increasing at lineage specific cis-elements (LSCE) (17). The latter
164 changes were correlated with cells entering the peripheral blood from the proliferation centre,
165 which could be labelled as activated-CLL cells (17).

166 Here, comparing H3K4me3 peak number evolution between our different time points,
167 we observed dynamic variations concordant with those seen for H3K27ac. These variations
168 reached a maximum of 16% peak loss observed at 6M compared to 2M for IbruR3 and 30%
169 loss between IbruR1 and IbruR1 +3M (Fig. 3A). To further characterise these evolutions, we
170 performed a correlation study using REMI analysis first looking at this peak evolution
171 compared to IbruR3 before treatment (IbruR3 BT) and then versus IbruR3 2M. As observed
172 previously, the fluctuation in peak content at 2M did not exceed 20% of what is observed
173 before treatment, with the number of bivalent peaks (EZH2, SUZ12) going down and LSCE
174 peaks going up and this fraction being enriched for cis-elements containing functional binding
175 sites for SOX2 and PRDM1 (Fig. 3B). Overall, the score evolution could be separated into 4
176 subsets, (1) LSCE, (2) bivalent, (3) PRDM1/SOX2 and (4) the other transcription factors (TFs)
177 of the ReMap database (Fig. 3C-I). In all samples analysed including IbruR3 2M, bivalent
178 peaks were down and SOX2/PRDM1 high suggesting that it could be a characteristic of
179 ibrutinib-treated CLL cells (Fig. 3C-I). Furthermore, we identified several types of CLL cell
180 populations based on the evolution of both LSCE and the rest of the TFs. First, as expected
181 for the three subsets showing a reduction of the total H3K4me3 peak number, 6M, 9M, and
182 IbruR1 +3M (Fig. 3A), an increased proportion of all the other TF-associated peaks (subset 4)
183 was observed, suggesting a loss of non-promoter cis elements (small) and a proportional
184 enrichment for promoters (larger cis-elements containing more TF-binding sites) (Fig. 3C, D
185 and G). In addition, the number of H3K4me3 LSCE-associated peaks could be separated into
186 (1) high, between 10% and 30% increase for IbruR1 and (2) low, less than 20% increase for
187 the other samples (Fig. 3C, D and G). Furthermore, IbruR1 peak score was highly correlated
188 with a sample previously characterised as activated CLL-cells (TN_7d, R^2 = 0.95). These cells
189 came from a front-line donor after 7 days on ibrutinib and characterised by a sharp increase

190 in CLL cell count in the PB in the first days of treatment (17) (Fig. 3I). Therefore, by merging
191 these two observations combined with the global level of H3K27me3, we could separate these
192 samples into four distinct groups; newly generated activated CLL cells (IbruR1, TN_7d), early
193 (2M and 33M, the correlation between both peak populations being R^2 = 0.67), intermediate
194 (9M) and late/oldest (6M, IbruR1 +3M) quiescent-like cells (Fig. 3C-I).
195

196 **The difference in H3K4me3 peak intensity is indicative of variations in transcriptional**
197 **activity in these cells**

198 The completed analyses described above suggested that CLL cells might have
199 different phenotypes, from activated CLL cells and progressing toward different degrees of
200 quiescence with a progressive disappearance of H3K27ac genome-wide and H3K4me3 at
201 LSCE-associated peaks, the oldest cells also losing H3K27me3.

202 First, to confirm that our classification of the different subcategories of cis-elements
203 containing identified TFs was correct, we took advantage of a new study which classified each
204 open chromatin region in CLL into 12 chromatin states depending of their content in specific
205 histone marks (20). Each H3K4me3 peak identified in our analysis was marked in red if they
206 were present in a specific chromatin state and in white if not. Finally, we ranked these peaks
207 for each analysed sample based on fold change between this sample and IbruR3 BT (Fig. 4A-
208 G). The same analysis was performed with 60 selected TFs from the ReMap database
209 (Supplementary Fig. S3A). By comparing both analyses, we confirmed that EZH2 and SUZ12
210 were enriched in the decreased fraction of IbruR1, which were enriched for the chromatin
211 states E5, E6, E7 representing polycomb-dependent silenced and bivalent cis-elements.
212 Similarly, H3K4me3 peaks associated with lineage specific transcription factors including
213 SOX2, PRDM1, BCL6 and BATF were enriched in the increased fraction, which contain E1,
214 E8 and E9 non-promoter cis-elements (Fig. 4, Supplementary Fig. S3A). Moreover, the
215 number of peaks in this increased fraction for each CLL sample was correlated with the level
216 of activation of these cells (Figs. 3 and 4).

217 To fine tune the analysis of the changes of peak population in these different samples,
218 we separated these H3K4me3 peaks in 4 subsets depending on fold change versus IbruR3
219 BT and performed the same analysis as described in Fig. 3 (Supplementary Fig. S3B). Overall,
220 the peak content for the different subsets was highly correlated between all samples with few
221 noticeable variations (Supplementary Fig. S3C-F). As expected, bivalent regions (SUZ12,
222 EZH2) were mainly found in the decreased subset, with IbruR1 +3M being the only sample
223 showing a reduced number of these elements compensated by an accumulation of SUZ12-
224 associated cis-element in the increased subset (Supplementary Fig. S3C and S3E). Moreover,
225 further loss of bivalent cis-elements with time on ibrutinib and the reciprocal gain of SOX2-
226 associated regions were observable for the stable and increased subsets when comparing
227 samples collected after 6 months treatment and IbruR3 2M (Supplementary Fig. S3C and D).
228 The most important variations could be seen when analysing the peak content of the increased
229 subset, probably due to the small number of peaks in it (Supplementary Fig. S3C). Notably,
230 PRDM1-containing validated binding sites were enriched at 6M, 9M and for IbruR1 and 6M,
231 9M, 33M and IbruR1 +3M were associated with a decrease in TAp73A-containing cis-
232 elements (Supplementary Fig. S3C). In contrast at 33M, the increased subset was enriched
233 for cis-elements containing validated ONECUT1- and MEIS1-binding sites and depleted from
234 BCL6 sites suggesting a possible change in the TF network regulating proliferation after
235 disease progression for this patient (Supplementary Fig. S3C). Finally, 6M and IbruR1 were
236 the two samples showing no correlation when compared to IbruR3 2M in the absent subset,
237 with the observed peak scores being indicative of an important loss of H3K4me3 at promoters
238 (large regions targeted by multiple TFs) (Supplementary Fig. S3F). At 6M, it could be
239 explained by the loss of almost 3000 peaks compared to 33M. In contrast, further analysis
240 revealed that, for IbruR1, it was dependent upon promoter reorganisation and nucleosome
241 loss as seen for selected samples (Supplementary Fig. S4A). Therefore, activated-CLL cells
242 were characterised by an accumulation of H3K4me3 at non-promoter elements and by
243 chromatin reorganisation leading to nucleosome loss at active promoters.

244 To conclude this analysis of H3K4me3 quantitative changes, we also compared the
245 evolution of the intensity of these peaks with time to determine if these changes were random
246 or conserved between samples (Fig. 5A). We determined that 57% of the enriched peaks seen
247 at 9M are also enriched at 6M and 33M. Similarly, 81% of the decreased peaks at 33M are
248 also decreased at 6M and 9M suggesting a significant level of stability between untreated and
249 on ibrutinib treated samples (Fig. 5A). Moreover, 98% of the peaks higher in IbruR1 +3M than
250 in IbruR3 BT were also higher three months before (Fig. 5A). We have associated those cis-
251 elements conserved in 6M, 9M and 33M with their closest genes and determined functional
252 associations between these genes by gene ontology analysis (Supplementary Fig. S4A, S5A,
253 B and table 4). However, the meaning of these changes is unclear because it occurred in the
254 context of a global decrease in H3K27ac and therefore reduced transcriptional activity.
255 Similarly, focusing on the 98% of peak high in both IbruR1 samples, non-canonical, positive
256 regulation of differentiation and actin cytoskeleton organisation were among the function
257 enriched for IbruR1 compared to IbruR3 BT (Supplementary Fig. S4A, Supplementary table
258 4). Interestingly, genes associated with actin cytoskeleton organisation (*PROX1, ACTN1,*
259 *ATR, COBLL1*) were found to be enriched before treatment compared to 6M-33M and reduced
260 compared to IbruR1 (Supplementary Fig. S4B). This revealed alternative H3K4me3 peaks in
261 regions corresponding to potential alternative promoters for IbruR1 suggesting a possible
262 aberrant regulation of these genes in CLL cells for this first relapse patient (Supplementary
263 Fig. S5B). To conclude, gene ontology analysis comparing genes closest to peaks enriched
264 in IbruR1 +3M compared to IbruR3 at 6M, 9M and 33M identified several pathways specifically
265 activated in quiescent-like IbruR1 including JNK, non-canonical WNT and pro-inflammatory
266 signalling pathways (Supplementary Fig. S5B). JNK signalling pathway has been shown to
267 mediate apoptosis in response to various treatment in CLL (21-23), but is inactivated by NF-
268 κB (24).
269
270 **Activation of the non-canonical Wnt signalling pathway might participate in disease**
271 **progression**

272 To identify chromatin structure specifically associated with IbruR1 and considering the
273 fact that this first CLL sample was showing characteristics of activated-CLL cells, we
274 compared H3K4me3 for this patient with 5 previously analysed patients for whom we had
275 chromatin at a time point corresponding to ibrutinib-dependent lymphocytosis (17). We
276 performed gene ontology analysis for the closest genes associated with H3K4me3 peaks
277 found 2.5-fold higher for IbruR1 compared to all 5 other samples. Only 15 genes were found
278 associated with specific functions, including regulation of growth and the non-canonical Wnt
279 signalling pathways (Fig. 5B and C). Of particular interest among these genes was *WNT5A*,
280 a gene previously associated with ibrutinib resistance (25), and the fibroblast growth factor 20
281 (*FGF20*), a ligand also associated with proliferation and developmental growth (Fig. 5C).
282 H3K4me3 data confirmed that this mark was specifically high at *WNT5A* promoter for both
283 IbruR1 samples and that a cis-element upstream of the *FGF20* promoter was also specifically
284 enriched for this histone mark in IbruR1, but lower at +3M (Fig. 5D).
285 To determine if the changes observed in chromatin structure were supported by similar
286 changes in gene expression, we extracted mRNA from IbruR1, IbruR1 +3M, IbruR3 BT and
287 IbruR3 33M as well as from an additional relapse patient IbruR2 and from 14 patients on
288 ibrutinib for which we collected CLL cells from before treatment and up to 18 months post
289 treatment initiation (Fig. 6A, Supplementary Fig S7 and S8). None of these later patients
290 showed any sign of disease progression at the time of the study. We compared gene
291 expression for two proliferation markers (*Ki67, PCNA*), *FGF20* and *WNT5A*. Among the
292 samples from relapse patients, only IbruR1 expressed both proliferation markers reinforcing
293 the view that these CLL cells were characteristic of activated cells and that IbruR2, IbruR3
294 33M and IbruR1 +3M were in a more resting/dormant stage (Fig. 6A).
295 Unexpectedly, *PCNA* and *Ki67* were also expressed in patients 7, 8, 9, 13 and 14 at
296 various time points (Fig. 6A). A more extensive gene expression analysis indicated a close
297 correlation between expression of these proliferation markers and genes including *BCL2*,
298 *EZH1/2, SOX11, CD38*, a surface protein for which enzymatic activity has been correlated
299 with enhanced growth and cellular mobility and poor risk in CLL (26), interferon and chemokine

300 receptors (Supplementary Fig. S7 and S8). Similarly, expression of pro-inflammatory related
301 genes including *TLR4*, *TLR9* and *TNFA* was also closely correlated, with the expression of
302 these genes overlapping partially with the proliferative-associated ones as seen when
303 comparing *TNFA* expression with *SOX11* or *PCNA* (Supplementary Fig. S7 and S8, Fig. 6A).
304 Finally, the gene expression profile of IbruR1 +3M was unique showing specific expression of
305 *WNT5A* and *FGFR1* (Fig. 6A, Supplementary Fig. S8A, right part) and substantial variations
306 compared to 3 months earlier (Supplementary Fig. S9). *FGFR1* expression was observed for
307 IbruR1 +3M and its ligand *FGF20* was expressed in IbruR1 with a gene expression profile
308 closely correlated to *VDR, RXRA, ELK1* and *ILB1* (Supplementary Fig. S8A). These data
309 indicated that expression of two potential drivers of resistance were only detected in CLL cells
310 after a prolonged phase of quiescence (H3K27me3 low).
311
312 **Discussion**
313
314 CLL cells present significant phenotypic variations depending on their age and
315 compartment that they come from (27). In response to ibrutinib, after an initial phase of
316 lymphocytosis, the chromatin structure of CLL cells changes progressively from activated cells
317 toward a quiescent-like phenotype losing both H3K27ac and H3K27me3 genome-wide as well
318 as H3K4me3 primarily at bivalent cis-elements characteristic of resting CLL cells (14, 17).
319 Here, we have shown an expected chromatin and gene expression profile of activated CLL
320 cells in one patient on ibrutinib showing disease progression, whereas two other “relapse”
321 samples have chromatin characteristics of quiescent-like cells. These data suggest that
322 relapse is not a continuous process and as observed in the absence of treatment, that these
323 cells could be either in a proliferating or a quiescent phase (Fig. 6B).
324 Interestingly, CLL cells from patients on ibrutinib display some similar chromatin
325 characteristics, independent of disease progression. These include loss of H3K4me3 at
326 bivalent cis-elements and, reciprocally, gain of the same histone PTM at regions containing
327 validated SOX2-binding motifs. SOX2 is of interest as a key regulator of both the early

328 development and maintenance of undifferentiated embryonic stem cells and has been
329 characterised as an oncogene in multiple cancers (28). EZH2-, SUZ12- and SOX2-validated
330 binding sites included into the ReMap database come from studies performed in Embryonic
331 Stem Cells (ESCs), including studies by the ENCODE consortium (29-32). Our results
332 suggest that in response to ibrutinib CLL cells switch on or off cis-elements which are activated
333 or repressed in ESCs respectively, linking a quiescence-like phenotype with increased
334 'stemness' as described in disseminating tumour cells (33, 34).

335 Furthermore, the maintenance of this signature after relapse suggests that disease
336 progression, at least in its initial phase, occurs while cells are still in some degree sensitive to
337 ibrutinib. For the C481S *BTK* mutant (IbruR3), these data agree with the fact that more than
338 50% of the cells do not carry the mutation in the initial phase of disease progression. This
339 discrepancy between disease progression and the percentage of *BTK* mutants in the growing
340 cell population is characteristic of ibrutinib treatment in CLL (12).

341 Moreover, our data suggest additional chromatin structure variations in quiescent-like
342 CLL cells, possibly dependent on two parameters - (i) disease progression and (ii) age of the
343 cells. EZH2 expression is restricted to proliferative cells in cancer and in non-transformed
344 human cells (35) and, accordingly, to proliferation centres in CLL and to the germinal centre
345 in B cells (36-38). Consequently, loss of H3K27me3 in quiescent-like CLL cells can be
346 interpreted as a prolonged exit of these cells from the cell cycle, characteristic of aging cells.
347 Here, two CLL samples amongst those investigated at greatest depth, IbruR3 6M and IbruR1
348 +3M, share a profile of drastic reduction in H3K27me3 suggesting that both have been
349 maintained in G0 phase for an extended period of time. Interestingly, these samples also show
350 common characteristics associated with quiescent-like cells including loss and gain of
351 H3K4me3 positive cis-elements associated with *Tap73* and *PRDM1*, respectively. Tap73
352 promotes the Warburg effect and proliferation in cancer (39); therefore, silencing of *Tap73*-
353 associated cis-elements might be a characteristic of this quiescence. Similarly, PRDM1/Blimp-
354 1, a master regulator of B cell differentiation, has been shown to actively repress a number of

functions in B cells including cell-cell adhesion (40), which we show to be associated with an increase in H3K4me3 at 6M, 9M and 33M compared to pre-treatment.

Whilst the IbruR1 +3M epigenome is comparable to that of IbruR3 6M, suggesting a prolonged exit from the cell cycle, the level of H3K27ac is significantly higher in these cells, indicative of enhanced transcriptional activity matched by a gene expression profile which is distinct from other quiescent-like cells. Additionally, these cells show an enrichment for H3K4me3 at bivalent regions, which could be due to the concomitant loss of the repressive mark (H3K27me3) and an increase in global transcriptional activity. In these cells, *WNT5A* and *FGFR1*, which have been described as potential candidates to mediate ibrutinib-resistance, show 44- and 17-fold higher expression compared to cells three months earlier, which have an activated phenotype. WNT5A can enhance the proliferation and migration of CLL cells via activation of the non-canonical WNT pathway (25, 41), a mechanism which is not inhibited by ibrutinib (42). Similarly, activation of FGFR targets four internal signalling pathways RAS-MAPK, PI3K-AKT, PLCγ, and STAT (43), suggesting that it might also induce proliferation in CLL cells by bypassing BTK inhibition. These pathways may act synergistically, with canonical WNT5A signalling increasing the transcription of *FGF20*, an FGFR1 ligand (44).

Detecting transient expression of proliferation markers, including *Ki67* and *EZH2*, in cells from four patients showing no clinical sign of disease progression reinforced the view that CLL cells are still able to enter cell cycle under ibrutinib-dependent repression of the BCR signalling pathway, as shown by analysis of IbruR3 9M epigenome. These data argue that CLL cells from patients treated with ibrutinib are still able to enter cell cycle in the absence of detectable disease progression.

Conclusions

We have shown that the chromatin structure of CLL cells from patients on ibrutinib changes dynamically for reasons independent of sensitivity to the treatment. These changes are coordinated and consistently correlated with the age of the cells and cannot be explained by random variations due to technical inconsistency. Accordingly, CLL cells from relapse

383 patients can be either in a quiescent-like or proliferating state. In addition, our data suggest
384 that older cells are the ones which might be responding to signalling which is capable of
385 bypassing BTK inhibition. As mentioned in the introduction, these phenotype-associated
386 variations are relevant for the particular individuals included in this study and suggest that any
387 mechanistic analysis aimed at identifying the phenotypic specificity of relapsing cells would
388 have to take the inherent epigenomic variation into consideration.
389
390 **Methods**
391
392 **Isolation of CLL Cells**
393 Peripheral blood was collected from patients with informed written consent (St.
394 James's University Hospital, approval number 14/YH/0034; IcICLLe EudraCT number: 2012-
395 003608-11). Peripheral blood mononuclear cells (PMBCs) were isolated by density gradient
396 centrifugation using Lymphoprep^{TM} (Axis-Shield). CLL cells were further purified using CD19+
397 MicroBeads (Miltenyi Biotec).
398
399 **BTK and PLCγ2 Mutation Detection**
400 100 ng of patient DNA extracted from CD19+ selected peripheral blood was used for
401 PCR. A 527 bp fragment encompassing the *BTKCys481* region was amplified using the
402 following primers, GACTGCTCTGATTCCCACCA and TACTCCTAGGTCAGCCCCTTC.
403 Similarly, three regions of the *PLCγ2* gene were amplified, a 190 bp fragment which
404 encompassed the *PLCγ2S707* region using the primers ACCAGGATCTTGGCATGTCA and
405 GGGTAGCGCAGTCTCATCTT, A 323 bp fragment encompassing the *PLCγ2L845* region
406 using the primers, TCCCCATGGACGTATCTGGT and TGGCATTCAAGACAGGACCC and a
407 212 bp fragment covering the *PLCγ2R665* region using the primers
408 AGGTGTCACTGGTGCCATTA and AGGTGATGGCATAGGAGTC. The amplified products
409 were isolated using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA) and then sent for
410 Sanger sequencing (Source Bioscience, Nottingham, UK).

411

412 **Immunoblot Analysis**

413 Immunoblot analysis was conducted as published previously (17). Primary antibodies
414 were as follows: H3K4me3 (Millipore; 04-745), H3K27me3 (Millipore; 07-449) and H3K27ac
415 (Millipore; 07-360) were used as primary antibodies and anti-Total H3 (Abcam; ab1791) was
416 used as loading controls.

417

418 **Chromatin Immunoprecipitation (ChIP) and ChIP-seq**

419 ChIP was performed as previously described (45, 46) using Dynabeads protein G
420 (Invitrogen) with 2.4 mg per 10 ml beads with 1 µg anti-H3K4me3 (Millipore; 04-745), anti-
421 H3K27ac (Millipore; 07-360), anti-H3K27me3 (Millipore; 07-449). For sequencing (ChIP-seq),
422 libraries were prepared using NEBNext® Ultra™ II DNA Library Prep Kit (New England
423 Biolabs). See Supplementary Methods for sequencing run details.

424

425 **Bioinformatics Analysis**

426 See Supplementary Methods for further details. Sequence data from this study have
427 been submitted to ArrayExpress (https://www.ebi.ac.uk/arrayexpress/) under accession
428 number E-MTAB-8220.

429

430 **Gene Expression Analysis**

431 Total RNA was extracted from patient CLL samples using Direct-zol™ RNA MiniPrep
432 Kit (Zymo Research) and cDNA was prepare using M-MLV Reverse Transcriptase
433 (Invitrogen). Expression of target genes (Supplementary Table 2) was determined using
434 quantitative real-time polymerase chain reaction (qPCR) with GoTaq® qPCR Master Mix
435 (Promega) on a QuantStudio™ 7 Flex Real-Time PCR System (Applied Biosystems). Relative
436 expression (ΔCt) was calculated from the Ct value of the average of 5 housekeeping genes
437 (*PPP6C, GAPDH, RPL13A, β-Actin, B2M*) – Ct value of the gene of interest.

438

439 **list of abbreviations**

440

441 **ACTN1** actinin alpha 1

442 **BATF** basic leucine zipper ATF-like transcription factor

443 **BT** before treatment

444 **BTK** bruton's tyrosine kinase

445 **B2M** beta 2 microglobulin

446 **CDK8** cyclin dependent kinase 8

447 **CLL** chronic lymphocytic leukaemia

448 **COBLL1** cordon-bleu WH2 repeat protein like 1

449 **ESC** embryonic stem cell

450 **EZH2** enhancer of zeste 2 polycomb repressive complex 2

451 **FGF20** fibroblast growth factor 20

452 **FGFR1** fibroblast growth factor receptor 1

453 **ILB1** interleukin 1 beta

454 **GAPDH** glyceraldehyde 3 phosphate dehydrogenase

455 **GO** gene ontology

456 **IbruR** ibrutinib resistant

457 **JNK** c-Jun N-terminal kinase

458 **Ki67** marker of proliferation Ki-67

459 **KLF4** kruppel like factor 4

460 **LSCE** lineage specific cis-elements

461 **MAPK** map kinase

462 **MED12** mediator complex subunit 12

463 **NF-κB** nuclear factor kappa B

464 **ONECUT1** one cut homeobox 1

465 **PB** peripheral blood

466 **PCNA** proliferating cell nuclear antigen

467 **PI3K** phosphatidylinositol 3 kinase

468 **PLCγ2** phospholipase C gamma 2

469 **PMBC** peripheral blood mononuclear cell

470 **PPP6C** protein phosphatase 6 catalytic subunit

471 **PRC2** polycomb receptor complex 2

472 **PRDM1** PR/SET domain 1

473 **PROX1** prospero homeobox 1

474 **PTM** post-translational modification

475 **qPCR** quantitative real-time polymerase chain reaction

476 **RPL13A** ribosomal protein L13a

477 **REMI** reMap and epigenetic mark intersection

478 **RXRA** retinoid X receptor alpha

479 **SOX2** SRY-box transcription factor 2

480 **SOX11** SRY-Box transcription factor 11

481 **STAT** signal transducer and activator of transcription

482 **Tap73** transformation related protein 73

483 **TGFβ** transforming growth factor beta

484 **TF** transcription factor

485 **TLR** Ttoll like receptor

486 **TN** treatment naive

487 **TNFA** tumor necrosis factor

488 **VDR** vitamin D receptor

489 **WNT5A** wnt family member 5A

490

491 **Declarations**

492

493 **Ethical approval and consent to participate**

494 Peripheral blood was collected from patients with informed written consent (St. James's
495 University Hospital, approval number 14/YH/0034; IcICLLe EudraCT number: 2012-003608-
496 11).

497

498 **Consent for publication**

499 Not applicable

500

501 **Availability of supporting data**

502 Sequence data from this study have been submitted to ArrayExpress
503 (https://www.ebi.ac.uk/arrayexpress/) under accession number E-MTAB-8220.

504

505 **Competing interests**

506 The authors declare no competing financial interests.

507

508 **Funding**

509 This work was supported by Bloodwise trial associated research project (14016).

510

511 **Authors' contribution**

512 KH, FT, CE and AR performed the experiments and generated figures. PL, IS analysed the
513 data and PL wrote the paper. PH was involved in the initial design of the project and
514 contributed to provide essential research material. DN participated to the data analysis. KH,
515 IS, PH and DN participated to the redaction of the manuscript.

516

517 **Acknowledgments**

518 Not applicable

519

520 **Authors' information**

521 **Affiliations**

522 Division of Haematology and Immunology, Leeds Institute of Medical Research at St.
523 James's, University of Leeds, Leeds, UK

524 Katie B. Holmes, Ildar I. Sadreev, Charlotte L. Evans, Fraser Thomas, Darren J. Newton[1],
525 Peter Hillmen and Pascal F. Lefevre

526 Haematological Malignancy Diagnostic Service (HMDS), St. James's Institute of Oncology,
527 Bexley Wing, Beckett Street, Leeds LS9 7TF, UK

528 Andy C. Rawstron

529 **Corresponding authors**

530 Correspondence to Pascal F. Lefevre

531

532 **References**

533

534 1. Howlader N NA, Krapcho M, Garshell J, Neyman N, Altekruse SF, Kosary CL, Yu M, Ruhl
535 J, Tatalovich Z, Cho H, Mariotto A, Lewis DR, Chen HS, Feuer EJ, Cronin KA (eds). SEER Cancer
536 Statistics Review, 1975-2010. Bethesda, MD: National Cancer Institute. 2013.

537 2. Fabbri G, Dalla-Favera R. The molecular pathogenesis of chronic lymphocytic
538 leukaemia. Nature Reviews Cancer. 2016;16(3).

539 3. Byrd JC, Brown JR, O'Brien S, Barrientos JC, Kay NE, Reddy NM, et al. Ibrutinib versus
540 ofatumumab in previously treated chronic lymphoid leukemia. The New England journal of
541 medicine. 2014;371(3):213-23.

542 4. Burger JA, Tedeschi A, Barr PM, Robak T, Owen C, Ghia P, et al. Ibrutinib as Initial
543 Therapy for Patients with Chronic Lymphocytic Leukemia. The New England journal of
544 medicine. 2015;373(25):2425-37.

545 5. Collett L, Howard DR, Munir T, McParland L, Oughton JB, Rawstron AC, et al.
546 Assessment of ibrutinib plus rituximab in front-line CLL (FLAIR trial): study protocol for a phase
547 III randomised controlled trial. Trials. 2017;18(1):387.
548 6. Hillmen P, Rawstron AC, Brock K, Munoz-Vicente S, Yates FJ, Bishop R, et al. Ibrutinib
549 Plus Venetoclax in Relapsed/Refractory Chronic Lymphocytic Leukemia: The CLARITY Study. J
550 Clin Oncol. 2019:JCO1900894.
551 7. Kater AP, Levin MD, Niemann CU. Ibrutinib and Venetoclax for First-Line Treatment of
552 CLL. The New England journal of medicine. 2019;381(8):788-9.
553 8. Kaur V, Swami A. Ibrutinib in CLL: a focus on adverse events, resistance, and novel
554 approaches beyond ibrutinib. Annals of hematology. 2017.
555 9. Ahn IE, Underbayev C, Albitar A, Herman SE, Tian X, Maric I, et al. Clonal evolution
556 leading to ibrutinib resistance in chronic lymphocytic leukemia. Blood. 2017;129(11):1469-79.
557 10. Jain P, Thompson PA, Keating M, Estrov Z, Ferrajoli A, Jain N, et al. Long-term
558 outcomes for patients with chronic lymphocytic leukemia who discontinue ibrutinib. Cancer.
559 2017;123(12):2268-73.
560 11. Herman SE, Mustafa RZ, Gyamfi JA, Pittaluga S, Chang S, Chang B, et al. Ibrutinib
561 inhibits BCR and NF-kappaB signaling and reduces tumor proliferation in tissue-resident cells
562 of patients with CLL. Blood. 2014;123(21):3286-95.
563 12. Landau DA, Sun C, Rosebrock D, Herman SEM, Fein J, Sivina M, et al. The evolutionary
564 landscape of chronic lymphocytic leukemia treated with ibrutinib targeted therapy. Nature
565 Communications. 2017;8(1):2185.
566 13. Schmidl C, Vladimer GI, Rendeiro AFF, Schnabl S, Krausgruber T, Taubert C, et al.
567 Combined chemosensitivity and chromatin profiling prioritizes drug combinations in CLL.
568 Nature chemical biology. 2019;15(3):232-40.

569 14. Rendeiro AF, Krausgruber T, Fortelny N, Zhao F, Penz T, Farlik M, et al. Chromatin
570 mapping and single-cell immune profiling define the temporal dynamics of ibrutinib drug
571 response in chronic lymphocytic leukemia. bioRxiv. 2019:597005.
572 15. de Rooij MF, Kuil A, Geest CR, Eldering E, Chang BY, Buggy JJ, et al. The clinically active
573 BTK inhibitor PCI-32765 targets B-cell receptor- and chemokine-controlled adhesion and
574 migration in chronic lymphocytic leukemia. Blood. 2012;119(11):2590-4.
575 16. Pastore A, Gaiti F, Lu SX, Brand RM, Kulm S, Chaligne R, et al. Corrupted coordination
576 of epigenetic modifications leads to diverging chromatin states and transcriptional
577 heterogeneity in CLL. Nature Communications. 2019;10(1):1874.
578 17. Holmes KB, Sadreev, II, Rawstron AC, Munir T, Westhead DR, Hillmen P, et al. Ibrutinib
579 induces chromatin reorganisation of chronic lymphocytic leukaemia cells. Oncogenesis.
580 2019;8(5):32.
581 18. Woyach JA, Furman RR, Liu T-MM, Ozer HG, Zapatka M, Ruppert AS, et al. Resistance
582 Mechanisms for the Bruton's Tyrosine Kinase Inhibitor Ibrutinib. The New England journal of
583 medicine. 2014.
584 19. Furman RR, Cheng S, Lu P, Setty M, Perez AR, Guo A, et al. Ibrutinib Resistance in
585 Chronic Lymphocytic Leukemia. The New England journal of medicine. 2014.
586 20. Mallm JP, Iskar M, Ishaque N, Klett LC, Kugler SJ, Muino JM, et al. Linking aberrant
587 chromatin features in chronic lymphocytic leukemia to transcription factor networks. Mol
588 Syst Biol. 2019;15(5):e8339.
589 21. Peng K, Pan Y, Li J, Khan Z, Fan M, Yin H, et al. 11beta-Hydroxysteroid Dehydrogenase
590 Type 1(11beta-HSD1) mediates insulin resistance through JNK activation in adipocytes. Sci
591 Rep. 2016;6:37160.

592 22. Bates D, Feris EJ, Danilov AV, Eastman A. Rapid induction of apoptosis in chronic
593 lymphocytic leukemia cells by the microtubule disrupting agent BNC105. Cancer biology &
594 therapy. 2016;17(3):291-9.
595 23. Paiva C, Godbersen JC, Soderquist RS, Rowland T, Kilmarx S, Spurgeon SE, et al. Cyclin-
596 Dependent Kinase Inhibitor P1446A Induces Apoptosis in a JNK/p38 MAPK-Dependent
597 Manner in Chronic Lymphocytic Leukemia B-Cells. PLoS One. 2015;10(11):e0143685.
598 24. Bubici C, Papa S, Pham CG, Zazzeroni F, Franzoso G. NF-kappaB and JNK: an intricate
599 affair. Cell Cycle. 2004;3(12):1524-9.
600 25. Yu J, Chen L, Cui B, Wu C, Choi MY, Chen Y, et al. Cirmtuzumab inhibits Wnt5a-induced
601 Rac1 activation in chronic lymphocytic leukemia treated with ibrutinib. Leukemia.
602 2017;31(6):1333.
603 26. Vaisitti T, Audrito V, Serra S, Buonincontri R, Sociali G, Mannino E, et al. The enzymatic
604 activities of CD38 enhance CLL growth and trafficking: implications for therapeutic targeting.
605 Leukemia. 2015;29(2):356-68.
606 27. Herndon TM, Chen SS, Saba NS, Valdez J, Emson C, Gatmaitan M, et al. Direct in vivo
607 evidence for increased proliferation of CLL cells in lymph nodes compared to bone marrow
608 and peripheral blood. Leukemia. 2017;31(6):1340-7.
609 28. Wuebben EL, Rizzino A. The dark side of SOX2: cancer - a comprehensive overview.
610 Oncotarget. 2017;8(27):44917-43.
611 29. Lister R, Pelizzola M, Dowen RH, Hawkins RD, Hon G, Tonti-Filippini J, et al. Human
612 DNA methylomes at base resolution show widespread epigenomic differences. Nature.
613 2009;462(7271):315-22.
614 30. Soufi A, Donahue G, Zaret KS. Facilitators and impediments of the pluripotency
615 reprogramming factors' initial engagement with the genome. Cell. 2012;151(5):994-1004.

616 31. Xu K, Wu ZJ, Groner AC, He HH, Cai C, Lis RT, et al. EZH2 oncogenic activity in
617 castration-resistant prostate cancer cells is Polycomb-independent. Science.
618 2012;338(6113):1465-9.
619 32. Abayakoon P, Lingford JP, Jin Y, Bengt C, Davies GJ, Yao S, et al. Discovery and
620 characterization of a sulfoquinovose mutarotase using kinetic analysis at equilibrium by
621 exchange spectroscopy. The Biochemical journal. 2018;475(7):1371-83.
622 33. Gay LJ, Malanchi I. The sleeping ugly: Tumour microenvironment's act to make or
623 break the spell of dormancy. Biochim Biophys Acta Rev Cancer. 2017;1868(1):231-8.
624 34. Weidenfeld K, Barkan D. EMT and Stemness in Tumor Dormancy and Outgrowth: Are
625 They Intertwined Processes? Front Oncol. 2018;8:381.
626 35. Bracken AP, Pasini D, Capra M, Prosperini E, Colli E, Helin K. EZH2 is downstream of
627 the pRB-E2F pathway, essential for proliferation and amplified in cancer. The EMBO Journal.
628 2003;22(20):5323-35.
629 36. van Galen JC, Dukers DF, Giroth C, Sewalt RG, Otte AP, Meijer CJ, et al. Distinct
630 expression patterns of polycomb oncoproteins and their binding partners during the germinal
631 center reaction. European journal of immunology. 2004;34(7):1870-81.
632 37. Velichutina I, Shaknovich R, Geng H, Blood J-NA. EZH2-mediated epigenetic silencing
633 in germinal center B cells contributes to proliferation and lymphomagenesis. Blood. 2010.
634 38. Szurian K, Csala I, Marosvari D, Rajnai H, Dezso K, Bodor C, et al. EZH2 is upregulated
635 in the proliferation centers of CLL/SLL lymph nodes. Exp Mol Pathol. 2018;105(2):161-5.
636 39. Li L, Li L, Li W, Chen T, Bin Z, Zhao L, et al. TAp73-induced phosphofructokinase-1
637 transcription promotes the Warburg effect and enhances cell proliferation. Nat Commun.
638 2018;9(1):4683.

639 40. Minnich M, Tagoh H, Bonelt P, Axelsson E, Fischer M, Cebolla B, et al. Multifunctional
640 role of the transcription factor Blimp-1 in coordinating plasma cell differentiation. Nat
641 Immunol. 2016;17(3):331-43.
642 41. Yu J, Chen L, Cui B, Widhopf GF, 2nd, Shen Z, Wu R, et al. Wnt5a induces ROR1/ROR2
643 heterooligomerization to enhance leukemia chemotaxis and proliferation. J Clin Invest.
644 2016;126(2):585-98.
645 42. Yu J, Chen Y, Chen L, Zhang L, Rassenti LZ, Widhopf GF, et al. Cirmtuzumab inhibits
646 ibrutinib-resistant, Wnt5a-induced Rac1 activation and proliferation in mantle cell lymphoma.
647 Oncotarget. 2018;9(37):24731-6.
648 43. Ornitz DM, Itoh N. The Fibroblast Growth Factor signaling pathway. Wiley
649 Interdisciplinary Reviews: Developmental Biology. 2015;4(3).
650 44. Katoh M, Katoh M. STAT3-induced WNT5A signaling loop in embryonic stem cells,
651 adult normal tissues, chronic persistent inflammation, rheumatoid arthritis and cancer
652 (Review). Int J Mol Med. 2007;19(2):273-8.
653 45. Lefevre P, Melnik S, Wilson N, Riggs AD, Bonifer C. Developmentally regulated
654 recruitment of transcription factors and chromatin modification activities to chicken lysozyme
655 cis-regulatory elements in vivo. Mol Cell Biol. 2003;23(12):4386-400.
656 46. Lefevre P, Witham J, Lacroix CE, Cockerill PN, Bonifer C. The LPS-induced
657 transcriptional upregulation of the chicken lysozyme locus involves CTCF eviction and
658 noncoding RNA transcription. Mol Cell. 2008;32(1):129-39.
659

660 **Figure legend**

661

662 **Fig. 1: Overview of chromatin structure changes with time on ibrutinib.** (**A**) Identification
663 of the BTK C481S mutation by PCR after 32 months of ibrutinib treatment. The black arrows
664 indicate the position of the cysteine codon (UGC) mutated into serine (UCC) (**B**) Summary of
665 the ChIP-seq data focusing on two regions (chr18:27mb-37mb and Chr11:91,000-98,000)
666 representative of the obtained data and including a gene rich region surrounded by two gene
667 poor regions for which H3K27m3 enrichment is at background level. ChIP-seq has been
668 performed for H3K27me3, H3K4me3 and H3K27ac looking at two patients (1) IbruR3 after 6,
669 9 and 33 months treatment and (2) IbruR1 at the time of disease progression detection (a)
670 and 3 months later (+3M).
671
672 **Fig. 2: Global level of H3K27ac does not follow disease progression.** (**A**) Differential
673 H3K27ac, as presented in Fig. 1 and defined as $\log_2$(H3K27ac fold-change) and $\log_{10}$(-p-
674 value) between IbruR3 before treatment and at 6, 9 and 33 months as well as between IbruR1
675 and IbruR1 +3 months. Each H3K27ac peak was associated with its closest gene. (**B**) Same
676 as A, but comparing IbruR3 at 6 and 9 months. (**C**) REMI analysis, cis-elements enriched for
677 the H3K27ac mark were screened for the presence of validated binding sites for transcription
678 factors and co-regulators (ReMap database). For each protein of the database, data were
679 expressed as a score (equation (2) in Supplementary methods) corresponding to the number
680 of peaks associated with a specific TF at 9M versus 6M. (**D**) Same as D but comparing IbruR3
681 BT and IbruR1.
682
683 **Fig. 3: IbruR1 is presenting a H3K4me3 profile of activated-CLL cells.** (A) Total H3K4me3
684 peak number between the different analysed subsets including IbruR3 BT, IbruR3 2M and TN
685 7d from a previous study (17). (**B**) REMI analysis, score variation for IbruR3 2M compared to
686 before treatment. (**C-I**) Scatter diagrams representing a comparison between REMI scores for
687 the different analysed samples (y axes) and reference profile (**C-E, G and H**) IbruR3 2M, (**F**)
688 IbruR3 6M and (**I**) TN 7d (x axes).
689

690 **Fig. 4: Changes in chromatin structure depending of the chromatin state.** (**A-G**) Bar
691 coding presentation: chromatin states as defined previously (20) (E1 and E9,
692 H3K4me1/H3K27ac; E8, H3K4me1; E10, H3K4me3; E11, H3K27ac/H3K9ac; E5 and E6,
693 H3K27me3; E7, H3K27me3/H3K4me1/3) have been attributed a colour code depending of
694 their overlap with identified H3K4me3 peaks in IbruR1 and/or IbruR3 samples (red = present,
695 white = absent). H3K4me3 peaks have been ranked based on fold-change between (**A**) TN
696 7d, (**B**) IbruR3 2M, (**C**) IbruR3 6M, (**D**) IbruR3 9M, (**E**) IbruR3 33M, (**F**) IbruR1 and (**G**) IbruR1
697 +3M compared to IbruR3 BT.
698
699 **Fig. 5: H3K4me3 peak intensity variation showed a significant degree of time dependent**
700 **conservation.** (**A**) Differential H3K4me3, as presented in Fig. 1 and defined as log_2(H3K4me3
701 fold-change) and log_{10}(-p-value) between IbruR3 before treatment and at 6, 9, 33 months and
702 IbruR1 as well as between IbruR1 and IbruR1 +3 months. Each H3K4me3 peak was
703 associated with its closest gene. The percentage of peaks conserved at all time points 6M,
704 9M and 33M are indicated for the decreased (red rectangle) and increased (green rectangle)
705 fractions. Similarly, the percentage of peaks conserved in both IbruR1 and IbruR1 +3M are
706 indicated for the increased (grey rectangle) fractions. (**B-C**) Biological processes enriched at
707 genes associated with differentially regulated H3K4me3 peaks (IbruR1 compared to 5 CLL
708 samples from patients which received up to 7 days ibrutinib treatment (17); hypergeometric
709 test BH-FDR < 0.05). (**D**) Example of differential H3K4me3 peak intensity between the
710 analysed samples (*WNT5A* and *FGF20*).
711
712 **Fig. 6: WNT5A is specifically expressed in IbruR1.** (A) gene expression analysis of CLL
713 cells from PB for 14 patients collected before treatment or at 6, 9, 12 and 18M (as indicated
714 on the Figure) compared to IbruR1 (R1), IbruR2 (R2) and IbruR (R3). *PCNA, Ki67, FGF20*
715 and *WNT5A*. Data are expressed relative to an average of 5 different control genes. (B)
716 Simplified model highlighting different level of transcriptional activity in CLL cells on ibrutinib.
717