

HB01 is required for the maintenance of leukaemia stem cells

Author

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1 **KAT7 / HBO1 is required for the maintenance of leukaemia stem cells.**

2

3 Laura MacPherson^{1,2}, Juliana Anokye^{1,2}, Miriam M. Yeung^{1,2}, Enid Y. N. Lam^{1,2},
4 Yih-Chih Chan^{1,2}, Chen-Fang Weng^{1,2}, Paul Yeh^{1,2}, Kathy Knezevic^{1,2}, Miriam S.
5 Butler^{1,2}, Annabelle Hoegl³, Kah-Lok Chan^{1,2}, Marian L. Burr^{1,2}, Linden J.
6 Gearing^{4,5}, Tracy Willson^{4,5}, Joy Liu⁴, Jarny Choi^{4,5}, Yuqing Yang^{4,5}, Rebecca A.
7 Bilardi⁴, Hendrik Falk^{4,5,6}, Nghi Nguyen⁷, Paul Stuppel^{6,7}, Thomas S. Peat^{6,8}, Ming
8 Zhang^{4,5,6}, Melanie de Silva^{4,5,6}, Catalina Carrasco-Pozo^{6,9}, Vicky M. Avery^{6,9}, Poh
9 Sim Khoo^{6,10}, Olan Dolezal^{6,8}, Matthew L. Dennis^{6,8}, Stewart Nuttall^{6,8}, Regina
10 Surjadi^{6,8}, Janet Newman^{6,8}, Bin Ren^{6,8}, David J. Leaver⁷, Yuxin Sun⁷, Jonathan B.
11 Baell^{7,11}, Oliver Dovey¹², George S. Vassiliou¹², Florian Grebien¹³, Sarah-Jane
12 Dawson^{1,2,14}, Ian P. Street^{4,5,6}, Brendon J. Monahan^{4,5,6}, Christopher J. Burns^{4,5},
13 Chunaram Choudhary³, Marnie E. Blewitt^{4,5}, Anne K. Voss^{4,5}, Tim Thomas^{4,5}, Mark
14 A. Dawson^{1,2,14,15}

15

16 ¹Cancer Research Division, Peter MacCallum Cancer Centre, Melbourne, VIC, Australia
17 ²Sir Peter MacCallum Department of Oncology, University of Melbourne, VIC, Australia
18 ³Department of Proteomics, The Novo Nordisk Foundation Center for Protein Research,
19 Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen,
20 Denmark
21 ⁴The Walter and Eliza Hall Institute of Medical Research, Melbourne, VIC, Australia
22 ⁵The Department of Medical Biology, The University of Melbourne, Melbourne, VIC,
23 Australia
24 ⁶Cancer Therapeutics CRC, Parkville, Victoria, Australia.
25 ⁷Medicinal Chemistry Theme, Monash Institute of Pharmaceutical Sciences, Monash
26 University, Melbourne, Victoria, Australia.
27 ⁸Commonwealth Scientific and Industrial Research Organisation (CSIRO), Biomedical
28 Program, Parkville, Victoria, Australia
29 ⁹Discovery Biology, Griffith Institute for Drug Discovery, Griffith University, Nathan,
30 QLD, Australia
31 ¹⁰Children's Cancer Institute, Kensington, NSW, Australia
32 ¹¹School of Pharmaceutical Sciences, Nanjing Tech University, Nanjing, China
33 ¹²Haematological Cancer Genetics, Wellcome Trust Sanger Institute, Hinxton, Cambridge
34 CB10 1SA, UK
35 ¹³Institute for Medical Biochemistry, University of Veterinary Medicine, Vienna,
36 Veterinaerplatz 1, 1210, Vienna, Austria
37 ¹⁴Centre for Cancer Research, University of Melbourne, VIC, Australia.
38 ¹⁵Department of Haematology, Peter MacCallum Cancer Centre, Melbourne, VIC,
39 Australia

40

41 **Corresponding Author:**

42 Professor Mark Dawson
43 Peter MacCallum Cancer Centre
44 305 Grattan Street
45 Melbourne VIC 3000 Australia
46 Email: Mark.Dawson@petermac.org
47 Phone: +61 3 8559 7131 / Fax: +61 3 8559 8054

48 **Abstract:**

49

50 Acute myeloid leukaemia (AML) is a heterogeneous disease characterised by
51 transcriptional dysregulation resulting in a block in differentiation and increased
52 malignant self-renewal. Various epigenetic therapies aimed at reversing these
53 hallmarks of AML have progressed into clinical trials, with most showing modest
54 efficacy due to an inability to effectively eradicate leukaemia stem cells (LSC)¹.
55 To specifically identify novel dependencies in LSC we screened a bespoke library
56 of small hairpin RNAs (shRNAs) targeting chromatin regulators in a unique *ex*
57 *vivo* model of LSC. We identified the MYST acetyltransferase HBO1 (KAT7 /
58 MYST2) and several known members of the HBO1 protein complex as critical
59 regulators of LSC maintenance. CRISPR domain screening and quantitative mass
60 spectrometry identified the HBO1 histone acetyltransferase (HAT) domain as
61 being essential to acetylate histone H3K14. H3K14ac facilitates the processivity
62 of RNA polymerase II to maintain the high expression of key genes including
63 *HOXA9* and *HOXA10* that helps sustain the functional properties of LSC. To
64 leverage this dependency therapeutically we developed highly a potent small
65 molecule inhibitor of HBO1 and demonstrate its mode of activity as a
66 competitive analogue of acetyl-CoA. Inhibition of HBO1 phenocopied our genetic
67 data and showed efficacy in a broad range of human cell lines and primary
68 patient AML cells. Together these biological, structural and chemical insights into
69 a novel therapeutic target in AML will enable the clinical translation of these
70 findings.

71 **Manuscript**

72

73 AML is organized in a loose hierarchy whereby a small population of self-
74 renewing LSC give rise to a large population of more mature leukemic blasts².
75 Whilst several human and mouse AML cell lines have undergone chemical and
76 genetic screens to identify targetable dependencies in this disease³⁻⁵, the
77 majority of these models do not replicate the functional properties of LSC.
78 Analogous to the effective maintenance of embryonic stem cells with therapeutic
79 pressure to decrease differentiation⁶, we serendipitously established a method
80 to sustain cells with the transcriptional and functional properties of LSC in liquid
81 culture⁷. Importantly, we concurrently established an isogenic population of
82 AML blasts.

83

84 As regulators of transcription are the most frequent mutational targets in AML⁸,
85 we performed a pooled negative selection screen with a customized shRNA
86 library against 270 known chromatin modifiers to uncover new transcriptional
87 regulators required for the maintenance of functionally validated LSC⁷. The
88 screen was highly reproducible and clearly identified shared and unique
89 dependencies in LSC and AML blasts (Extended Data Fig. 1a). Interestingly, we
90 observed far fewer dependencies in LSC; with less than one-third of shRNAs
91 depleted in the LSC compared to the blasts (Figure 1a, Supplementary Table 1).
92 We previously showed that the BET bromodomain proteins (BRD2/3/4) were
93 not a major dependency in this LSC model⁷ and in addition we found that most of
94 the hitherto identified epigenetic dependencies, including DOT1L, LSD1, EZH2
95 and PRMT5 that have been the focus of clinical therapies¹ selectively eradicate
96 only the blasts and not the LSC (Extended Data Fig. 1b-c). Of the few
97 dependencies identified in the LSC, we chose to focus on HBO1 as it is not a
98 recognised essential gene and it was equally effective in eradicating the blast and
99 LSC populations (Figure 1a-b and Extended Data Fig. 1d). HBO1 is one of five
100 mammalian members of the highly conserved MYST acetyltransferase family.
101 Recent efforts to identify unique and global genetic dependencies in human cells
102 have highlighted the fact that MOF (KAT8) and TIP60 (KAT5) are pan-essential
103 genes⁹, whereas HBO1 is highly expressed in human AML (Extended Data Fig. 2),
104 where it shows a clear and unique dependency⁹ (Figure 1 b-c, Extended Data Fig.
105 3a-b). HBO1 has been reported to function as a major transcriptional regulator
106 primarily via histone acetylation and although various histone modifications
107 have been attributed to HBO1¹⁰⁻¹², these conflicting reports are likely influenced
108 by the specificity of the antibodies used. Therefore, to precisely identify the
109 major histone modifications regulated by HBO1 we coupled conditional deletion
110 of HBO1 in AML cells with quantitative mass spectrometry¹³. These data clearly
111 demonstrate that acetylation of histone H3K14 (H3K14ac) is the major non-
112 redundant chromatin modification mediated by HBO1 (Supplementary Table 2
113 and Figure 1c).

114
115 HBO1, like most HATs can interact with several scaffolding proteins to form
116 functionally distinct catalytically active complexes¹⁴. Therefore, to identify the
117 major complex members required for the maintenance of LSC we assessed the
118 functional impact of genetic depletion of the HBO1 complex members. We found
119 that knockdown of BRPF2 (BRD1), mEAF6 and PHF16 (JADE3) phenocopied the
120 functional and biochemical effects of HBO1 reduction (Figure 1d-e and Extended
121 Data Fig. 3c). As all of the HBO1 complex components which resulted in
122 impaired maintenance of LSC, also caused a global reduction in H3K14ac, we
123 reasoned that the catalytic domain of HBO1 might be the critical target. CRISPR
124 domain screening with five separate sgRNAs against the MYST domain
125 confirmed that this domain was essential for H3K14ac and LSC survival (Figure
126 1f-g). While CRISPR domain screening is an effective approach to identify
127 functional domains for drug discovery¹⁵, our rescue experiments with wildtype
128 and catalytically inactive HBO1 provided the highest level of confidence that the
129 HAT domain of HBO1 was the critical therapeutic target in the complex (Figure
130 1h-i).

131
132 The dominant cellular phenotypes that resulted from HBO1 loss included an
133 induction of apoptosis, a prominent G0/G1 cell cycle arrest and a marked
134 differentiation of the immature LSC population (Figure 2a-b, Extended Data Fig.
135 3d-h). Together these data highlighted the importance of HBO1 in LSC
136 maintenance in an *ex vivo* model system. To address the broader application of
137 our findings in the absence of therapeutic pressure to maintain the LSC state⁷,
138 we generated an enriched population of LSC *in vivo*¹⁶ and performed a
139 competition assay to assess the requirement of HBO1 for LSC maintenance *in*
140 *vivo*. Here we transplanted a fixed ratio of 90% shRNA expressing cells and
141 followed the percentage of shRNA expressing cells contributing to the leukaemia
142 *in vivo*. Despite 90% of HBO1 shRNA expressing LSC being transplanted <5% of
143 them remain at the time of death from leukaemia demonstrating a marked
144 negative selection (Figure 2c). In contrast, both the non-targeting shRNA and
145 shRNA against the closely related MYST family member MOZ (KAT6A) show no
146 detrimental effect to LSC. Similar results were also seen in the NPM1c and FLT3-
147 ITD mouse model¹⁷ (Figure 2d). Moreover, the mice transplanted with HBO1
148 expressing shRNAs showed a significant survival benefit (Figure 2e, Extended
149 Data Fig. 4a) raising the prospect that HBO1 null LSC are incapable of
150 perpetuating the disease.

151
152 To explore this possibility further we generated leukaemias using an Mx1-Cre
153 model for conditional deletion of HBO1 (Extended Data Fig. 4b). The resulting
154 leukaemia was then transplanted into secondary recipient mice and
155 polyinosinic-polycytidylic (pIpC) acid was administered following engraftment.
156 Neither pIpC injection nor heterozygous deletion of HBO1 significantly impact

157 survival or leukaemia latency (Extended Data Fig. 4c). Leukemic cells derived
158 from *Hbo1^{flox/flox} Mx1-Cre* mice show a marked survival advantage and none of
159 the fatal leukaemia that occurred in plpC treated mice contained complete loss of
160 HBO1 (Figure 2f). In contrast, homozygous deletion of MOZ showed no
161 significant effects in two separate AML mouse models (Extended Data Fig. 4d-f).
162 Together, these data confirm the results from our *ex vivo* model and provide
163 compelling evidence that HBO1 is an essential requirement for LSC maintenance.

164

165 To assess the generality of our findings beyond mouse models of AML we chose
166 to delete HBO1 using CRISPR/Cas9 in a range of human AML cell lines
167 encompassing a variety of oncogenic drivers prevalent in AML⁸. We found the
168 majority of AML cell lines recapitulate our results in the murine LSC and show
169 impaired survival of HBO1 deleted cells resulting from an induction of apoptosis,
170 a G0/G1 cell cycle arrest and prominent differentiation (Figure 2g-k, Extended
171 Data Fig. 5). In contrast, very few non-AML cell lines show a similar dependency
172 on HBO1 (Extended Data Fig. 6). Having established the requirement of HBO1 in
173 mouse and human AML models we next wanted to understand the molecular
174 events underpinning its role in LSC maintenance. Consistent with the major
175 cellular phenotype of myeloid differentiation, we found that HBO1 loss results in
176 the marked enrichment of a myeloid differentiation gene expression program
177 (Extended Data Fig. 7a). The established role of HBO1 as a facilitator of
178 transcription led us to examine the top downregulated genes following HBO1
179 deletion. Interestingly, these downregulated genes are some of the most highly
180 expressed (Extended Data Fig. 7b) and include several homeobox genes (Figure
181 3a), which are known to be important in LSC maintenance and are commonly
182 upregulated in poor prognosis AML¹⁸. The requirement of HBO1 to sustain the
183 expression of the essential LSC genes within the 5'-HoxA cluster is conserved in
184 human AML cells (Extended Data Fig. 7c) and the dominant role of these genes in
185 mediating the cellular phenotypes of HBO1 loss is highlighted by the fact that
186 overexpression of *HoxA9* or *HoxA10* significantly rescues the myeloid
187 differentiation and loss of viability observed following HBO1 depletion
188 (Extended Data Fig. 7d-f).

189

190 Many of the genes downregulated following HBO1 loss, particularly the
191 homeobox genes, are established targets of both wildtype MLL1 and MLL1-
192 fusion proteins¹⁹. Using quantitative proteomics in an isogenic leukaemia cell
193 line designed to express a single copy of seven distinct MLL1-fusion proteins,²⁰
194 we identified the HBO1 complex functionally required to maintain LSC (Figure
195 1d) as strong interactors with the N-terminus of MLL1 (Figure 3b-c). Whilst
196 these findings provide molecular insights into how the HBO1 complex is
197 recruited to specific gene loci, to further understand the role of HBO1 in
198 regulating these genes we performed ChIP-Seq analyses for H3K14ac and RNA
199 polymerase-II (RNA POL-II). These data show that H3K14ac deposited by HBO1

200 is widespread throughout the genome but interestingly at the highly expressed
201 genes repressed by HBO1 loss, H3K14ac and RNA POL-II blanket the entire
202 coding region of the gene (Fig. 3d, Extended Data Fig. 7g). H3K14ac is an
203 evolutionarily conserved histone modification and recent evidence suggests that
204 H3K14ac may regulate transcriptional elongation²¹. Consistent with this, we find
205 markedly increased RNA POL-II levels within the coding region of highly
206 expressed genes containing the highest H3K14ac levels (Fig. 3e). Furthermore,
207 expressed genes with the highest level of H3K14ac have the lowest RNA POL-II
208 travelling ratio and HBO1 loss leads to a more prominent loss of RNA POL-II
209 within the body these genes (Fig. 3f, Extended Data Fig. 7h). The processivity of
210 RNA POL-II is greatly facilitated by chromatin remodelling complexes and
211 H3K14ac has been shown to be specifically bound by SMARCA4²², DPF2²³ and
212 several members of the ISWI family where it markedly potentiates their
213 remodelling activity²⁴. Remarkably, these chromatin remodelling complex
214 members show a similar cancer cell line dependency profile to HBO1 with a
215 predilection for AML⁹ and also phenocopy the effects of HBO1 loss in LSC (Fig.
216 3g-i, Extended Data Fig. 7i).

217 Our genetic data in both mouse and human AML clearly identified the catalytic
218 activity of HBO1 as the central therapeutic target. A long-standing challenge in
219 the field has been the ability to develop highly selective small molecule HAT
220 inhibitors that discriminate between the major families of histone
221 acetyltransferases. We have recently demonstrated that the
222 acylsulfonylhydrazide backbone provides a simple chemical scaffold for the
223 generation of selective MYST-family inhibitors²⁵. Using this template, we
224 generated WM-3835 (N'-(4-fluoro-5-methyl-[1,1'-biphenyl]-3-carbonyl)-3-
225 hydroxybenzenesulfonylhydrazide; Figure 4a), which retains specificity for the
226 MYST acetyltransferases but has increased potency against HBO1 compared to
227 WM-1119 (Figure 4b and Extended Data Fig. 8a). Crystal structure of HBO1 with
228 WM-3835 bound in the acetyl-CoA binding site was solved to 2.14 Å (Figure 4c,
229 Extended Data Fig. 9). Overlay of this crystal structure with WM-1119 in
230 MYST^{Cryst}²⁵ shows that WM-3835 makes additional interactions with the protein
231 surface, which may explain the increased activity of WM-3835 against HBO1.
232 Specifically, the WM-3835 phenol forms a hydrogen-bonding network to Glu525
233 and Lys488, neither of which is conserved throughout the MYST family.

234 WM-3835 is a cell permeable small molecule that results in a rapid and selective
235 reduction in H3K14ac levels (Figure 4d). Treatment of a diverse set of AML cell
236 lines with WM-3835 resulted in a marked reduction in tumour cell viability
237 (Figure 4e, Extended Data Fig. 10a) that was not observed following treatment
238 with the inactive analogue WM-2474²⁵ (Extended Data Fig. 8b). Notably, we
239 observed an excellent dose-response relationship between a reduction of
240 H3K14ac and cell viability (Extended Fig. 8c-d). Although WM-3835 retains

241 potency against MOZ and QKF/MORF (KAT6B), CRISPR/CAS9 mediated deletion
242 of these enzymes do not alter the activity of WM-3835 (Figure 4f), highlighting
243 that the efficacy of WM-3835 in AML is primarily via HBO1 inhibition. Moreover,
244 treatment of cells with WM-3835 phenocopied the molecular and cellular effects
245 of genetic depletion of HBO1 by inducing apoptosis, a G0/G1 cell cycle arrest,
246 differentiation of human AML cells and transcriptional repression on *HOXA9* and
247 *HOXA10* (Figure 4g-i, Extended Data Fig. 8e-h). Similar to our genetic studies,
248 overexpression of *HoxA9* and *HoxA10* ameliorated the effects of WM-3835
249 (Extended Data Fig. 8i). Although the rapid metabolism, including
250 glucuronidation of WM-3835 precluded efficacy experiments *in vivo* (Extended
251 Data Fig. 10b-c), the compound showed a prominent reduction of clonogenic
252 potential in primary human AML cells derived from several patients harbouring
253 different driver mutations, highlighting the therapeutic potential of catalytic
254 inhibitors against HBO1 in AML (Figure 4j).

255 Central to the ambition to alter the natural history of AML is the requirement for
256 new therapies that effectively target LSC from the outset. LSC serve as the
257 reservoir for evolving resistance to conventional and targeted therapies and our
258 clinical experience has clearly proven that monotherapies are incapable to
259 subvert the vast adaptive potential of LSC. Therefore, the future lies in
260 identifying key therapeutic targets in LSC that can be leveraged in combination
261 with other effective agents including conventional chemotherapy. Here we
262 identify HBO1 as a targetable dependency in LSC. Our molecular insights suggest
263 that MLL1 recruits HBO1 to regulate highly expressed LSC genes including the
264 HOXA cluster through H3K14ac, which potentiates the activity of specific
265 chromatin remodelling complexes enabling greater processivity of RNA POL-II
266 (Figure 4k). The blueprint for selective and potent inhibition of HBO1, together
267 with these new biological insights provide the impetus and platform for the
268 translation of these findings into the clinical setting.

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296
297 **AUTHOR CONTRIBUTIONS**

298 M.A.D developed the overall concept behind the study and supervised the project
299 with important advice from L.M, M.E.B, C.J.B, A.K.V, T.T, C.C, F.G and B.J.M. The
300 manuscript was written by L.M, B.J.M and M.A.D with input from all the authors.
301 L.M performed the majority of the experiments with help from J.A, E.Y.N.L, Y-C.C,
302 C-F.W, M.M.Y, P.Y, K-L. C, K.K, M.S.B, M.L.B, J.L, Y.Y, A.D, R.A.B, T.T, H.F, M.D.S, N.N,
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306 and M.E.B. A.H. and C.C. performed mass spectrometry analysis of histone
307 acetylation sites. Protein production, SPR and structural studies were done by

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310 **COMPETING INTERESTS**

311 M.A.D. has been a member of advisory boards for CTX CRC, Storm Therapeutics,
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314

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378 senescence and arrest tumour growth. *Nature* **560**, 253-257 (2018).
- 379
380
381

382 **Figure Legends**

383

384 **Figure 1: HBO1 is an essential dependency in LSC.**

385 **a.** Pooled negative-selection screening in MLL-AF9 bulk blasts (left) and
386 leukaemic stem cells (LSC, right). Volcano plot depicting changes in
387 representation of shRNAs in the screen between days 2 and 14. One dot
388 represents the mean of two independent experiments for each gene. **b.** Negative
389 selection competition assays depicting the percentage of shRNA over time in
390 blasts and LSC expressing *Hbo1* shRNAs. n=3 (mean + s.e.m.). **c.** Immunoblot of
391 HBO1, H3K14Ac and H3K27Ac n=3 and **d.** Negative selection competition assays
392 n=3 (mean + s.e.m.) and **e.** Immunoblot of H3K14ac in LSC expressing shRNAs
393 targeting *Hbo1* complex members. n=3. **f.** Negative selection competition assays
394 n=3 (mean + s.e.m.) and **g.** Immunoblot of HBO1, H3K14Ac and H3K27Ac in LSC
395 expressing Cas9 and sgRNAs against *Rosa26* (control) or the *Hbo1* catalytic
396 domain. n=3. **h.** Schematic outline of *Hbo1* rescue experiments. Wildtype (wt) or
397 catalytic mutant *Hbo1* (E508Q) resistant to sgHbo1 e12.2 or GFP were
398 overexpressed in LSC expressing Cas9 and then transduced with sgRNAs
399 targeting *Hbo1*. **i.** *Hbo1* rescue negative selection competition assays. n=3 (mean
400 + s.e.m.). sgHbo1 e11.1 targets endogenous (END) and overexpressed (OE) *Hbo1*.
401 Right panel: immunoblot of overexpressed wildtype and catalytic mutant HBO1.

402

403 **Figure 2: Loss of HBO1 impairs LSC maintenance *in vivo*.**

404 **a.** Cell cycle profile n=3 (mean + s.e.m.) and **b.** Surface expression of Gr1 and
405 CD11b in LSC expressing Cas9 transduced with *Hbo1* sgRNAs. n=3. **c.** Percentage
406 of shRNA positive cells in bone marrow (BM) and spleen (SP) at endpoint in
407 recipients transplanted with 5° MLL-AF9 cells. (mean ±s.d.) n= 5/group. **d.**
408 Percentage of shRNA positive cells (mean ±s.e.m.) in bone marrow (BM) at
409 endpoint in recipients transplanted with 3° NPM1c/FLT3-ITD cells. n=6/group.
410 **e.** Kaplan-Meier curves of recipients injected with NPM1c/FLT3-ITD cells
411 expressing hairpins targeting *Hbo1*. n=5/group. Inset: immunoblot of HBO1 in
412 NPM1c/FLT3-ITD cells expressing *Hbo1* hairpin from a recipient that died
413 showed that the hairpin was inactivated. *Hbo1* levels from parental
414 NPM1c/FLT3-ITD cells are shown for comparison. **f.** Kaplan-Meier curves of
415 C57BL/6 mice injected with 10³ *Hbo1*^{flox/flox} *Mx1-Cre* 1° MLL-AF9 leukaemic cells.
416 Mice were treated with saline or pIpC (arrowheads). Inset: representative
417 genotyping of recipient bone marrow at endpoint. n=6/group. **g.** Negative
418 selection competition assays n=3 (mean + s.e.m.) and **h.** Immunoblot of HBO1
419 and H3K14Ac n=3. and **i.** Cell cycle profile n=3 (mean + s.e.m.) and **j.** Apoptosis
420 n=3 (mean + s.e.m.). and **k.** Surface expression of CD11b in Molm13 cells
421 expressing Cas9 and HBO1 sgRNAs. n=3.

422

423 **Figure 3: HBO1 regulates expression of the 5' end of the HoxA cluster in**
424 **AML.**

425 **a.** Heat map depicting normalised expression of the top 25 down-regulated
426 genes in LSC with HBO1 loss. **b.** Heat map displaying the mean spectral counts
427 from AP-MS data of 7 MLL1 translocation partners for all MYST family members.
428 **c.** Network analysis of known protein interaction partners of HBO1 (thick grey
429 lines) linking to known members of the HBO1 complex and HBO1 network to 7
430 MLL-fusions (thin red line). Nodes size represents the mean spectral counts for
431 each interaction. Node border width is scaled to the number of interactions with
432 the MLL-fusions. **d.** CHIP-seq profiles of H3K14Ac and RNA Polymerase II (RNA
433 POL-II) at *HOXA* gene cluster in LSC expressing Cas9 and Hbo1 sgRNA.
434 Representative of n=3 biological replicates. **e.** RNA POLII coverage across highly
435 expressed genes (high) divided according to H3K14ac levels. **f.** Waterfall plots of
436 change in RNA POLII (POL-II LFC) binding throughout the gene promoter (-30 to
437 +300 bp) and gene body (+300 bp to TES) following HBO1 loss for highly
438 expressed genes divided according to H3K14ac levels. **g.** Negative selection
439 competition assays n=3 (mean + s.e.m.) and **h.** *Hoxa9* and *Hoxa10* mRNA
440 expression in LSC expressing Cas9 and *Smarca4*, *Dpf2* or *Smarca5* sgRNAs. n=3
441 (mean + s.e.m.) **i.** Surface expression of Gr1 and CD11b in LSC expressing Cas9
442 and *Dpf2* sgRNAs. n=2.

443

444 **Figure 4: Treatment with WM-3835 reduces AML growth.**

445 **a.** Chemical structure of WM-3835. **b.** Selectivity and potency profile of WM-
446 3835 and WM-1119 superimposed on the KAT family dendrogram as measured
447 by histone acetyltransferase inhibition assay. Values listed in Extended Data
448 Figure 8A. **c.** Ribbon representation of the HBO1-BPRF2 crystal structure(cyan)
449 with WM-3835 bound (yellow with element colouring; PDB code 6MAJ) overlaid
450 with the corresponding region of MYST^{CRYST} with WM-1119 bound (magenta
451 with element colouring; PDB code 6BA4), where non-conserved residues of
452 MYST^{CRYST} are shown in (blue). Key residues highlighted show hydrogen bond
453 with the phenol of WM-3935 **d.** Immunoblot of H3K14Ac in LSC treated with
454 WM-3835. n=2. **e.** LSC and human AML cell proliferation with 1 μ M WM-3835.
455 n=3 (mean + s.e.m.). **f.** LSC with *Moz* or *Qkf/Morf* deletion proliferation with WM-
456 3835 n=3 (mean \pm s.e.m.). Right, immunoblot of H3K23ac and H3K14ac in LSC
457 with *Moz* or *Qkf/Morf* deletion. n=2. **g.** Cell cycle profile n=3 (mean + s.e.m.) and
458 **h.** Surface expression of CD11b in Molm13 cells with WM-3835. n=3. **i.** *Hoxa9*
459 expression in murine LSC and Molm13 cells with WM-3835. n=2. **j.** Clonogenic
460 assays with primary patient AML cells treated with WM-3835. n=5 (mean +
461 s.e.m.). **k.** Schematic overview of AML gene regulation by the HBO1 complex.

462 **Extended Data Figure Legends**

463

464 **Extended Data Figure 1. Hbo1 is an AML specific dependency.**

465 **a.** RNAi screen multidimensional scaling plot of shRNA sequences from bulk
466 leukaemic blasts (bulk) and leukaemic stem cells (LSC) over 14 days of
467 screening. Screens were performed in duplicate (R1 and R2). **b.** Ezh2, Lsd1 and
468 Prmt5 mRNA expression relative to non-targeting (NT) in LSC expressing
469 shRNAs. Validation of the shRNA's against DOT1L have previously been
470 published²⁶. n=3 (mean + s.e.m.) **c.** Negative selection competition assays in bulk
471 leukaemic blast and LSC expressing shRNAs against Ezh2, Lsd1 and Prmt5. n=3
472 (mean + s.e.m.). **d.** Hbo1 mRNA expression relative to non-targeting (NT) in LSC
473 expressing shRNAs. n=3 (mean + s.e.m.).

474

475 **Extended Data Figure 2. Expression levels of HBO1.**

476 **a.** Box plot of HBO1 expression levels in different cancer types from TCGA²⁷. The
477 upper limit, centre and lower limit of each box denotes the upper quartile,
478 median and lower quartile of the data respectively **b.** Dot plot of HBO1
479 expression levels in normal haematopoietic and AML cells from BloodSpot²⁸, line
480 indicates mean expression.

481

482 **Extended Data Figure 3. Hbo1 depletion increases apoptosis, cell cycle**
483 **arrest and myeloid differentiation in murine LSC.**

484 **a.** Negative selection competition assays in LSC expressing shRNA's against *Moz*,
485 *Qkf* or *Hbo1*. n=3 (mean + s.e.m.). **b.** Negative selection competition assays in LSC
486 expressing Cas9 transduced with sgRNAs targeting *Moz* or *Qkf*. n=3 (mean +
487 s.e.m.). **c.** Hbo1 complex member expression relative to non-targeting (NT) in
488 LSC expressing shRNAs targeting individual complex members. n=3 (mean +
489 s.e.m.) **d.** Apoptosis of LSC expressing shRNAs targeting *Hbo1*. n=3 (mean +
490 s.e.m.). **e.** Cell cycle profile of LSC expressing shRNAs targeting *Hbo1*. n=3 (mean
491 + s.e.m.). **f.** Surface expression of Gr1 and CD11b in LSC expressing shRNAs
492 against *Hbo1*. n=3. **g.** Apoptosis of LSC expressing Cas9 and sgRNAs targeting
493 *Hbo1*. n=3 (mean + s.e.m.). **h.** Surface expression of cKit (CD117) in LSC
494 expressing sgRNAs targeting *Hbo1*. Representative of n=2 biological replicates.

495

496 **Extended Data Figure 4. *in vivo* depletion of Hbo1 increases disease latency.**

497 **a.** Kaplan-Meier curves of NSG mice transplanted with quinary MLL-AF9
498 leukaemic cells expressing shRNAs targeting *Hbo1* and *Moz*. n=6 per group. **b.**
499 Schematic of wildtype and *Hbo1* mutant alleles with numbered black boxes
500 representing exons. Genotyping primers are indicated (adapted from Kueh et.,
501 al). **c.** Kaplan-Meier curves of C57BL/6 mice injected with 10⁶ *Hbo1*^{fllox/+} *Mx1-Cre*
502 primary MLL-AF9 leukaemic cells. Mice were treated with saline or pIpC. n=12
503 per group. Right: representative genotyping of recipient bone marrow at
504 endpoint. **d.** Kaplan-Meier curves of C57BL/6 mice injected with *Moz*^{+/+}, *Moz*^{+/-} or

505 *Moz*^{-/-} MLL-AF9 leukaemic cells. n=5 per group. **e.** Kaplan-Meier curves of Balb/c
506 mice injected with *Moz*^{+/+} or *Moz*^{-/-} HoxA9/Meis1 leukaemic cells. n = 5 per
507 group.

508
509

510 **Extended Data Figure 5. HBO1 is a dependency in various AML subtypes.**

511 **a.** Negative selection competition assays in human AML cell lines expressing
512 Cas9 and sgRNAs targeting *HBO1*. n=3 (mean + s.e.m.). Driver mutations are in
513 parentheses. **b.** Immunoblot of HBO1 and H3K14Ac in OCI-AML3 cells expressing
514 Cas9 and sgRNAs targeting *HBO1*. n=3 (mean + s.e.m.). **c.** Cell cycle profile of OCI-
515 AML3 cells expressing Cas9 and sgRNAs targeting HBO1. n=3 (mean + s.e.m.). **d.**
516 Apoptosis of OCI-AML3 cells expressing Cas9 and sgRNAs targeting *HBO1*. n=3
517 (mean + s.e.m.). **e.** Surface expression of CD11b in OCI-AML3 cells expressing
518 Cas9 and sgRNAs targeting *HBO1*. n=3.

519

520 **Extended Data Figure 6. HBO1 dependency in other cancers.**

521 Negative selection competition assays in human cancer cell lines expressing Cas9
522 and sgRNAs targeting *HBO1*. n=3 (mean + s.e.m.). Cancer type is in parentheses.

523

524 **Extended Data Figure 7. Hbo1 depletion increases myeloid signature and
525 decreases global H3K14Ac**

526 **a.** Barcode plot evaluating changes in myeloid development signature following
527 HBO1 depletion with sgHbo1 e11.1 and e12.2 in LSC. n=3. **b.** Bar plot of changes
528 in genes expression following HBO1 deletion in LSC ranked by expression levels.
529 Green bars show the top 25 most down-regulated genes following *Hbo1* deletion.
530 **c.** HOXA9 and HOXA10 mRNA expression in Molm13 and OCI-AML3 cells
531 expressing Cas9 and sgRNA targeting *HBO1*. n=3 (mean ± s.e.m.). **d.** Surface
532 expression of CD11b in LSC overexpressing *Hoxa9* or *Hoxa10* and sgRNAs
533 targeting Hbo1. **e.** Immunoblot of overexpressed Hoxa9 or Hoxa10 in LSC cells
534 expressing Cas9. Representative of n=3 biological replicates. **f.** *Hoxa9* and
535 *Hoxa10* rescue negative selection competition assays. Representative of n=3
536 biological replicates. **g.** ChIP-seq profiles of H3K14ac and RNA Polymerase II
537 (RNA POL-II) at the *Pbx3* locus in LSC expressing Cas9 and an sgRNA targeting
538 *Hbo1*. Representative of n=3 biological replicates. **h.** RNA PolII traveling ratio
539 distribution for highly expressed genes divided according to H3K14ac levels
540 from ChIP-seq. **i.** Surface expression of Gr1 in LSC overexpressing sgRNAs
541 targeting *Smarca5*. Representative of n=3 biological replicates.

542

543 **Extended Data Figure 8. WM-3835 inhibits cell growth and HOXA
544 expression in AML.**

545 **a.** KAT biochemical and SPR values for WM-3835 compared to WM-1119.
546 Biochemical assay was done at 1 μM acetyl-CoA, *K_m* of HBO1. **b.** Proliferation
547 assays of human AML cells treated with 1 μM WM-2474. n=3 (mean + s.e.m.) **c.**

548 Cellular H3K14Ac biomarker assay dose response curves for WM-3835 (blue)
549 and WM-1119 (red) (mean \pm s.e.m.). n=6 **d.** Growth inhibition assays of MLL-
550 AF9 AML cell line Molm13 treated with WM-3835 at doses indicated. Boxes
551 represent minimum and maximum values. n=11 (mean \pm s.e.m.) **e.** Cell cycle
552 profile of OCI-AML3 cells treated with WM-3835 or vehicle. n=3 (mean + s.e.m.)
553 **f.** Apoptosis of OCI-AML3 cells treated with WM-3835 or vehicle. n=3 (mean +
554 s.e.m.). **g.** Surface expression of CD11b in OCI-AML3 cells treated with WM-3835
555 or vehicle. n=3 **h.** *HOXA10* mRNA expression in LSC and Molm13 cells treated
556 with WM-3835 or vehicle. n=3 (mean + s.e.m.) **i.** *HOXA9* and *HOXA10* mRNA
557 expression in OCI-AML3 cells treated with WM-3835. n=3 (mean + s.e.m.). **j.**
558 *Hoxa9* and *Hoxa10* rescue proliferation assays with 1 μ M WM-3835 in LSC. n=3
559 (mean \pm s.e.m.).
560

561 **Extended Data Figure 9. HBO1 Crystal Structure.**

562 **a.** Data collection and refinement statistics of WM-3835 HBO1-BRPF2 co-crystal
563 structure. **b.** WM-3835 binding site in HBO1-BRPF2. WM-3835 shown in silver
564 with element colouring and the OMIT electron density map contoured to 3 σ
565 shown in green. **c.** Overlay of WM-3835 and acetyl-coA (purple with element
566 colouring), showing that WM-3835 binds in the acetyl-coA binding site of HBO1.
567 **d.** Ribbon diagram of HBO1-BRPF2 showing WM-3835 bound to the acetyl-coA
568 binding site. **e.** Space filling model showing WM-3835 (yellow with element
569 colouring) in the acetyl-coA binding pocket of HBO1-BRPF2.
570

571 **Extended Data Figure 10. High *in vitro* metabolism and poor *in vivo* oral**
572 **exposure of WM-3835.**

573 **a.** Proliferation assays of human AML cell lines treated with 1 μ M WM-3835. **b.**
574 WM-3835 demonstrates high clearance in both human and mouse liver
575 microsome assays. The use of dual cofactors (UDPGA and NADPH) results in an
576 increased rate of clearance in human liver microsomes, which is consistent with
577 glucuronidation having a role in the clearance of this compound. **c.** BALB/c
578 female mice were dosed with WM-3835 at 100 mg/kg *p.o. b.i.d.* formulated in
579 20% PEG400/10% Solutol or vehicle. 4 hours after the third dose blood samples
580 were collected. An average total drug concentration of 1860 nM was observed.
581 The free drug level was determined to be 2.6 nM after accounting for mouse
582 plasma protein binding (*f_u* 0.0014). This free drug level was considered too low
583 to affect H3K14 acetylation based on the *in vitro* H3K14ac cellular biomarker
584 data (Figure 4). An additional chromatographic peak eluting earlier than WM-
585 3835 was detected in the plasma samples from the treatment group. Subsequent
586 analyses using predicted multiple-reaction monitoring and accurate mass
587 measurement indicated that it is likely to be a glucuronide conjugate of WM-
588 3835, consistent with the *in vitro* metabolism data.

589 **Methods**

590 **Cell Culture**

591 MLL-AF9 bulk blasts and LSC were generated as previously described⁷. Murine
592 and human cell lines (NOMO-1, Molm13, MV4;11, HL-60, OCI-M2, OCI-AML3,
593 K562, NB4, SKM-1, and KG-1) were maintained in RPMI-1640 supplemented
594 with 10% FCS, 2 mM GlutaMAX, 100 IU ml⁻¹ penicillin, 100 ug ml⁻¹ streptomycin
595 under standard culture conditions (5% CO₂, 37°C). Blasts and LSC were
596 maintained in the presence of 0.1% DMSO or 1 μM I-BET151, respectively, and
597 IL-3 (10 ng ml⁻¹). HEK293T cells were maintained in DMEM supplemented with
598 10% FCS, 100 IU ml⁻¹ penicillin, 100 ug ml⁻¹ streptomycin in 10% CO₂ at 37°C.
599 All cell lines were regularly tested and verified to be mycoplasma negative by
600 PCR analysis by in-house genotyping. Human cell lines were authenticated by
601 STR profiling through the Australian Genome Research Facility (Melbourne,
602 Victoria).

603

604 **Virus production and transduction**

605 Retrovirus was produced by triple transfection of HEK293T cells with a
606 retroviral LMP-BFP transfer vector and structural pMD1-gag-pol plasmid and
607 Vsv-g envelope plasmid at a 0.75:0.22:0.03 ratio, as previously described²⁹.
608 Lentivirus was produced by triple transfection of HEK293T cells with a lentiviral
609 transfer vector, and the packaging plasmids psPAX2 and Vsv-g at a 0.5:0.35:15
610 ratio. All transfections were performed using polyethylenimine (PEI). Viral
611 supernatants were collected 48 h following transfection, filtered through a 0.45
612 μm filter and added to target cells.

613

614 To examine the effect of loss of MOZ function on progression of leukaemia foetal
615 liver cells were isolated from E13 embryos with a germline deletion of *Moz* or
616 littermate controls³⁰. E13 embryos were used because *Moz* null embryos die by
617 E14. Foetal liver cells (C57B/6; CD45.2 cell surface phenotype) were transfected
618 with MSCV expressing either MLL-AFP and GFP or Meis1, Hoxa9 and GFP or
619 control viruses: empty vector (Gfp) or Meis1-Gfp or Hoxa9-Gfp alone (not
620 shown); prepared for infection as above except the ECO envelope protein was
621 used. After overnight culture infected foetal liver cells were injected into C57B/6
622 CD45.1 recipient mice, which had been irradiated with a single dose of 700 rads.

623

624 **Pooled negative-selection RNAi screening**

625 A custom shRNA library targeting 270 murine epigenetic enzymatic genes was
626 designed using the Designer of Small Interfering RNA Website and subcloned
627 into the LMP-blue fluorescent protein (BFP) vector with selectable markers
628 EBFP/puromycin as previously described³¹. After sequence verification, 1922
629 shRNAs (6-8 per gene) were combined with several positive- and negative
630 control shRNAs at equal concentration in one pool. This pool was used to
631 produce retrovirus, which was then transduced into 4 x 10⁶ MLL-AF9 bulk blasts

632 and LSC at a multiplicity of infection of 0.3 and selected with 3 and 5 $\mu\text{g ml}^{-1}$
633 puromycin, respectively, commencing 30 h after transduction. Throughout 14
634 days of puromycin selection more than 20 million cells were maintained at each
635 passage to preserve 10 000-fold library representation. Genomic DNA from D2 to
636 D14 was isolated (DNeasy Blood & Tissue Kit, Qiagen) from both blasts and LSC.
637 shRNA sequences were amplified by PCR with primers containing adaptors for
638 Illumina sequencing as previously described³². The resulting libraries were
639 sequenced with single-end 50 bp reads on a HiSeq2500. The shRNA sequences
640 were mapped to the shRNAs within the pool, and the shRNA counts were
641 analysed as previously described³³. The likelihood ratio test was used to
642 determine the hairpins significantly depleted over the timecourse of the
643 experiment. Genes with at least two hairpins depleted by greater than 10-fold
644 were considered to be significant dependencies.

645

646 **CRISPR-Cas9-mediated gene disruption**

647 sgRNA oligonucleotides (Sigma-Aldrich) were phosphorylated, annealed and
648 cloned into lentiviral expression vectors, pKLV-U6gRNA(BbsI)-PGKpuro2ABFP
649 (Addgene 50946, deposited by K. Yusa). Cells were first transduced with the
650 FUCas9Cherry (Addgene 70182, deposited by M. Herold) and FACS sorted for
651 high mCherry expression and then subsequently transduced with the pKLV
652 sgRNA expression vector.

653

654 **shRNA and sgRNA Competitive Proliferation Assay**

655 Bulk blast and LSC were transduced with retrovirus expressing a gene specific
656 shRNA and the percentage of BFP-positive cells was measured between days 1
657 and 13 post-transduction and normalised to the percentage of BFP positive cells
658 at day 1. For sgRNA, Cas9-expressing cells were transduced with a lentivirus
659 expressing an *Hbo1* sgRNA and the percentage of double positive BFP and
660 mCherry cells was measured between days 2 and 14 post-transduction and
661 normalised to the percentage BFP/mCherry positive cells at day 2 or 4. All
662 shRNA and sgRNA sequences are provided in Supplementary Table 3.

663

664 **Antibodies**

665 Immunoblotting: rabbit anti-acetyl-histone H3 (Lys 14) (D4B9, Cell Signalling
666 Technology), mouse anti-histone H3K14Ac (13HH3-1A5, Active Motif), rabbit
667 anti-histone H3 (acetyl K27) (ab4729, Abcam), rabbit anti-KAT7/Hbo1/MYST2
668 (ab70183, Abcam), rabbit anti-histone H3 (ab1791, Abcam), mouse anti-HSP60
669 (C10, Santa Cruz), rabbit anti-HSP60 (H-300, Santa Cruz), mouse anti-FLAG (M2,
670 Sigma), mouse anti-RNA polymerase II (CTD4H8, Millipore). Flow Cytometry:
671 Alexa Fluor 700 anti-Gr1 (108422, BioLegend) and Brilliant Violet 605 anti-
672 CD11b (101237, Biolegend), APC/Cy7 anti-mouse CD117 (c-kit) (313228,
673 Biolegend).

674

675 **Flow Cytometry**

676 Cell apoptosis, hairpin or sgRNA positive cells were washed once with PBS and
677 assessed using FITC conjugated Annexin V (640906, Biolegend) and DAPI
678 (D9542, Sigma) staining according to manufacturer's instructions. For cell cycle
679 analysis, hairpin or sgRNA positive cells were washed with PBS and fixed for at
680 least 2 hours at -20°C in 70% ethanol. Fixed cells were PBS washed and
681 incubated at 4°C in 4',6-diamidino-2-phenylindole (DAPI) staining solution (1 mg
682 ml⁻¹ DAPI, 0.05% (v/v) Triton X-100 in PBS) for 30 min. For surface expression
683 of myeloid markers, hairpin or sgRNA positive cells were washed in PBS and
684 stained for Gr1 or CD11b on ice for 30 min in PBS plus 5% FCS. All flow
685 cytometry analyses were performed on a LSR Fortessa X-20 flow cytometer (BD
686 Biosciences) and all data analysed with FlowJo. Cell sorting was performed on a
687 FACS Aria Fusion 5 (BD Biosciences).

688

689 **Immunoblotting**

690 Hairpin or sgRNA positive cells were lysed in 20 mM HEPES pH7.9, 0.5 mM
691 EDTA, 2% SDS plus 1X protease inhibitor cocktail (Roche) by brief sonication.
692 Lysates were heated to 95°C in SDS sample buffer with 50 mM DTT for 5 min,
693 separated by SDS-PAGE and transferred to PVDF membrane (Millipore).
694 Membranes were blocked in 5% milk in TBS +0.1% Tween-20, probed with the
695 indicated antibodies, and reactive bands visualised using ECL Prime (GE).

696

697 **Analysis of HBO1-regulated acetylation of core histones**

698 Murine MLL-AF9 *CreERT2 Hbo1^{f/f}* conditional knockout cells were SILAC-
699 labelled with "light" (¹²C₆,¹⁴N₄-arginine and ¹²C₆,¹⁴N₂-lysine) and "heavy"
700 ((¹³C₆,¹⁵N₄-arginine and ¹³C₆,¹⁵N₂-lysine, Cambridge Isotope Laboratories). To
701 delete HBO1, the heavy-labelled cells were treated with 4-hydroxytamoxifen
702 (200 nM) for ~40 hours, and light-labelled control cells were treated with
703 vehicle control. Histones were extracted as described previously.³⁴ Briefly, cells
704 were lysed mechanically in ice-cold hypotonic lysis buffer (10 mM Tris pH 8.0, 1
705 mM KCl, 1.5 mM MgCl₂, 1 mM DTT and 1× complete protease inhibitor cocktail
706 (Roche)) and intact nuclei were harvested by centrifugation. Histones were acid-
707 extracted with H₂SO₄ (0.4 N) and precipitated with TCA (33% final
708 concentration). Purified histones from knockout and control cells were mixed in
709 equal amounts, separated on SDS-PAGE, in-gel digested with Trypsin or LysC.
710 (Sigma). The histone peptides were analysed by online nanoflow liquid
711 chromatography coupled tandem mass spectrometry (LC-MS/MS) using a
712 Proxeon easy nLC system connected to a Q-Exactive HFX mass spectrometer
713 (Thermo Scientific). The raw data was computationally processed using
714 MaxQuant³⁵ (Version 1.5.6.5) and searched against the UniProt database
715 (downloaded Jan 23, 2014) using the integrated Andromeda search engine
716 (<http://www.maxquant.org/>). The data were searched with: 3 missed cleavages,

717 minimum peptide length of 6 amino acids, re-quantify option selected,
718 acetylation was included as variable modification.

719

720 **Rescue Assays**

721 cDNA of *Hbo1* and *Hoxa10* were PCR amplified from the cDNA library of murine
722 MLL-AF9 cells with primers containing a FLAG. *Hoxa9* cDNA was amplified from
723 pTRE rtTA FLAG HoxA9 GFP³⁶. Catalytic mutant HBO1 E508Q was generated by
724 site-directed mutagenesis. Wildtype and mutant *Hbo1* were made resistant to
725 sgHbo1 e12.2 by silent point mutation of the PAM site corresponding this sgRNA
726 by site-directed mutagenesis. All cDNAs were cloned into the lentiviral pHRISIN-
727 P_{SFFV}-GFP-P_{PGK}-Puro vector³⁷. LSC expressing Cas9 were transduced with
728 expression vectors and selected with 5 µg ml⁻¹ puromycin for one week.
729 Overexpression lines were then subsequently transduced with *Hbo1* sgRNA.

730

731 **Animal details**

732 All animal work was performed at the Peter MacCallum Cancer Centre animal
733 facility, under approval E530 from the Peter MacCallum Cancer Centre animal
734 ethics committee and at the Walter and Eliza Hall Institute of Medical Research
735 with approval from the Walter and Eliza Hall Institute Animal Ethics Committee
736 under approval 2015.015. *Mx1-Cre Hbo1^{flox/flox}* mice¹⁰ and *Moz^{+/-}* mice were as
737 previously described³⁰.

738

739 **In vivo Competition Assay**

740 6° MLL-AF9 cells were transduced with non-targeting (NT), shHbo1 or shMoz
741 hairpins at 90% transduction efficiency. 100 000 cells were transplanted 48 h
742 post-transduction into 8 week old female NSG mice. BFP positive hairpin positive
743 cells were determined by flow cytometry.

744

745 **Leukaemia Maintenance**

746 The generation of *Mx1-Cre Hbo1^{f/f}* conditional knockout mice have been
747 previously described¹⁰. c-kit positive cells from whole bone marrow were
748 selected through magnetic bead selection (Miltenyi Biotec) and retrovirally
749 transduced with the MSCV-MLL-AF9-IRES-YFP construct. Cells were
750 transplanted in sublethally irradiated 6-8 week-old female C57BL/6 recipients.
751 100 000 leukaemic cells from the bone marrow was collected and subsequently
752 transplanted into sublethally irradiated 11-week-old female C57BL/6 recipients.
753 Polyinosinic:polycytidylic (pIpC, GE) was i.p. administered 6, 10, 14 days post-
754 transplantation at 7.5 mg/kg. Amplification of wildtype and floxed alleles of
755 leukaemic cells from bone marrow has been previously described¹⁰.

756

757 **RNA sequencing and analysis**

758 RNA from sgRNA positive cells was prepared using the Qiagen RNeasy kit. RNA
759 concentration was quantified with a NanoDrop spectrophotometer (Thermo

760 Scientific). Libraries were prepared using QuantaSeq 3' mRNA Library Prep kit
761 (Lexogen). Libraries were sequenced on a NextSeq500 with 75 bp single end
762 reads. All RNA-seq experiments were performed in triplicate. Following
763 trimming of poly-A tails with cutadapt³⁸ (v.1.14). Reads were aligned to the
764 mouse genome (ensembl_GRC38.78) using hisat2³⁹, and assigned to genes using
765 htseq-count⁴⁰. Differential gene expression analysis was performed using the
766 edgeR⁴¹ package in R (<http://www.R-project.org/>), adjusted *p*-values were
767 calculated using the Benjamini-Hochberg method.⁴² Genes with log fold-changes
768 below -1 and adjusted *p*-values below 0.05 were considered to be significantly
769 down-regulated genes. Count data was voom-transformed using the voom
770 function before performing gene set testing with the mroast function⁴³, both
771 from the limma package⁴⁴.

772

773 **Chromatin immunoprecipitation sequencing (ChIP-seq) and analysis**

774 10-20 million sgRNA positive cells were cross-linked with 1% formaldehyde for
775 10 min at room temperature and cross-linking was quenched by addition of
776 0.125 M glycine. Cells then lysed in 1% SDS, 10 mM EDTA, 50 mM Tris-HCl pH
777 8.0 and protease inhibitors. Lysates were sonicated in a Covaris ultrasonicator to
778 achieve a mean DNA fragment size of 500 bp. Immunoprecipitation with anti-
779 H3K14Ac (Cell Signalling Technologies) or anti-RNA polymerase II (Millipore) was
780 performed overnight at 4°C in modified RIPA buffer (10 mM Tris-HCl pH 8.0, 90
781 mM NaCl, 1% Triton X-100, 0.1% deoxycholate). Protein A or G magnetic beads
782 (Life Technologies) were used to bind antibody and associated chromatin.
783 Reverse crosslinking of DNA was followed by DNA purification using the
784 QIAquick PCR purification kit (Qiagen). Sequencing libraries were prepared from
785 eluted DNA using ThruPLEX DNA-seq kit (Rubicon). Libraries were size selected
786 between 200-500 bp and sequenced on a NextSeq500 with 75 bp single end
787 reads. Following the removal of Illumina adaptors using cutadapt³⁸. Reads were
788 aligned to a joint reference genome of mouse (ensemble_GRCh38.78) and
789 drosophila (ensemble_BDGP5.78) with bwa-mem (v. 0.7.13). SAM files were
790 converted to BAM files using samtools⁴⁵ (v. 1.4.1). A scaling factor was calculated
791 using the drosophila spike-in, as previously described⁴⁶. The scaling factor was
792 used to normalise the coverage across the genome, when calculated using
793 *bamCoverage* from deepTools⁴⁷ (v. 2.5.3) with binsizes of 10 bp and filtered with
794 ENCODE project ChIP blacklist regions for mm10
795 (<https://www.encodeproject.org/annotations/ENCSR636HFF/>). Genome-
796 browser images were generated from the conversion of BAM files to TDF using
797 igvtools⁴⁸ (v. 2.3.95). Heatmap plots were generated using deepTools⁴⁷ over the
798 region, 5 kb upstream to 5 kb downstream of the genebody of all genes.
799 Coverage across the length of the genebody was scaled to 5 kb, and regions with
800 no coverage were excluded from the plot.

801

802 **qRT-PCR**

803 RNA from sgRNA positive cells 4-5 days post-transduction or cells treated with
804 WM-3835 for 6-12 h was extracted using the Qiagen RNAeasy kit. cDNA was
805 prepared using SuperScript VILO (Life Technologies) according to
806 manufacturer's instructions. Quantitative real-time PCR was performed on an
807 Applied Biosystems StepOnePlus using Fast SYBR green reagents (Thermo
808 Scientific). Expression levels were determined using the $\Delta\Delta C_t$ method
809 normalised to $\beta 2$ -microglobulin. All mRNA primer sequences are provided in
810 Supplementary Table 3.

811

812 **Cell Proliferation Assays**

813 Cells were seeded at a constant density prior to treatment in triplicate and
814 treated with either 1 μ M WM-3835, 1 μ M WM-2474 or DMSO (0.1%) over the
815 indicated time period. Drug was refreshed at least every two days. Cells were
816 stained with DAPI and live cell number was calculated using the BD FACSVerser
817 (BD Biosciences). To determine the IC₅₀ for the WM-3835, four hours after
818 seeding the cells at a constant density in duplicate, they were treated with WM-
819 3835, DMSO or positive control (3 μ M puromycin) for 10 days. Drug and media
820 were refreshed at day 4 and 7. At day 10, after incubating the cells with 600 μ M
821 of resazurin for 6 h, fluorescence was measured at λ_{ex} 530 nm and λ_{em} 590 nm,
822 using a Microplate Reader (EnSpire, Perkin Elmer). Relative fluorescence units
823 were converted to percent of inhibition relative to controls on the same plate
824 and the data fitted against a four-parameter logistic model to determine the 50%
825 inhibitory concentration (IC₅₀).

826

827 **Clonogenic Assays in Methylcellulose**

828 Clonogenic potential was assessed through colony growth of AML patient bone
829 marrow plated in cytokine-supplemented methylcellulose (MethoCult H4434,
830 Stemcell Technologies). Bone marrow was plated in duplicate at a cell dose of 2 X
831 10⁴ cells per plate in the presence of vehicle (0.1% DMSO) or 1 μ M WM-3835.
832 Cells were incubated at 37°C and 5% CO₂ for 12 days at which time colonies
833 were counted.

834

835 **Patient Material**

836 Bone marrow containing >80% blasts was obtained from patients following
837 consent and under full ethical approval by the Peter MacCallum Cancer Centre
838 Research Ethics Committee (Reference number: HREC/17/PMCC/69).

839

840 **Lysine acetyltransferase biochemical assays**

841 KAT enzymes were either produced or purchased as previously described²⁵.
842 Lysine acetyltransferase assays were run as described previously²⁵ with two
843 modifications. Firstly, 100 nM of full-length biotinylated histone H3 (for MOZ,
844 QKF, HBO1) or histone H4 (for KAT5, KAT8) proteins were used as the substrate,

845 as indicated. Secondly, assays were run with 1 μ M acetyl-coA concentration, the
846 approximate K_m for acetyl-coA for these enzymes in this assay format.

847

848 **HBO1 H3K14ac biomarker assay**

849 The cell line U2OS was seeded at a density of 3,000 cells per well in 384-well
850 optical quality tissue culture plates in RPMI medium supplemented with 10%
851 foetal bovine serum and 10 mM HEPES. The cells were allowed to adhere for 24
852 hours under standard culture conditions (37°C, 5% CO₂). At the end of this
853 period the cells were washed with medium. Compound dilutions prepared in
854 DMSO were added to the medium, with negative control wells reserved for
855 treatment with DMSO only and 100% inhibition positive controls at 10 μ M
856 concentration. After incubation for 24 hours, the cells were fixed with 4%
857 formaldehyde in PBS for 15 minutes at room temperature, washed with
858 phosphate buffer saline and blocked with blocking buffer containing 0.2%
859 TritonX100 and 2% BSA. Anti-H3K14ac antibody (Cell Signalling Technologies)
860 in blocking buffer was added and incubated overnight at 4°C. After washing, a
861 secondary antibody labelled with AlexaFluor 488 dye (ThermoFisher) and
862 Hoechst 33342 (1 μ g/mL, Life Technologies) were added for 2 hours incubation
863 at room temperature. Plates were washed and read on a PerkinElmer Opera HCS
864 high content imaging platform. Using a Columbus image analysis pipeline,
865 individual nuclei were located by Hoechst 33342 stain and the level of H3K14ac
866 was calculated from the AlexaFluor 488-related intensity in the same area. The
867 resulting mean intensity per cell was converted to percent inhibition relative to
868 controls on the same plate and the data fitted against a four-parameter logistic
869 model to determine the 50% inhibitory concentration (IC₅₀).

870

871 **HBO1-BPRF2 protein production, SPR, and structural biology**

872 HBO1-BPRF2 protein was produced as described previously⁴⁹. SPR for WM-3835
873 was done as described²⁵. HBO1-BPRF2 protein was produced as described
874 previously⁴⁹. SPR for WM-3835 was done as described²⁵. Crystals were grown at
875 the CSIRO C3 crystallisation centre in SD2 sitting drop plates at 20 °C with equal
876 volumes of protein and crystallant (200 nL plus 200 nL drops) with the reservoir
877 consisting of 244 mM diammonium tartrate and 20% PEG 3350. Crystals started
878 to form overnight and were harvested 3 days later using 20% glycerol as a
879 cryoprotectant. Data were obtained at the MX2 microfocus beamline at the
880 Australian Synchrotron. The space group was found to be H3 and the data and
881 refinement statistics can be found in Extended Data Fig. 6. The data were
882 indexed with DIALS⁵⁰ (WM-3835) or XDS⁵¹ (acetyl-CoA), scaled and integrated
883 with Aimless⁵², the structure was solved with Phaser⁵³ using PDB entry 5GK9 as
884 the initial model, manual refined with Coot⁵⁴ and full refinement was done with
885 Phenix.refine⁵⁵ (WM-3835) or REFMAC⁵⁶ (acetyl-CoA). Crystal structure data for
886 HBO1-BPRF2 in complex with WM-3835 and acetyl-CoA have been submitted to
887 the Protein Data Bank (PDB) under accession numbers 6MAJ (WM-3835) and

888 6MAK (acetyl-CoA). Crystallisation and refinement statistics are shown in
889 Extended Data Fig. 10.

890

891 ***In vitro* metabolic stability**

892 The metabolic stability assay was performed by incubating each test compound
893 in liver microsomes at 37 °C and a protein concentration of 0.4 mg/mL. The
894 metabolic reaction was initiated by the addition of either single cofactor (NADPH
895 only), or dual cofactors (NADPH and UDPGA), and quenched at various time
896 points over a 60-minute incubation period by the addition of acetonitrile
897 containing diazepam as internal standard. Control samples (containing no
898 NADPH) were included (and quenched at 2, 30 and 60 minutes) to monitor for
899 potential degradation in the absence of cofactor. The human liver microsomes
900 used in this experiment were supplied by XenoTech, lot # 1410230. The mouse
901 liver microsomes used in this experiment were supplied by XenoTech, lot #
902 1510256. Microsomal incubations were performed at a substrate concentration
903 of 1 µM.

904

905 **Code availability**

906 All code used in this study are publically available and are detailed in the method
907 section.

908

909 **Data availability**

910 The shRNA screen sequencing data have been deposited to the NCBI Sequence
911 Archive under the accession number GSE120813. Source Data are provided for
912 Figs 1, 2, 3, 4.

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914

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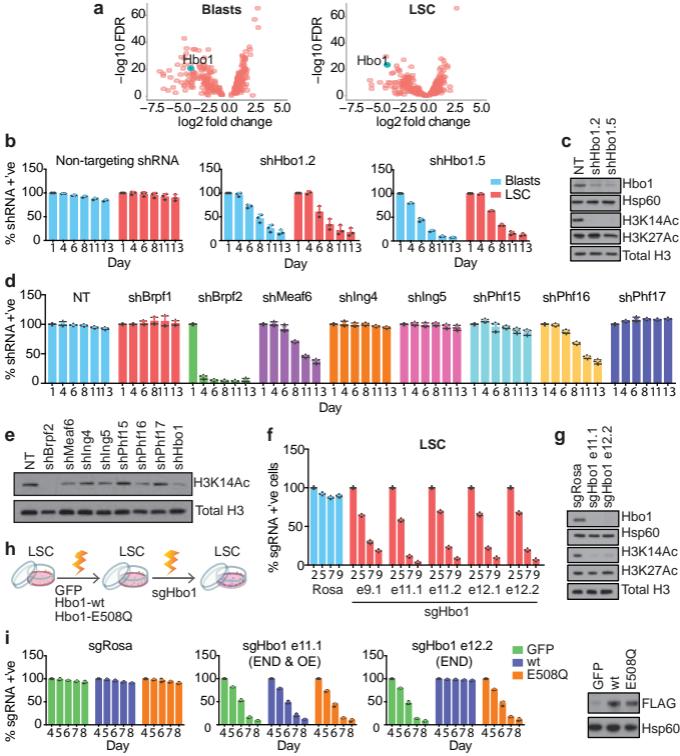
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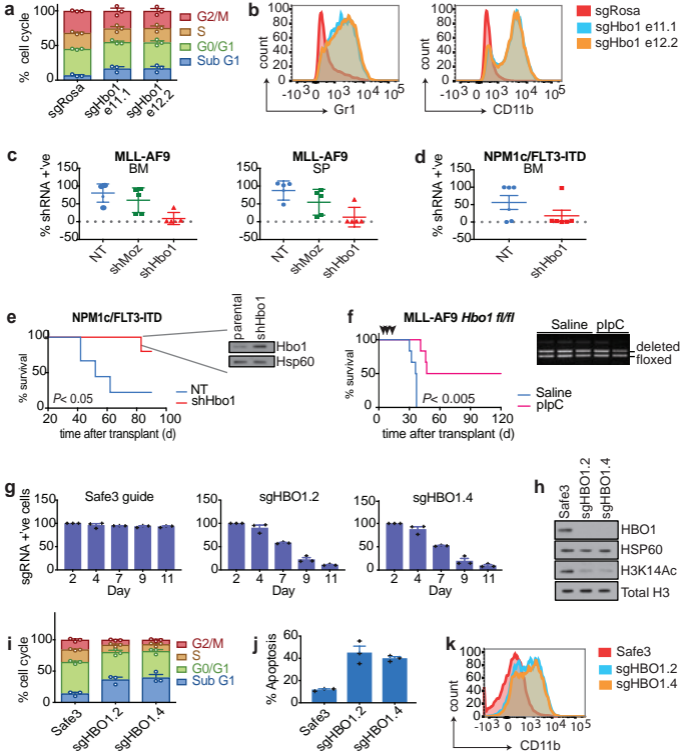
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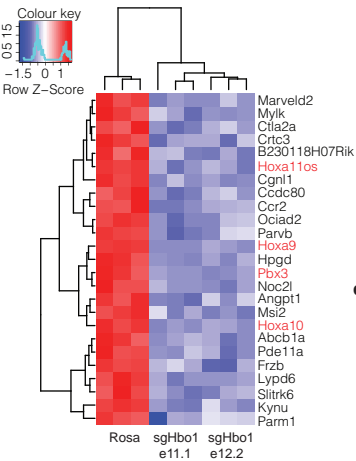
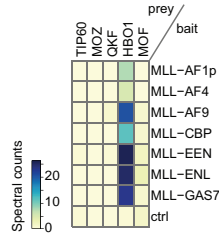
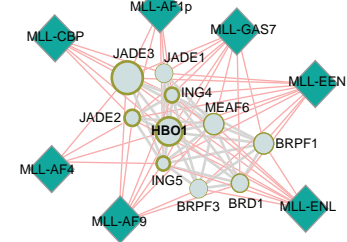
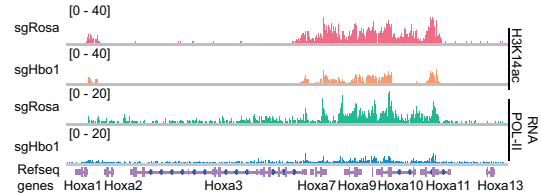
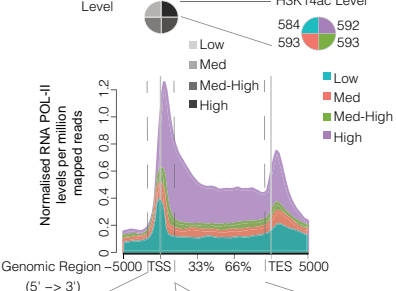
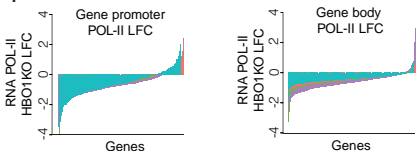
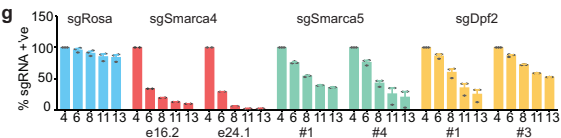
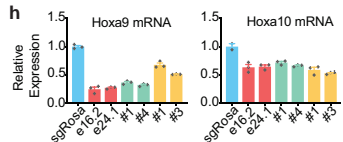
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a sgHbo1 top 25 down-regulated genes**b****c****d****e****f****g****h****i**