SUPPLEMENTARY APPENDIX

- **Supplementary Data**
- **Supplementary Discussion**
- **Supplementary References**
- **Supplementary Tables 1-2**
- **Supplementary Figures 1-3 and legend**

SUPPLEMENTARY DATA

Detailed clinical history of Case 1 developing cHL and AML

- May 2013: diagnosis of nodular sclerosis classic Hodgkin Lymphoma, stage IVB, EBV-negative.
- May 2013 – May 2014: As first-line therapy for cHL, the patient received 5 cycles of ABVD regimen (doxorubicin: 25 mg/m2, days 1, 15; bleomycin: 10 mg/m2, days 1, 15; vinblastine: 6 mg/m2, days 1, 15; dacarbazine: 375 mg/m2, days 1, 15)[1]. PET-CT carried out after two cycles documented partial remission; the fifth cycle was only partially administered due to stable disease revealed by PET-CT performed at the end of the fourth cycle. The patient then received 3 cycles of IGEV salvage regimen[2] (ifosfamide 2000 mg/m2 on days 1 to 4; gemcitabine 800 mg/m2 on days 1 and 4; vinorelbine 20 mg/m2 on day 1; prednisolone 100 mg on days 1 to 4), followed by collection of $10.9x10^6$/Kg CD34+ peripheral blood hematopoietic stem and progenitor cells (HSPCs) mobilized with G-CSF. The patient underwent autologous hematopoietic stem cell transplantation (ASCT) of $5.45x10^6$/Kg CD34+ HSPCs following the FEAM conditioning regimen (fotemustine 150 mg/m2 on days -7, -6, etoposide 200 mg/m2 and cytarabine 400 mg/m2 on days -5, - 4, -3, -2; melphalan 140 mg/m2 on day -1)[3]. G-CSF was administered at day +6 post-infusion and engraftment occurred at day +8 post-infusion. Toxicity included: grade 2 mucositis and an infectious episode at day +4. Mediastinal mass radiotherapy (total dose of 40 Gy) was performed after ASCT as consolidation. The patient achieved a complete remission of cHL by PET-CT, which was durable in the subsequent follow-up.
- February 2019: diagnosis of AML with normal karyotype carrying *NPM1*^{mut} and *FLT3*^{ITD} mutations, including a leukemia cutis presentation.

February – July 2019: AML induction therapy with "7+3" regimen (cytarabine 100 mg/m2 on days 1 to 7; daunorubicin 60 mg/m2 days 1, 2, 3). A complete remission was achieved. A consolidation course with high-dose cytarabine (cytarabine 1.5 g/m2 days 1, 3 and 5) plus midostaurin (50 mg orally twice daily, on days 8 through 21)[4] was then administered. Leukemia cutis relapsed about one month later and, despite subsequent treatment with etoposide (100 mg/m2, from days 1 to 7) and high-dose cytarabine (3 g/m2, days 1, 3, 5, 7), leukemic progression occurred followed by death shortly after.

SUPPLEMENTARY DISCUSSION

First description in cHL of mutations in epigenetic DNA-modifying genes

cHL Case 1, which was the only EBV+ case with extensive tissue CH in our cohort, carried the hotspot *DNMT3A* variant R882H and represents, to the best of our knowledge, the first description of mutant *DNMT3A* in the tumor cell clone of cHL.

In individuals with CHIP and in patients with AML, *DNMT3A* mutations at codon 882, which are the most prevalent *DNMT3A* mutations and likely result in loss-of-function[5-6], have been found in all blood cell lineages, including B cells, and are thus considered to occur in multipotent stem cells[5-9]. In mouse models conditionally knocking-out *Dnmt3a*[10-14] or knocking-in *Dnmt3a*^{R878H} (the murine homologue to human *DNMT3A*^{R882H})[15,16] in hematopoietic stem/progenitor cells, some biased differentiation potential through the B-cell lineage[10] and the development of mature B-cell tumors[13,14] have been reported.

However, in humans, mutations of *DNMT3A*, while relatively frequent in T-cell lymphomas, were very rarely reported in tumor cells of B-cell neoplasms, for example at frequencies <1%[17] and <3%[18] in diffuse large B-cell lymphoma (DLBCL) studied by WES (including 526/574 and 601/1001 cases, respectively, analyzed in the absence of matched non-neoplastic cells, whose sequencing could prevent the calling of CHIP-associated mutations). Intriguingly, in DLBCL presumably clonal *DNMT3A* mutations were found exclusively among EBV+ cases (no. 52 and 67 in ref. 19 and Table S9 therein) and were accompanied by mutations of the other epigenetic DNA modifier *TET2* in the absence of histone-modifying gene mutations. This genetic configuration is similar to our cHL Case 5, whose EBV-infected tumor cell clone also carried a non-sense *TET2* mutation (Q1274*) but had an otherwise almost null exome-wide somatic mutation burden (known to be low in EBV+ cHL[20]).

Considering that *Tet2* loss facilitates the neoplastic transformation of murine germinal center B cells[21], and that *TET2* mutations were observed more frequently in EBV+ than

EBV- DLBCL cases[19], it will be interesting to determine whether *DNMT3A* and *TET2* co-mutation may play a tumor-cell intrinsic role in the pathogenesis of some EBV+ Hodgkin (and non-Hodgkin) B cell lymphomas.

Post-therapy AML arising from a large pre-existing CH clone

Among myeloid neoplasms arising from CHIP after cytotoxic treatments, including ASCT for lymphoma, it is important to recognize the genetically distinct cases of *de novo* AML carrying *NPM1* mutations and normal karyotype (or anyway lacking therapy-related cytogenetic abnormalities) that can arise from a large pre-existing CHIP clone driven by *DNMT3A*, *TET2* and/or *ASXL1* mutations (this report and refs. 22, 23, 24). Such *de novo* AML may develop in a therapy-unrelated manner that depend just on the higher risk of neoplastic evolution intrinsic to a CHIP clone already considerably expanded (refs. 23, 25 and this report); or in a manner that may be influenced by the previous treatment but through mechanisms distinct from those at play in the more frequent and typical cases of therapy-related myeloid neoplasms. Indeed, the latter usually carry an abnormal karyotype (often complex and/or including loss of chromosome 5 and 7) and evolve from a frequently less extensive CHIP sustained by mutations disabling *TP53* and/or *PPM1D*, which confer resistance to cytotoxic treatments and are therefore often directly selected by therapy[24,26-29]. Although myeloablation during ASCT can promote the growth of pre-existing CHIP clones due to their higher fitness in reconstituting hematopoiesis[30], this phenomenon unlikely promoted AML development in our cHL patient (Case 1), as his CH was already massive before transplant. Anyway, further studies on larger number of cases are needed to better understand the specific pathogenetic mechanisms underlying the development of such atypical post-therapy myeloid neoplasms.

Finally, it is interesting to note that, in Case 1, AML developed from a massive CH (VAF 47%) 6 years after CH was identified in coincidence with cHL diagnosis at 45 years of age. Conversely, in another patient we recently described[23], AML developed just one year after identifying at the same age a similarly massive CH (VAF 49%) and an angioimmunoblastic T-cell lymphoma evolved from it. Such different lag time in AML development in these two patients, despite the very similar CH size identified at the same age, could be due at least in part to the fact that in the former case only one gene mutation underlay CH (*DNMT3A*^{R882H}), whereas in the latter case the CH clone originating AML had multiple mutations disrupting two genes[23,25, 31] (*TET2*, likely in a biallelic manner, and *ASXL1*[23]).

SUPPLEMENTARY REFERENCES

1. Canellos GP, Anderson JR, Propert KJ, Nissen N, Cooper MR, Henderson ES, et al. Chemotherapy of advanced Hodgkin's disease with MOPP, ABVD, or MOPP alternating with ABVD. *N Engl J Med*. 1992;327(21):1478-84.
2. Santoro A, Magagnoli M, Spina M, Pinotti G, Siracusano L, Michieli M, et al. Ifosfamide, gemcitabine, and vinorelbine: a new induction regimen for refractory and relapsed Hodgkin's lymphoma. *Haematologica*. 2007;92(1):35-41.
3. Musso M, Scalone R, Marcacci G, Lanza F, Di Renzo N, Cascavilla N, et al. Fotemustine plus etoposide, cytarabine and melphalan (FEAM) as a new conditioning regimen for lymphoma patients undergoing auto-SCT: a multicenter feasibility study. *Bone Marrow Transplant*. 2010;45(7):1147-1153.
4. Stone RM, Mandrekar SJ, Sanford BL, Laumann K, Geyer S, Bloomfield CD, et al. Midostaurin plus chemotherapy for acute myeloid leukemia with a FLT3 mutation. *N Engl J Med.* 2017;377(5):454-464.
5. Ley T, Ding L, Walter MJ, McLellan MD, Lamprecht T, Larson DE, et al. DNMT3A mutations in acute myeloid leukemia. *N Engl J Med*. 2010;363:2424-2433.
6. Russler-Germain Da, Spencer DH, Young MA, Lamprecht TL, Miller CA, Fulton R, et al. The R882H DNMT3A mutation associated with AML dominantly inhibits wild-type DNMT3A by blocking its ability to form active tetramers. *Cancer Cell*. 2014;25(4):442-54.
7. Buscarlet M, Provost S, Zada YF, Bourgoin V, Mollica L, Dubé MP, et al. Lineage restriction analyses in CHIP indicate myeloid bias for TET2 and multipotent stem cell origin for DNMT3A. *Blood*. 2018;132(3):277–280.
8. Buscarlet M, Provost S, Zada YF, Barhdadi A, Bourgoin V, Lépine G, et al. *DNMT3A* and *TET2* dominate clonal hematopoiesis and demonstrate benign phenotypes and different genetic predispositions. *Blood.* 2017;130(6):753-762.
9. Shlush LI, Zandi S, Mitchell A, Chen WC, Brandwein JM, Gupta V, et al. Identification of pre-leukaemic hematopoietic stem cells in acute leukaemia. *Nature.* 2014;20;506(7488):328-33.
10. Challen GA, Sun D, Jeong M, Luo M, Jelinek J, Berg JS, et al. Dnmt3a is essential for hematopoietic stem cell differentiation. *Nat Genet*. 2011;44(1):23-3.
11. Mayle A, Yang L, Rodriguez B, Zhou T, Chang E, Curry CV, et al. Dnmt3a loss predisposes murine hematopoietic stem cells to malignant transformation*. Blood.* 2015;125(4):629-638.
12. Celik H, Mallaney C, Kothari A, Ostrander EL, Eultgen E, Martens A, et al. Enforced differentiation of Dnmt3a-null bone marrow leads to failure with c-kit mutations driving leukemic transformation. *Blood*. 2015;125(4):619-28.
13. Peters SL, Hlady RA, Opavska J, Klinkebiel D, Pirruccello SJ, Talmon GA, et al. Tumor suppressor functions of Dnmt3a and Dnmt3b in the prevention of malignant mouse lymphopoiesis. *Leukemia*. 2014;28(5):1138-42.
14. Haney SL, Upchurch GM, Opavska J, Klinkebiel D, Kusi Appiah A, Smith LM, et al. Loss of Dnmt3a induces CLL and PTCL with distinct methylomes and transcriptomes in mice. *Sci Rep*. 2016;28;6:34222.
15. Guryanova OA, Shank K, Spitzer B, Luciani L, Koche RP, Garrett-Bakelman FE, et al. DNMT3A mutations promote anthracycline resistance in acute myeloid leukemia via impaired nucleosome remodeling. *Nat Med*. 2016;22(12):1488-1495.
16. Loberg MA, Bell RK, Goowing LO, Eudy E, Miles LA, SanMiguel JM, et al. Sequentially inducible mouse models reveal that NPM1 mutation causes malignant transformation of DNMT3a-mutant clonal hematopoiesis. *Leukemia*. 2019;33(7):1635-1649.
17. Schmitz R, Wright GW, Huang DW, Johnson CA, Phelan JD, Wang JQ, et al. Genetics and pathogenesis of diffuse large B-cell lymphoma. *N Engl J Med*.

2018;378(15):1396-1407.
18. Reddy A, Zhang J, Davis NS, Moffitt AB, Love CL, Waldrop A, et al. Genetic and functional drivers of diffuse large B-cell lymphoma. *Cell*. 2017;171(2):481-494.
19. Kataoka K, Miyoishi H, Sakata S, Dobashi A, Couronné L, Kogure Y. et al. Frequent structural variations involving programmed death ligands in Epstein-Barr virus-associated lymphomas. *Leukemia*. 2019:33(7):1687-1699.
20. Tiacci E, Ladewig E, Schiavoni G, Penson A, Fortini E, Pettirossi V, et al. Pervasive mutations of JAK-STAT pathway genes in classical Hodgkin lymphoma. *Blood.* 2018;131(22):2454-2465.
21. Dominguez PM, Ghamlouch H, Rosikiewicz W, Kumar P, Béguelin W, Fontán L, et al. TET2 deficiency causes germinal center hyperplasia, impairs plasma cell differentiation, and promotes B-cell lymphomagenesis. *Cancer Discov*. 2018;8(12):1632-1653.
22. Desai P, Mencia-Trinchant N, Savenkov O, Simon MS, Cheang G, Lee S, et al. Somatic mutations precede acute myeloid leukemia years before diagnosis. *Nat Med*. 2018;24(7):1015-1023.
23. Tiacci E, Venanzi A, Ascani S, Marra A, Cardinali V, Martino G, et al. High-Risk Clonal Hematopoiesis as the Origin of AITL and NPM1-Mutated AML. *N Engl J Med.* 2018;379(10):981- 984.
24. Young AL, Spencer Tong R, Birmann BM, Druley TE. Clonal hematopoiesis and risk of acute myeloid leukemia*. Haematologica.* 2019;104(12):2410-2417.
25. Tuval A, Shlush LI. Evolutionary trajectory of leukemic clones and its clinical implications. *Haematologica.* 2019;104(5):872-880
26. Gibson CJ, Lindsley RC, Tchekmedyian V, Mar BG, Shi J, Jaiswal S, et al. Clonal hematopoiesis associated with adverse outcomes after autologous stem-cell transplantation for lymphoma. *J Clin Oncol*. 2017;35(14):1598-1605.
27. Wong TN, Ramsingh G, Young AL, Miller CA, Touma W, Welch JS, et al. Role of TP53 mutations in the origin and evolution of therapy-related acute myeloid leukemia. *Nature*. 2014;518(7540):552- 555.
28. Wong TN, Miller CA, Jotte M, Bagegni N, Baty J, Schmidt A, et al. Cellular stressors contribute to the expansion of hematopoietic clones of varying leukemic potential. Nat Commun. 2018;9(1):455.
29. Husby S, Favero F, Nielsen C, Sørensen BS, Bæch J, Grell K, et al. Clinical impact of clonal hematopoiesis in patients with lymphoma undergoing ASCT: a national population-based cohort study. *Leukemia*. 2020;34(12):3256-3268.
30. Ortmann CA, Dorsheimer L, Abou-El-Ardat K, Hoffrichter J, Assmus B, Bonig H, et al. Functional dominance of CHIP- mutated hematopoietic stem cells in patients undergoing autologous transplantation. *Cell Rep*. 2019;27(7):2022-2028.
31. Abelson S, Collord G, Ng SWK, Wiessbrod O, Mendelson Cohen N, Niemeyer E, et al. Prediction of acute myeloid leukemia risk in healthy individuals. *Nature*. 2018;559(7714):400-404.

Supplementary Table 1. Genes subjected to targeted deep sequencing

Table S1A: QIAseq Targeted DNA Custom panel-QIAGEN (CDHS: 13640Z-1040)

Gene	Targeted exon(s)
ASXL1	12
BCOR	All exons
BCORL1	All exons
BRAF	15
CALR	9
CBL	8-9
CEBPA	All exons
CSF3R	10,14-18
DNMT3A	All exons
EZH2	All exons
FLT3	14, 15, 20
GATA1	2
GATA2	4-6
HRAS	2.3
IDH1	4
IDH2	4
JAK2	12.14
JAK3	13
KDM6A	14
KMT2A	1,3,5-8,27
KIT	2, 8-11, 13, 17
KRAS	2, 3
MPL	10
NPM1	12
NRAS	2, 3
PDGFRA	12, 14, 18
PHF6	All exons
PPM1D	5-6
PTEN	5, 7
PTPN11	3,13
RAD21	All exons
RUNX1	1-6
SETBP1	4 (partial)
SF3B1	13-16
SMC1A	2, 11, 16, 17
SMC3	10, 13, 19, 23, 25, 28
SRSF2	1
STAG2	All exons
TET2	3-11
TP53	2-11
U2AF1	2, 6
ZRSR2	All exons
WT1	7.9

Table S1B: QIAseq Targeted DNA Custom panel-QIAGEN (CDHS: 16895Z-163)

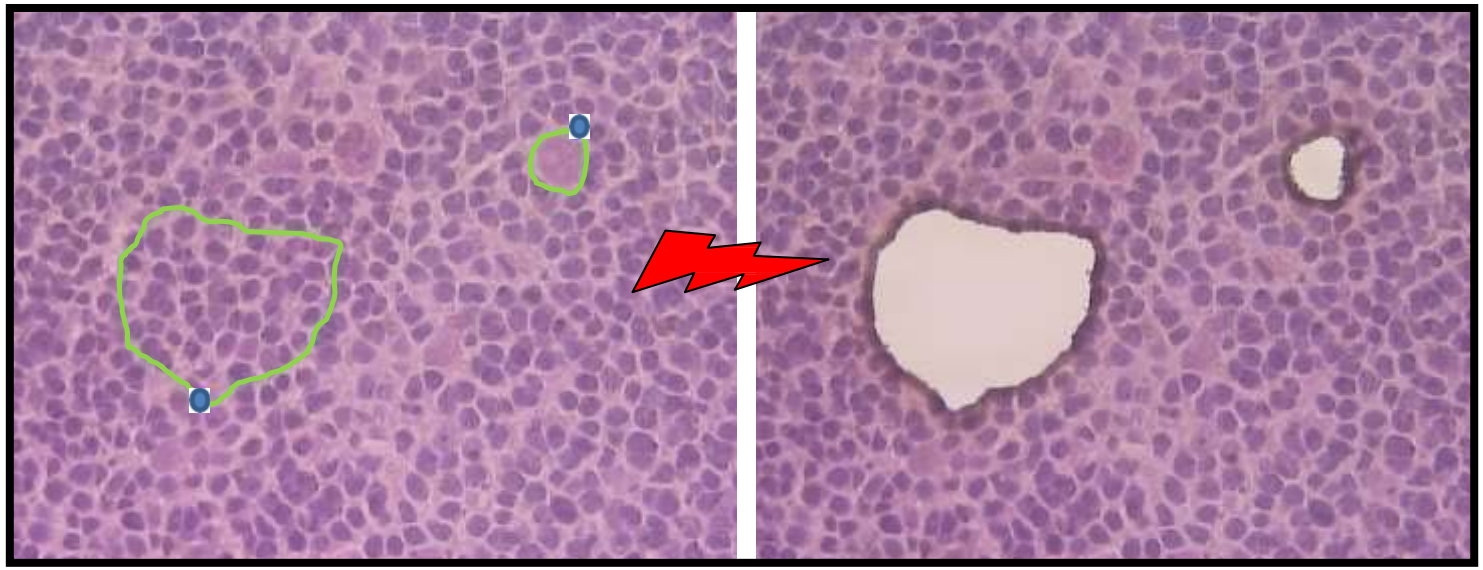
Gene	Targeted exon(s)
JAK1	14-26
STAT3	10-19, 20-21
STAT5b	9-14, 15-16
STAT6	9-14, 15-16
SOCS1	All exons
PTPN1	All exons

Supplementary Table 2. CHIP-associated mutations observed by NGS in 40 cHL patients

									WHOLE EXOME SEQUENCING					BARCODED TARGETED SEQUENCING				AML onset	AML remission	
									Variant allele frequency (Variant/Total Reads)					Variant allele frequency (Variant/Total Reads)				BARCODED TARGETED SEQUENCING		
																		Variant allele frequency (Variant/Total Reads)		
									Blood	Microdissected lymphoid cells				Blood	Microdissected lymphoid cells		Whole lymph node sections			
										Reactive		Neoplastic			Reactive	Neoplastic				
Pt. #	Age	Time of sampling	NGS technique	Gene	Chr	Coordinate	HGVS coding sequence name	HGVS protein sequence name		Replicate 1	Replicate 2	Replicate 1	Replicate 2		Pooled replicates	Pooled replicates		Bone marrow	Blood B cells	Blood T cells
Case 1	45	cHL Onset	Targeted	DNMT3A	2	25457242	NM_175629.2:c.2645G>A	NP_783328.1:p.Arg882His	-	-	-	-	-	47% (475/1010)	16.4 % (687/4193)	NC	12.2% (506/4152)	45.2% (509/1127)	48% (274/571)	6.9% (109/1584)
				NPM1	5	170837543	NM_002520.6:c.859_860insTCTG	NP_002511.1:p.Trp288CysfsTer12	-	-	-	-	-	NC	NC	NC	NC	40.6% (152/374)	NC	NC
				PTPN11	12	112888210	NM_002834.3:c.226G>A	NP_002825.3:p.Glu76Lys	-	-	-	-	-					25.7% (143/556)		
				STAT6	12	57496668	NM_003153.4:c.1249A>T	NP_003144.3:p.Asn417Tyr	-	-	-	-	-	NA	NC	36.9% (240/651)	NA	NA	NA	NA
				STAT6	12	57496662	NM_003153.4:c.1255G>C	NP_003144.3:p.Asp419His	-	-	-	-	-			35.7% (257/720)				
				SOCS1	16	11349064	NM_003745.1:c.246_271delGCCCCTGAGCGTGCACGGGGCGCACG	NP_003736.1:p.Pro83AlafsTer25	-	-	-	-	-			98.6% (72/73)*				
				PPM1D	17	58740593	NM_003620.3:c.1498C>T	NP_003611.1:p.Pro500Ser	-	-	-	-	-	NC		13.9% (1128/8124)	NC	NC	NC	NC
Case 2	30	2nd relapse	Whole-exome & targeted	KRAS	12	25380279	NM_033360.2:c.179G>A	NP_203524.1:p.Gly60Asp	NA	60.3% (35/58)	35.1% (27/77)	NC	NC	NA	NA	NA	22.9% (2243/10546)			
Case 3	83	Onset	Targeted	CBL	11	119148903	NM_005188.3:c.1123G>A	NP_005179.2:p.Gly375Ser	NA	NA				NA	2.5% (321/13420)	NC	NA			
Case 4	81	Onset	Whole-exome & targeted	TET2	4	106193995	NM_001127208.2:c.4460delA	NP_001120680.1:p.Asn1487IlefsTer84	3.2% (8/247)	-	-	NC	NC	2.6 % (51/2002)	NC					
Case 5	68	Onset	Targeted	DNMT3A	2	25457242	NM_175629:c.2645G>A	NP_783328.1:p.Arg882His	NA	NA				NA	NA	NA	32.4% (2524/7797)			
			Targeted	TET2	4	106180792	NM_001127208.2:c.3820C>T	NP_001120680.1:p.Gln1274Ter		NA							22.3% (1293/5799)			
	73	1st relapse	Whole-exome & targeted	DNMT3A	2	25457242	NM_175629:c.2645G>A	NP_783328.1:p.Arg882His		32.6% (30/92)	26.2% (16/61)	56.1% (55/98)	33.3% (44/132)	NA	30.6% (2560/8374)	37.3% (4359/11702)	37.9% (1492 /3934)			
			Whole-exome & targeted	TET2	4	106180792	NM_001127208.2:c.3820C>T	NP_001120680.1:p.Gln1274Ter		5% (7/141)	13.3% (13/98)	29.9% (40/134)	32.7% (64/104)		10.6% (431/4052)	33% (1671/5062)	26.9% (614/2287)			

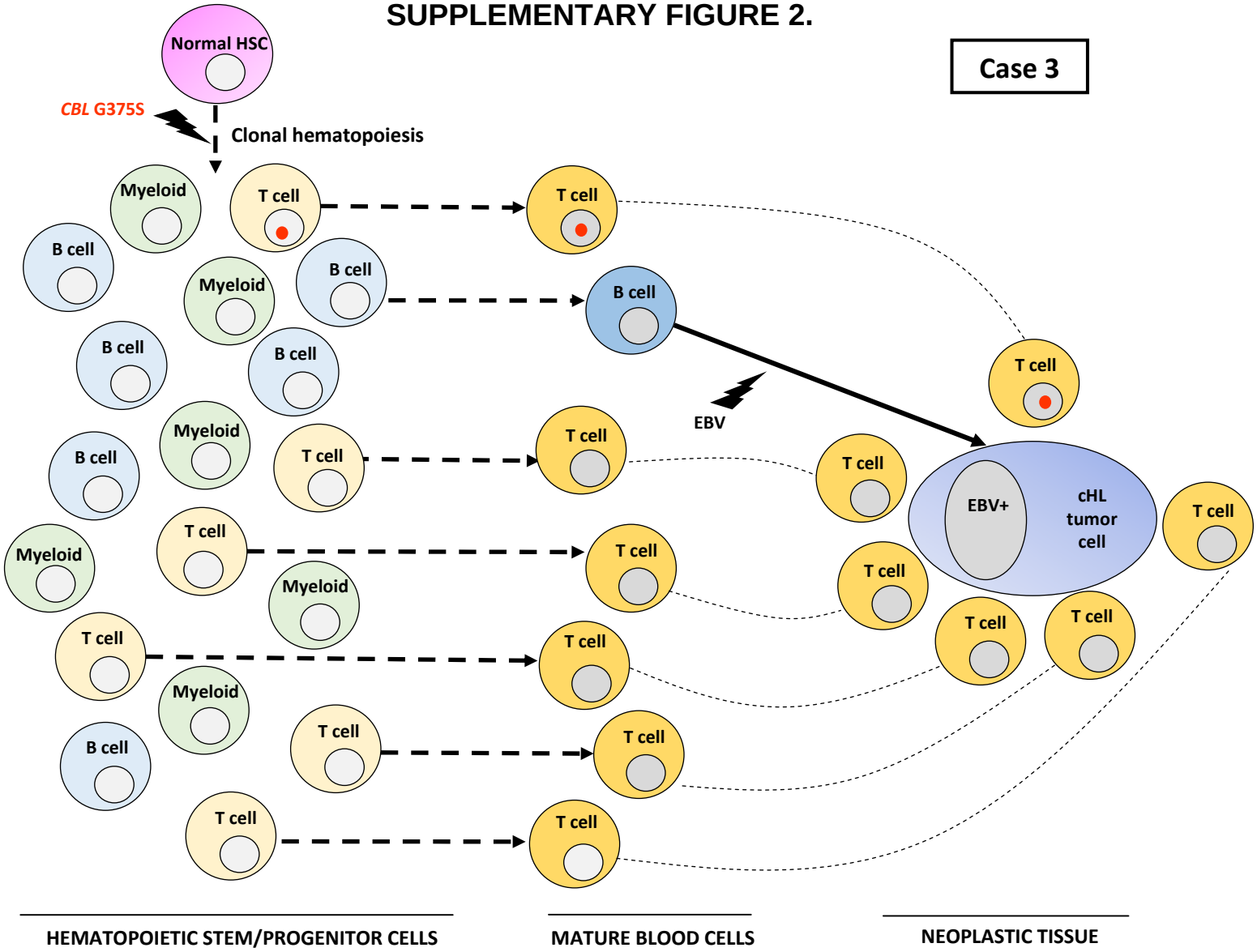
NA, sample not available
-, sample not analyzed
NC, variant not called
* Only one of the WGA replicates was evaluable (the coverage was too low in the other, but the mutation was then confirmed by Sanger Sequencing also in that replicate)

SUPPLEMENTARY FIGURE 1.



Microdissection of HRS cells and reactive cells from the cHL tissue.
(*Left panel*) Representative image of a frozen lymph node section stained with hematoxylin and eosin, showing one large HRS cell with its clear morphology (*left panel, right green circle*) among numerous small reactive cells, mostly of lymphoid morphology. A green line was drawn around both the HRS cell and reactive cells, to direct their laser cutting and subsequent catapulting (*red lightning*) out of the section (*right panel*).

SUPPLEMENTARY FIGURE 2.



Tissue involvement by clonal hematopoiesis (CH) in cHL Case 3.

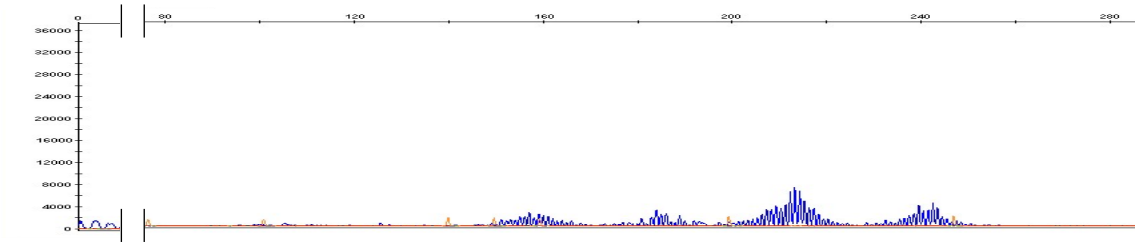
In this EBV-positive mixed cellularity cHL case, the reactive tissue microenvironment (abundant in T cells - not shown) showed the presence of a small clone featuring a *CBL*^{G375S} mutation at a VAF of 2.5%. The HRS cell clone developed from an EBV-infected B cell not belonging to the *CBL*-mutant reactive clone. Because we could not analyze samples from patient blood or bone marrow, the presence and cell lineage involvement of CH in these latter tissues as shown in the figure are hypothetical.

SUPPLEMENTARY FIGURE 3

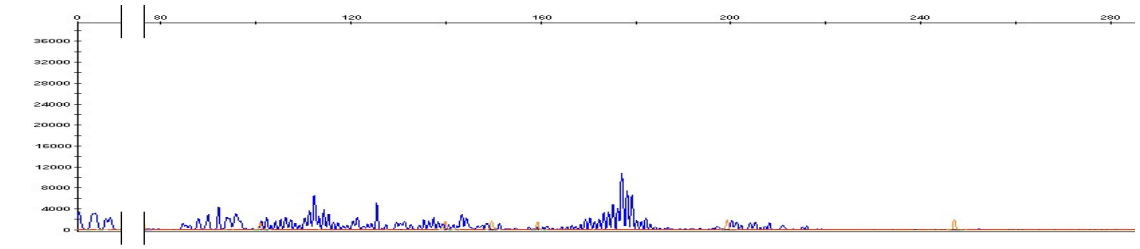
A

Case 1

VJ-A



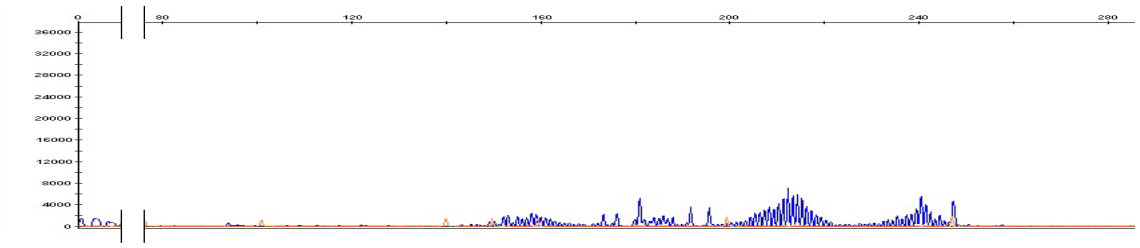
VJ-B



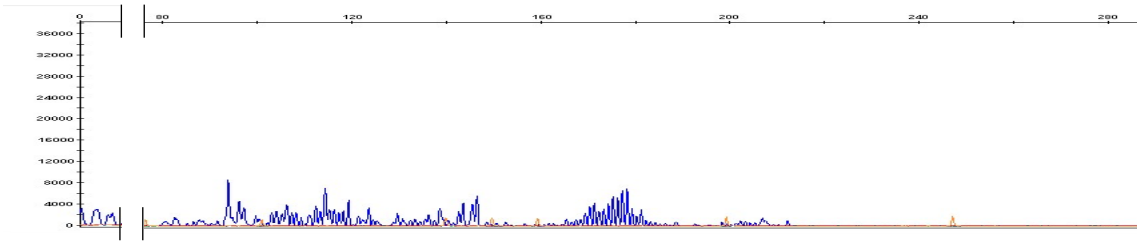
B

Case 3

VJ-A



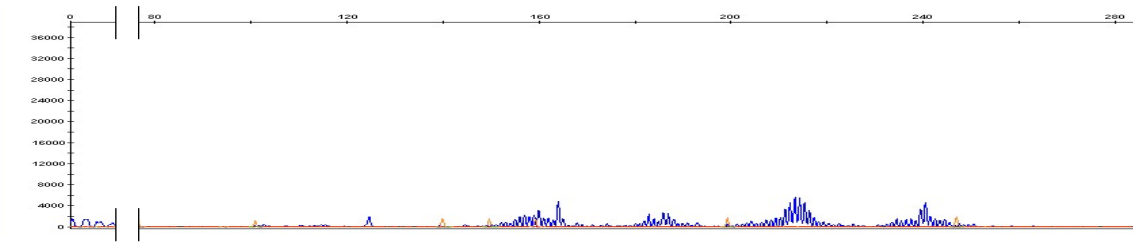
VJ-B



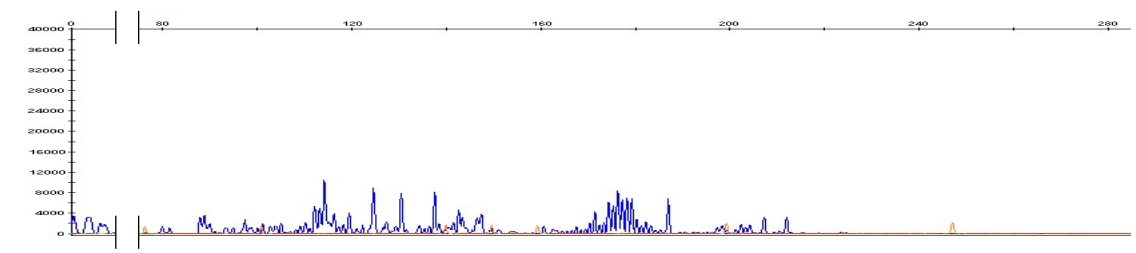
C

Case 5

VJ-A



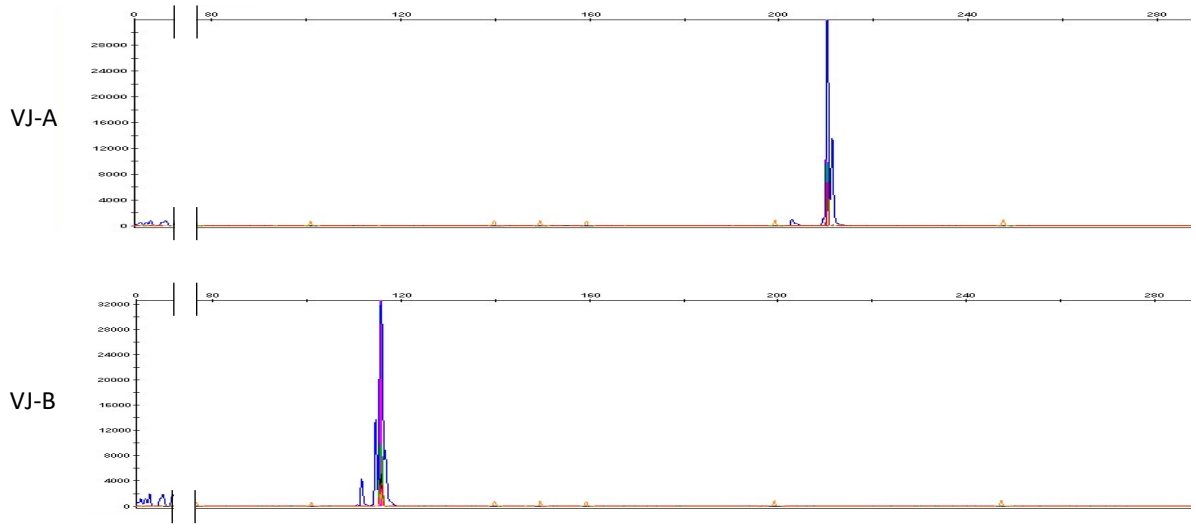
VJ-B



SUPPLEMENTARY FIGURE 3

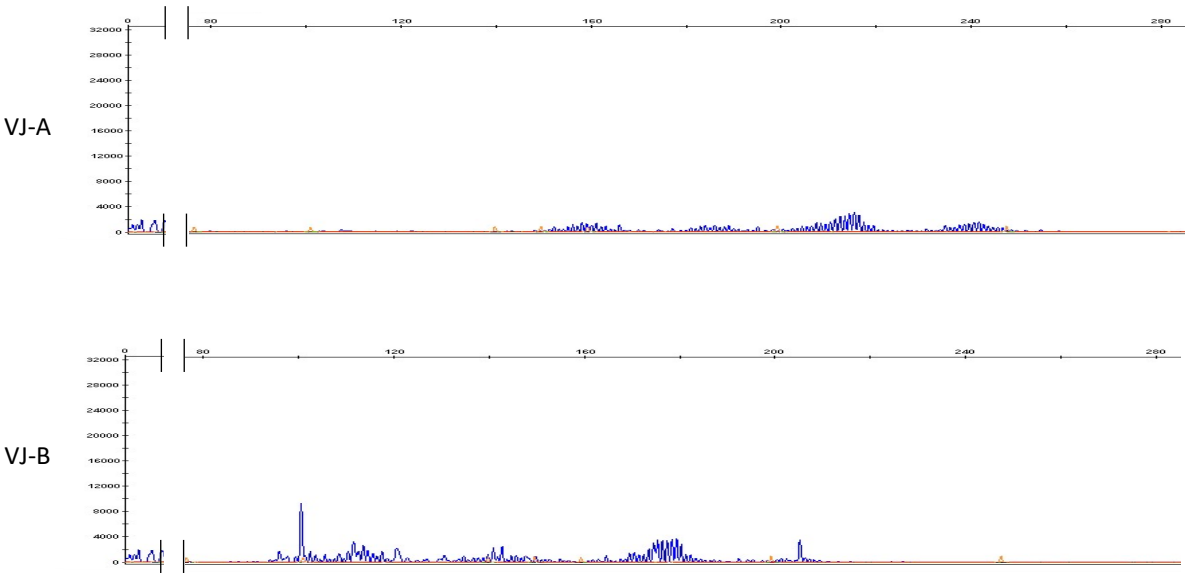
D

TCR monoclonal control



E

TCR polyclonal control



SUPPLEMENTARY FIGURE 3. T-cell receptor gamma gene rearrangement analysis in cHL tissues with extensive clonal hematopoiesis (CH).
In all three cHL cases with significant tissue CH (VAF >10%), fragment length analysis of whole sections showed polyclonal T-cell receptor gamma gene rearrangements (panels A-C). In panel D and E, monoclonal and polyclonal controls are shown, respectively. This result implies that CH-associated mutations detected in microdissected non-neoplastic cells (mainly of mature lymphoid morphology and of T-cell phenotype – representative example in Figures 2C and S1) had occurred in a bone marrow stem or progenitor cell before T-cell receptor gene rearrangement.