Classical Hodgkin's lymphoma shows epigenetic features of abortive plasma cell differentiation

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Citation: Seitz V, Thomas PE, Zimmermann K, Paul U, Ehlers A, Joosten M, Dimitrova L, Lenze D, Sommerfeld A, Oker E, Leser U, Stein H, and Hummel M. Classical Hodgkin's lymphoma shows epigenetic features of abortive plasma cell differentiation. Haematologica 2011;96(6):863-870. doi:10.3324/haematol.2010.031138

Online Supplementary Protocol S1

Chromatin immunoprecipitation with cultured suspension cells

I. Formaldehyde cross-linking of cells

- 1×10^8 cells were used for each immunoprecipitation;
- Cells were fixed with 4% formaldehyde solution for 20 min at room temperature. Fixation was stopped with 0.25 M glycine;
- Cells were washed three times with phosphate-buffered saline (4° C) and cell pellets were stored at -80° C in polypropylene tubes.

II. Preblock and binding of antibody to magnetic beads

- 50 µL Dynal magnetic beads coated with Protein A and 50 µL Dynal magnetic beads coated with Protein G were mixed and washed three times with 1 mL blocking solution (phosphate-buffered saline containing 0.5% bovine serum albumin);
- Beads were mixed with 10 µg of anti-acetyl histone H3 (Lys9 + Lys14) (06-599) (Upstate) in 250 µL blocking solution;
- Beads and antibody were incubated overnight under rotation at 4°C;
- Beads-antibody-complexes were washed three times with blocking solution and resuspended in 100 µL blocking solution.

III. Cell lysis and sonication

- Cell pellets were resuspended in 5 mL lysis buffer 1 (50 mM Hepes-KOH, pH 7.5; 140 mM NaCl; 1 mM EDTA; 10% glycerol; 0.5% NP-40; 0.25% TritonX-100) containing freshly added protease inhibitors (Complete, Roche), incubated at 4°C for 10 min and centrifuged at 1800 rpm for 5 min at 4 °C;
- Pellets were resuspended in 5 mL lysis buffer 2 (10 mM Tris-HCl, pH 8.0; 200 mM NaCl; 1 mM EDTA; 0.5 mM EGTA) containing freshly added protease inhibitors (Complete, Roche), incubated at room temperature for 10 min and spun down at 1800 rpm for 5 min at 4 °C;
- Pellets were resuspended in 3 mL lysis buffer 3 (10 mM Tris-HCl, pH 8.0; 100 mM NaCl; 1 mM EDTA; 0.5 mM EGTA; 0.1% Na-deoxycholate; 0.5% N-lauroylsarcosine) containing freshly added protease inhibitors (Complete, Roche);
- 2x 1.5 mL of the samples were separated into 2x 15 mL polypropylene tubes and sonicated with the Bioruptor employing Titan sticks (Diagenode, Liége, Belgium) for 3 x 15 min (changing the ice after each sonication round) at high power in pulsed mode (30 sec on, 30 sec off);
- 300 µL of 10% Triton X-100 were added to the pooled 2 x 1.5 mL sample;
- Centrifugation at 13000 rpm for 10 min at 4 °C to pellet debris;
- 50 µL from the supernatant of each sample were saved as input DNA (negative control) and stored at -20 °C. Furthermore, 50 µL from the supernatant were used to analyze the success of DNA sonication by gel analysis according to Young's protocol using 3 µg DNA;[1]
- Remaining supernatant was used for immunoprecipitation.

IV. Chromatin immunoprecipitation

- Beads-antibody-complexes were mixed with the supernatant samples and incubated overnight under rotation at 4 °C.

V. Washing, elution, and cross-link reversal

- Beads were separated from the supernatant by magnetic force;
- Beads were washed with RIPA buffer (50 mM Hepes-KOH, pH 7.6; 500 mM LiCl; 1 mM EDTA; 1% NP-40; 0.7% Na-deoxycholate) containing freshly added protease inhibitors (Complete, Roche) for four times;
- Beads were washed with TE containing 50 mM NaCl and freshly added protease inhibitors (Complete, Roche);
- Beads were incubated with 210 µL elution buffer (50 mM Tris-HCl, pH 8.0; 10 mM EDTA; 1% SDS) at 65 °C for 15 min and resuspended by vortexing every 2 min;
- Beads were spun down for 1 min at room temperature and supernatant with DNA was incubated at 65 °C overnight to reverse the cross-linking of the DNA. The 50 µL input DNA (negative control) was also dissolved in 150 µL elution buffer and incubated at 65 °C overnight.

VI. Digestion of cellular protein and RNA

- Samples were incubated with 200 µL 1 x TE buffer with freshly added 0.25 µg/mL RNaseA at 37°C for 2 h.
- Samples were incubated with proteinase K (0.2 µg/mL) at 55°C for 2 h;

- DNA was purified by phenol/chloroform extraction and precipitated by ethanol;
- Pellets were resuspended in 70 μL of 10 mM Tris-HCl, pH 8.0.

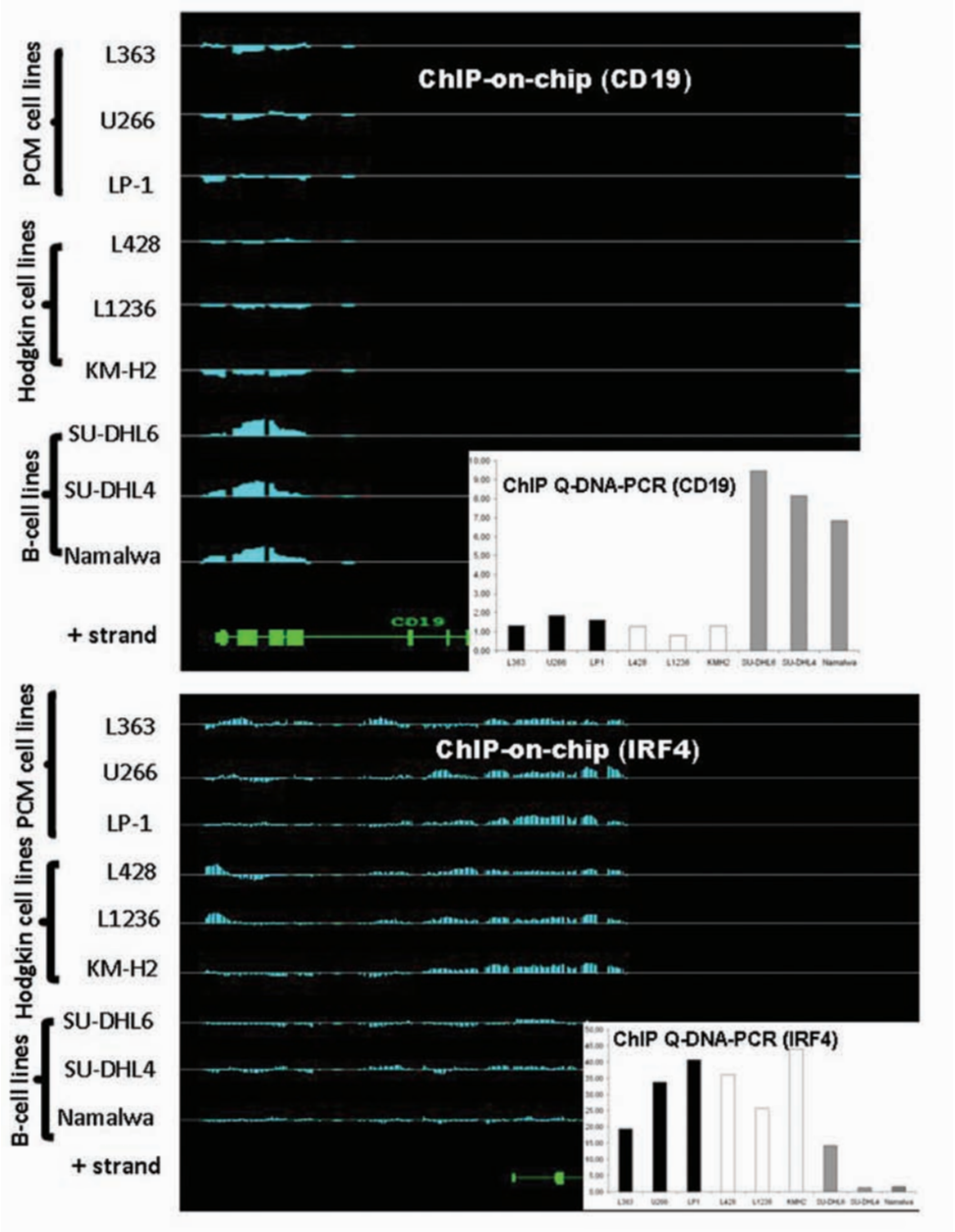
"Small scale" protocol for H3K27 trimethylation ChiP analysis

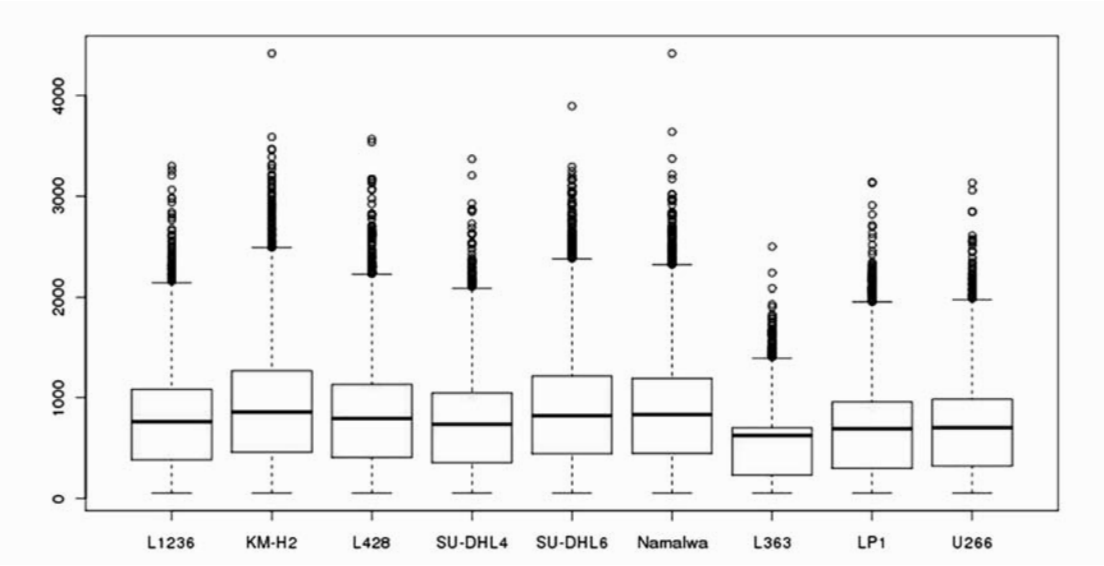
H3K27 trimethylation was analyzed by chromatin immunoprecipitation (ChIP) as described above, for selected genes in the three Hodgkin's lymphoma cell lines and one B-cell lymphoma cell line (Namalwa), employing anti-H3K27 trimethylation antibody (07-499, Millipore). However, instead of performing the ChIP in a 3 mL volume we worked with three volumes of 1 mL in a small scale approach. For each tube of 1 mL we used approximately the same amount of antibody coated G-beads originally recommended in Young's protocol for the large scale approach. Therefore, 100 μL beads were covalently linked overnight with 15 -20 μg antibody. After the washing steps the antibody-coated beads were resolved in 100 μL blocking solution.

Reference

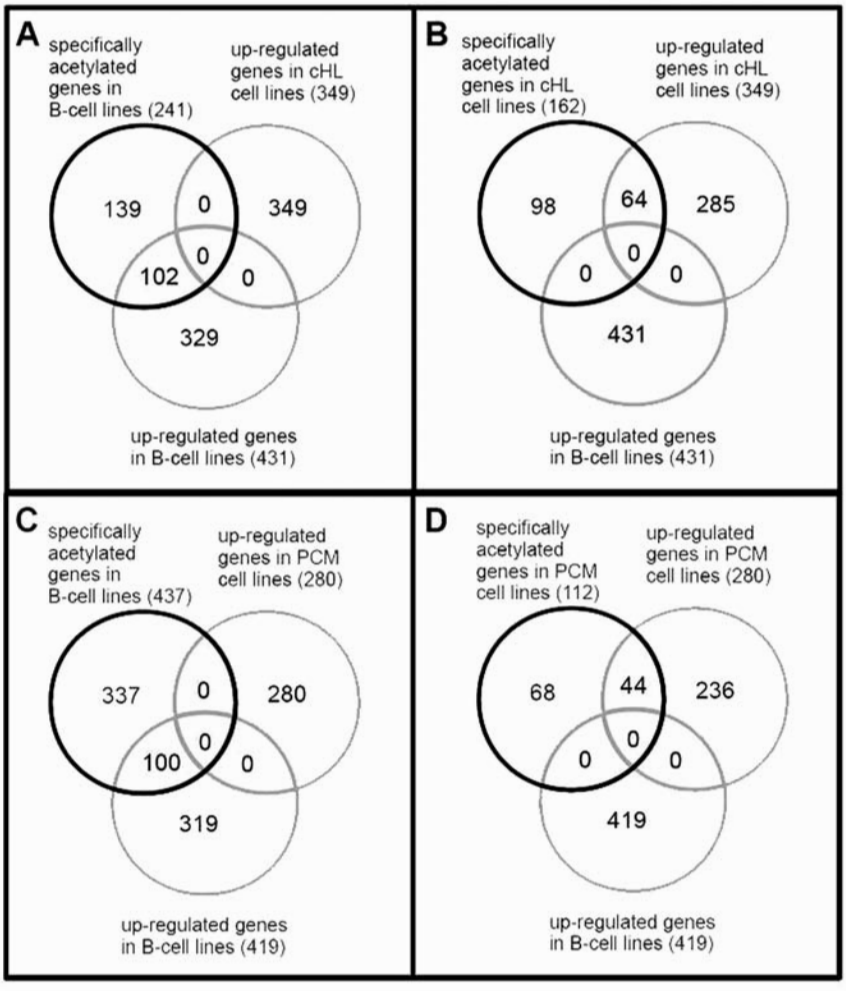
1. Lee TI, Johnstone SE, Young RA. Chromatin immunoprecipitation and microarray-based analysis of protein location. Nat Protoc. 2006;1(2):729-48.

Online Supplementary Figure S1. Exemplary validation of the ChIP-on-chip derived genomic intervals for *IRF4* and *CD19* by the Integrated Genomic Browser (IGB, Affymetrix) and by real-time DNA-PCR (inserts).



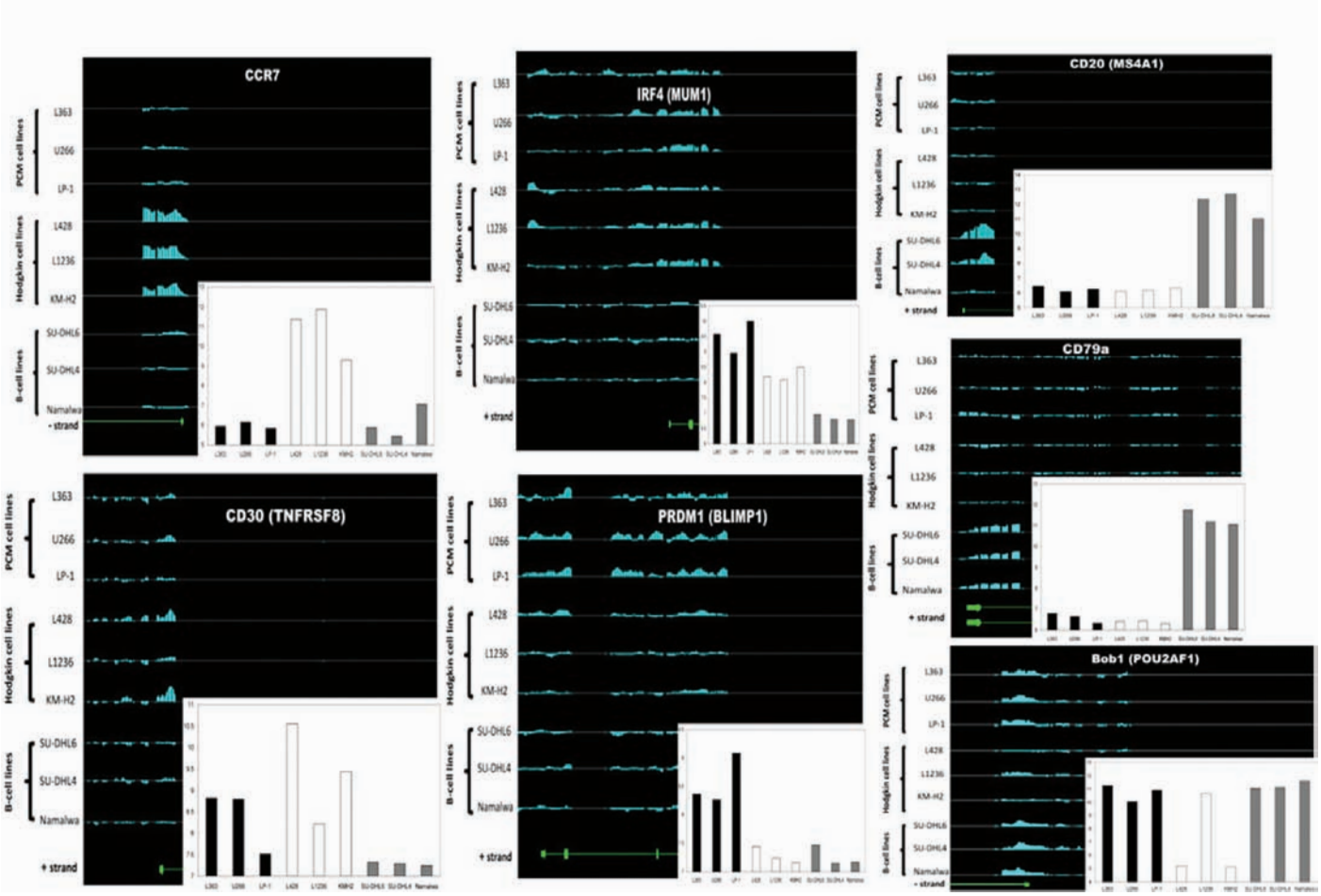


Online Supplementary Figure S2. Length of the most significant acetylated regions used for the analysis depicted as boxplots for each cell line after analysis employing the MAT algorithm. The y-axis shows the length of acetylated regions in base pairs (bp). The interquartile range of the length of this acetylated region is similar between the cell lines.

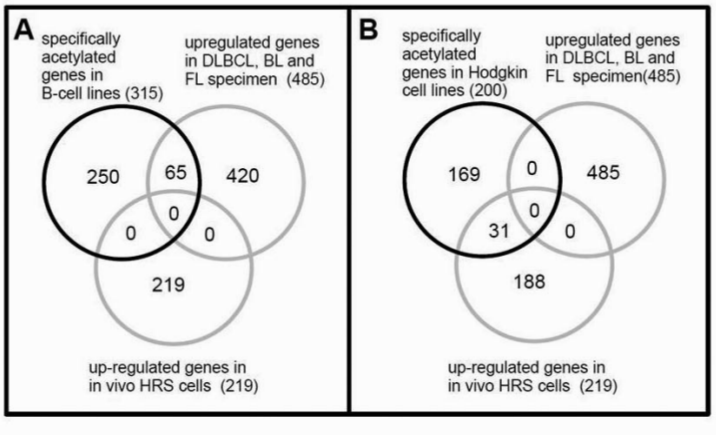


Online Supplementary Figure S3. Venn diagrams for comparison of acetylation (black circle) and gene expression (gray circles) data. A and B: Comparison of classical Hodgkin's lymphoma (cHL) and B-cell lines. C and D: Comparison of plasma cell myeloma (PCM) and B-cell lines. (A) An overlap of 102 genes (P=1.295205e-58) was found when the 431 genes up-regulated in B-cell lines and the 241 genes specifically acetylated in B-cell lines were compared. None of the 349 genes up-regulated in cHL cell lines was acetylated in B-cell lines. (B) An overlap of 64 genes (P=6.881182e-42) was found when the 349 genes up-regulated in cHL cell lines and the 162 genes specifically acetylated in cHL cell lines were compared. None of the 431 genes up-regulated in B-cell lines was acetylated in cHL cell lines. Note that only 241 of the 327 differentially acetylated B-cell genes ($B1_{Ac}$) and 162 of the 211 differentially acetylated cHL genes (Figure 2) were covered by the U133A Affymetrix GeneChips and thus used for comparison. Furthermore all 431 genes significantly expressed in B-cell lines and all 349 genes significantly expressed in cHL cell lines were covered by the GeneChip® Human Promoter 1.0R Tiling Array. (C) An overlap of 100 genes (P=1.789886e-38) was found when the 419 genes up-regulated in B-cell lines and the 437 genes specifically acetylated in B-cell lines were compared. None of the 280 genes up-regulated in PCM cell lines was acetylated in B-cell lines. (D) An overlap of 44 genes (P=2.434177e-33) was found when the 280 genes up-regulated in PCM cell lines and the 112 genes specifically acetylated in PCM cells were compared. None of the 419 genes up-regulated in B-cell lines was acetylated in PCM cell lines. Note that only 437 of the 591 differentially acetylated B-cell genes ($B2_{Ac}$) and 112 of the 143 differentially acetylated genes in PCM cell lines (Figure 2) were covered by the U133A Affymetrix GeneChips and thus used for comparison. Furthermore all 419 genes significantly expressed in B-cell lines and all 280 genes significantly expressed in PCM cell lines were covered by the GeneChip® Human Promoter 1.0R Tiling Array.

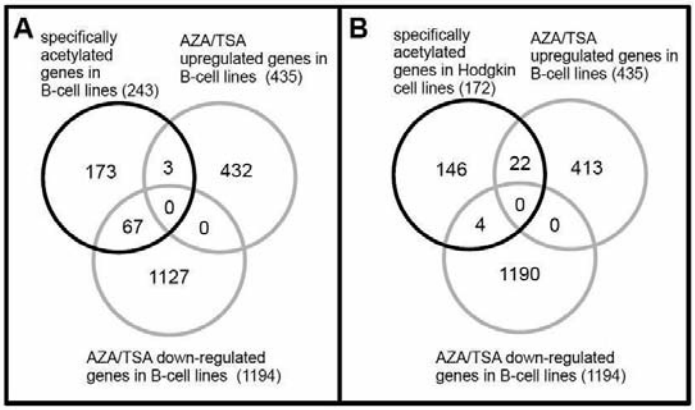
Online Supplementary Figure S4. Comparison of the acetylation pattern for selected genes with their gene expression (Affynetrix U133A). Mapping of the acetylation pattern of selected genes typical for cHL, PCM and B-cell lines (*CCR7*, *CD30*, *CD20*, *CD79a*, *IRF4*, *PRDM1, BOB1*) using the Integrated Genomic Browser (IGB) and their gene expression as determined by GeneChip U133A gene expression analysis (inserts).



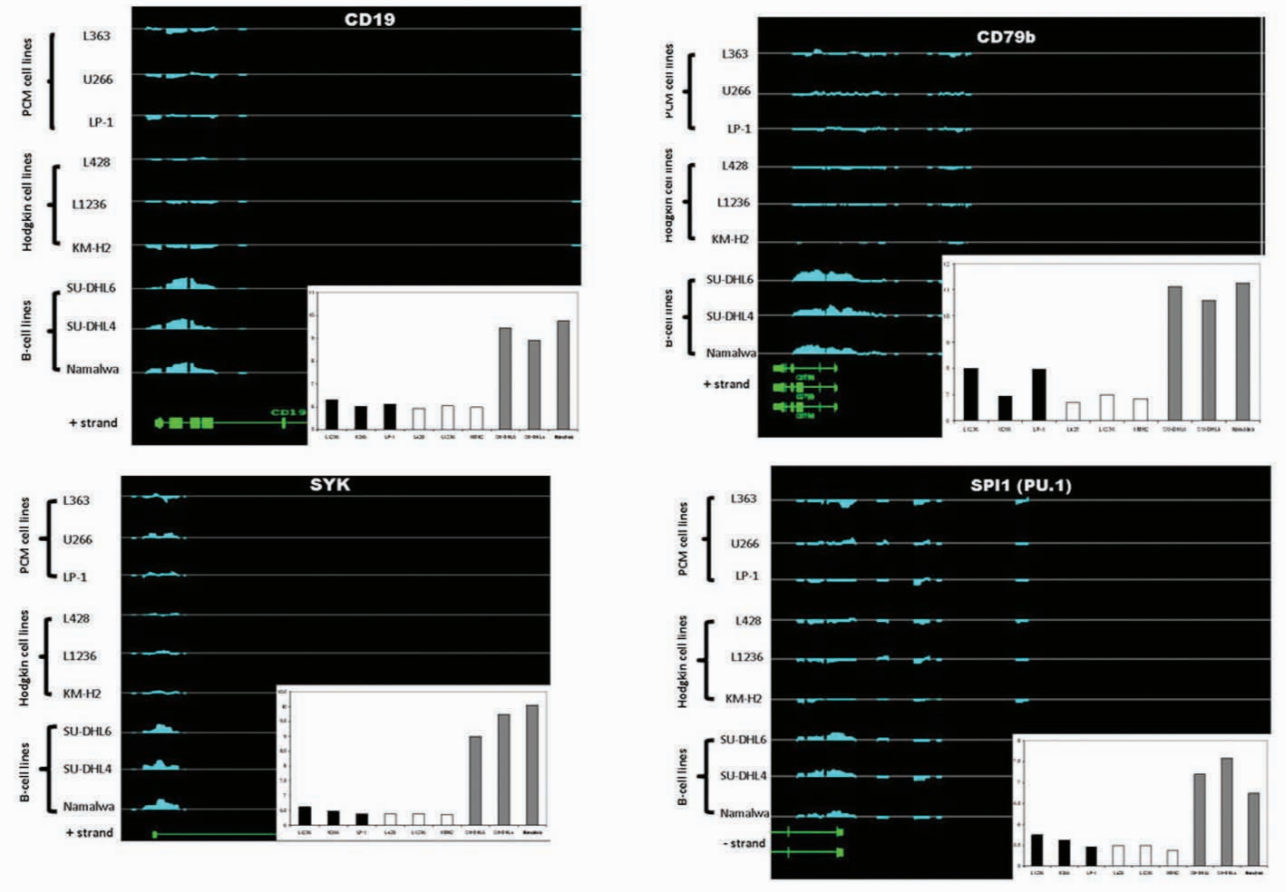
Online Supplementary Figure S5. Venn diagrams for comparison of acetylation (black circle) and gene expression (gray circles) data. (A) An overlap of 65 genes (*P*=8.810192e-37) was found when the 485 genes up-regulated in the primary tumor cells of 21 published DLBCL, BL and FL cases (available through GEO accession number GSE12453) and the 315 genes specifically acetylated in B-cell lines ($B1_{Ac}$ Figure 2) were compared. None of the 219 genes up-regulated in the HRS cells of cHL cases was found to be acetylated in B-cell lines. (B) An overlap of 31 genes (*P*=1.950429e-24) was found when the 219 genes up-regulated in the primary HRS cell of 11 published cHL cases (GEO accession number GSE12453) and the 200 genes specifically acetylated in cHL cell lines were compared. None of the 485 genes up-regulated in the primary tumor cells of the 21 published cases of diffuse large B-cell lymphoma (DLBCL), Burkitt's lymphoma (BL) and follicular lymphoma (FL)[1] was found to be acetylated in cHL cell lines. Note that only 315 of the 327 differentially acetylated B-cell genes and 211 of the 219 differentially acetylated cHL genes were covered by the U133 Plus 2.0 Affymetrix GeneChips. All 485 genes significantly expressed in the 21 published DLBCL, BL and FL cases[1] and all 219 genes significantly expressed in primary HRS cells[1] were covered by the GeneChip® Human Promoter 1.0R Tiling Array.



Online Supplementary Figure S6. Venn diagrams comparing specifically acetylated genes in cHL and B-cell lines (black circle) with genes up- and down-regulated in B-cell lines after epigenetic treatment with AZA/TSA (gray circles). (A) An overlap of 67 genes was identified when the 243 specifically acetylated genes in B-cell lines were compared to the 1194 genes down-regulated by AZA/TSA treatment of B-cell lines (*P*=6.047922e-16). In contrast only three genes were commonly specifically acetylated in B-cell lines and up-regulated in the AZA/TSA treated B-cell lines (n.s.; *P*=0.9920506). (B) An overlap of 22 genes was identified when the 172 specifically acetylated genes in cHL cell lines were compared with the 435 genes up-regulated by AZA/TSA treatment of B-cell lines (*P*=1.452767e-07). Only four genes were commonly specifically acetylated in cHL cell lines and down-regulated in the AZA/TSA-treated B-cell lines (n.s.; *P*=0.9999694). Note that only 243 of the 327 differentially acetylated B-cell genes (B1Ac) and 172 of the 211 differentially acetylated cHL genes (Figure 2) were covered by the U133A Affymetrix GeneChips and thus used for comparison. Furthermore only 1194 of 1236 genes down-regulated in AZA/TSA-treated B-cell lines and 435 of 472 genes up-regulated in AZA/TSA-treated B-cell lines were covered by the GeneChip® Human Promoter 1.0R Tiling Array.



Online Supplementary Figure S7. Mapping of the acetylation pattern of several genes expressed in cHL, PCM and B-cell lines using the Integrated Genomic Browser (IGB) and their gene expression as determined by Affymetrix U133A GeneChip hybridization.



Bioinformatic Algorithm S1

Our primary aim was the comparison of acetylation patterns of cHL, PCM and B-cell lines to assess the extent of their epigenetic alterations. We, therefore, established a bioinformatic workflow, which avoids binary decisions in early steps of the analysis. The analysis of the ChIP-on-chip data was performed using the MAT algorithm[1] resulting in a list of acetylated regions for each cell line. We calculated an acetylation score for each gene applying the formula in the insert. Using a moderated t-test the significance between the histone H3 acetylation of cHL and B-cell lines or PCM and B-cell lines was calculated. This resulted in a list of differentially acetylated genes which was used to perform enrichment analysis. The results from the ChIP-on-chip analysis were also compared to gene expression data obtained from the same cHL, PCM and B-cell lines. This figure exemplarily shows the comparison of three cHL cell lines (cHL1-3) and three B-cell lines (B1-3). The analysis pipeline was written in R and the code is available on personal request to PT.

References

1. Brune V, Tiacci E, Pfeil I, Doring C, Eckerle S, van Noesel CJ, et al. Origin and pathogenesis of nodular lymphocyte-predominant Hodgkin lymphoma as revealed by global gene expression analysis. J Exp Med. 2008;205(10): 2251-68.
2. Johnson WE, Li W, Meyer CA, Gottardo R, Carroll JS, Brown M, et al. Model-based analysis of tiling-arrays for ChIP-chip. Proc Natl Acad Sci USA. 2006;103(33):12457-62.

Online Supplementary Table S1 Primers used for ChIP DNA analyses. SEE EXCEL FILES

Online Supplementary Table S2. SEE EXCEL FILE

Online Supplementary Table S2A. 18546 Entrez gene identifiers covered by the ChIP-on-chip analysis (B-cell line in comparison to classical Hodgkin lymphoma (cHL) cell lines) are shown.

The columns can be divided in five sections.

1) Columns A to E describe different identifiers like Entrez Gene, HUGO gene name and RefSeq.
2) Columns F to O cover the results of the ChIP-on-chip analysis.
3) Columns P to R cover the results for the expression analysis between cHL and B-cell lines.
4) Columns S to U cover results from the expression analysis between AZA/TSA treated normal B-cell lines.
5) Columns V to Y cover information about the genomic location of a RefSeq transcript. Each row corresponds to a gene with at least one validated or reviewed RefSeq entry.

Column	Column name	Description
A	Entrez Gene	Entrez gene identifier
B	Name	HUGO gene name
C	Full name	Complete gene name. Information from NCBI
D	Ref Seq	RefSeq identifier used to derive the chromosomal location
E	Probe sets	Associated HGU133A probe sets
F	L1236	Acetylation score for cell line L1236
G	KM-H2	Acetylation score for cell line KM-H2
H	L428	Acetylation score for cell line L428
I	Mean cHL	Mean score over all three cHL cell lines
J	SU-DHL6	Acetylation score for cell line SU-DHL6
K	SU-DHL4	Acetylation score for cell line SU-DHL4
L	Namalwa	Acetylation score for cell line Namalwa
M	Mean B-cell lines	Mean score over all three B-cell lines
N	Ratio	log (region cHL+offset)/(region B+offset)
O	Acetylation p-value	Result of the two sided moderated t-test between column F, G, H and J, K, L
P	Probe set (cHL/B-cell)	Probe set used for the comparison between cHL vs. B-cell expression data
Q	BH (cHL/B-cell)	Benjamini Hochberg corrected p-value from two sided t-test between cHL and B-cell lines
R	Expression (cHL/B-cell)	Logarithmic (base 2) fold change between cHL and B-cell lines
S	Probe set (A/T)	Probe set used for the comparison between AZA/TSA treated vs. untreated B-cell lines (gene expression)
T	BH (A/T)	Benjamini Hochberg corrected p-value from two sided t-test between AZA/TSA treated vs. untreated B-cell lines (gene expression)
U	Expression (A/T)	Logarithmic (base 2) fold change between AZA/TSA treated vs. untreated B-cell lines (gene expression)
V	Chromosome	Chromosomal location of the gene
W	Start	Start position in terms of nucleotide bases (Based on NCBI 36.3)
X	End	End position in terms of nucleotide bases (Based on NCBI 36.3)
Y	Strand	Strand location of the gene (Forward (+) or reverse (-) strand)

Online Supplementary Table S2B. 18546 Entrez gene identifiers covered by the ChIP-on-chip analysis (B-cell lines in comparison to plasma cell myeloma (PCM) cell lines) are shown.

The columns can be divided in five sections.

1) Columns A to E describe different identifiers like Entrez Gene, HUGO gene name and RefSeq.
2) Columns F to O cover the results of the ChIP-on-chip analysis.
3) Columns P to R cover the results for the expression analysis between PCM and B-cell lines.
4) Columns S to V cover information about the genomic location of a RefSeq transcript. Each row corresponds to a gene with at least one validated or reviewed RefSeq entry.

Column	Column name	Description
A	Entrez Gene	Entrez gene identifier
B	Name	HUGO gene name
C	Full name	Complete gene name. Information from NCBI
D	Ref Seq	RefSeq identifier used to derive the chromosomal location
E	Probe sets	Associated HGU133A probe sets
F	L363	Acetylation score for cell line L363
G	U266	Acetylation score for cell line U266
H	LP-1	Acetylation score for cell line LP-1
I	Mean PCM	Mean score over all three PCM cell lines
J	SU-DHL6	Acetylation score for cell line SU-DHL6
K	SU-DHL4	Acetylation score for cell line SU-DHL4
L	Namalwa	Acetylation score for cell line Namalwa
M	Mean B-cell lines	Mean score over all three B-cell lines
N	Ratio	log (region PCM+offset)/(region B+offset)
O	Acetylation p-value	Result of the two sided moderated t-test between column F, G, H and J, K, L
P	Probe set (PCM/B-cell)	Probe set used for the comparison between PCM vs. B-cell expression data
Q	BH (PCM/B-cell)	Benjamini Hochberg corrected p-value from two sided t-test between PCM and B-cell lines
R	Expression (PCM/B-cell)	Logarithmic (base 2) fold change between PCM and B-cell lines
S	Chromosome	Chromosomal location of the gene
T	Start	Start position in terms of nucleotide bases (Based on NCBI 36.3)
U	End	End position in terms of nucleotide bases (Based on NCBI 36.3)
V	Strand	Strand location of the gene (Forward (+) or reverse (-) strand)

Online Supplementary Table S3. SEE EXCEL FILE

The colors in this table match the colored gene groups in Figure 2. Table sheet A: Specifically acetylated genes in cHL (n=211) and B-cell lines ($B1_{AC}$ in Figure 2: n=327) determined by the cHL versus B-cell line comparison. Table sheet B: The specifically acetylated genes in PCM cell lines (n=143) and B-cell lines ($B2_{AC}$ in Figure 2: n=591) determined by the comparison of PCM and B-cell lines is shown. The 17 genes which were collectively and significantly acetylated in the cHL and PCM cell lines are marked in red in the cHL and PCM specific gene lists. Furthermore the 141 genes which were found in the intersection of the two B-cell groups (which are therefore jointly hypoacetylated in cHL and PCM cell lines) are marked in blue in the two B-cell line gene lists.

Column description table sheet A:

Column	Column name	Description
A	Entrez Gene	Entrez gene identifier
B	Name	HUGO gene name
C	Full name	Complete gene name. Information from NCBI
D	Ref Seq	RefSeq identifier used to derive the chromosomal location
E	p-value (Acetylation)	Result of the two sided moderated t-test
F	Ratio	log (region cHL+offset)/(region B+offset)

Column description table sheet B:

Column	Column name	Description
A	Entrez Gene	Entrez gene identifier
B	Name	HUGO gene name
C	Full name	Complete gene name. Information from NCBI
D	Ref Seq	RefSeq identifier used to derive the chromosomal location
E	*P* value (Acetylation)	Result of the two sided moderated t-test
F	Ratio	log (region PCM+offset)/(region B+offset)

Online Supplementary Table S3. SEE EXCEL FILE

The colors in this table match the colored gene groups in Figure 2. Table sheet A: Specifically acetylated genes in cHL (n=211) and B-cell lines (B1AC in Figure 2: n=327) determined by the cHL versus B-cell line comparison. Table sheet B: The specifically acetylated genes in PCM cell lines (n=143) and B-cell lines (B2AC in Figure 2: n=591) determined by the comparison of PCM and B-cell lines is shown. The 17 genes which were collectively and significantly acetylated in the cHL and PCM cell lines are marked in red in the cHL and PCM specific gene lists. Furthermore the 141 genes which were found in the intersection of the two B-cell groups (which are therefore jointly hypoacetylated in cHL and PCM cell lines) are marked in blue in the two B-cell line gene lists.

Column description table sheet A:

Column	Column name	Description
A	Entrez Gene	Entrez gene identifier
B	Name	HUGO gene name
C	Full name	Complete gene name. Information from NCBI
D	Ref Seq	RefSeq identifier used to derive the chromosomal location
E	p-value (Acetylation)	Result of the two sided moderated t-test
F	Ratio	log (region cHL+offset)/(region B+offset)

Column description table sheet B:

Column	Column name	Description
A	Entrez Gene	Entrez gene identifier
B	Name	HUGO gene name
C	Full name	Complete gene name. Information from NCBI
D	Ref Seq	RefSeq identifier used to derive the chromosomal location
E	p-value (Acetylation)	Result of the two sided moderated t-test
F	Ratio	log (region PCM+offset)/(region B+offset)

Online Supplementary Table S4. Gene Ontology (GO) results. Interesting terms specifically enriched in one group are highlighted in red. The gene numbers of the different table sheets identify the groups shown in Figure 2. SEE EXCEL FILE

Online Supplementary Table S5 SEE EXCEL FILE

Table sheet A) Genes up-regulated in DLBCL, BL and FL cases (available through GEO Accession Nr. GSE12453) as well as specifically acetylated in B-cell lines (n=65). Table sheet B) Genes up-regulated in microdissected HRS cells of 11 cHL cases (GEO Accession Nr. GSE12453) as well as specifically acetylated in cHL cell lines (n=31).

Column description table sheet A and B:

Column	Column name	Description
A	Entrez Gene	Entrez gene identifier
B	Name	HUGO gene name
C	Full name	Complete gene name. Information from NCBI
D	Ref Seq	RefSeq identifier used to derive the chromosomal location
E	p-value (Acetylation)	Result of the two sided moderated t-test
F	Ratio (Acetylation)	log (region cHL+offset)/(region B+offset)
G	p-value (Expression)	Benjamini Hochberg corrected p-value from two sided t-test between cHL in comparison to DLBCL, BL and FL specimen
H	Fold change (Expression)	Logarithmic (base 2) fold change between cHL in comparison to DLBCL, BL and FL specimen
I	Probe set	Associated U133 Plus 2.0 Affymetrix GeneChip probe sets

Online Supplementary Table S6 SEE EXCEL FILE

Table sheet A) Specifically acetylated genes in cHL cell lines which are also up-regulated in B-cell lines upon AZA/TSA treatment (n=22). Table sheet B) Furthermore, the genes specifically acetylated in B-cell lines and down-regulated in B-cell lines upon AZA/TSA treatment are listed (n=67).

Column description table sheet A and B:

Column	Column name	Description
A	Entrez Gene	Entrez gene identifier
B	Name	HUGO gene name
C	Full name	Complete gene name. Information from NCBI
D	Ref Seq	RefSeq identifier used to derive the chromosomal location
E	p-value (Acetylation)	Result of the two sided moderated t-test
F	Ratio (Acetylation)	log (region cHL+offset)/(region B+offset)
G	BH (A/T)	Benjamini Hochberg corrected p-value from two sided t-test between AZA/TSA treated vs. untreated B-cell lines (gene expression)
H	Expression (A/T)	Logarithmic (base 2) fold change between AZA/TSA treated vs. untreated B-cell lines (gene expression)
I	Probe set (A/T)	GeneChip U133 A Probe sets used for the comparison between AZA/TSA treated vs. untreated B-cell lines (gene expression)