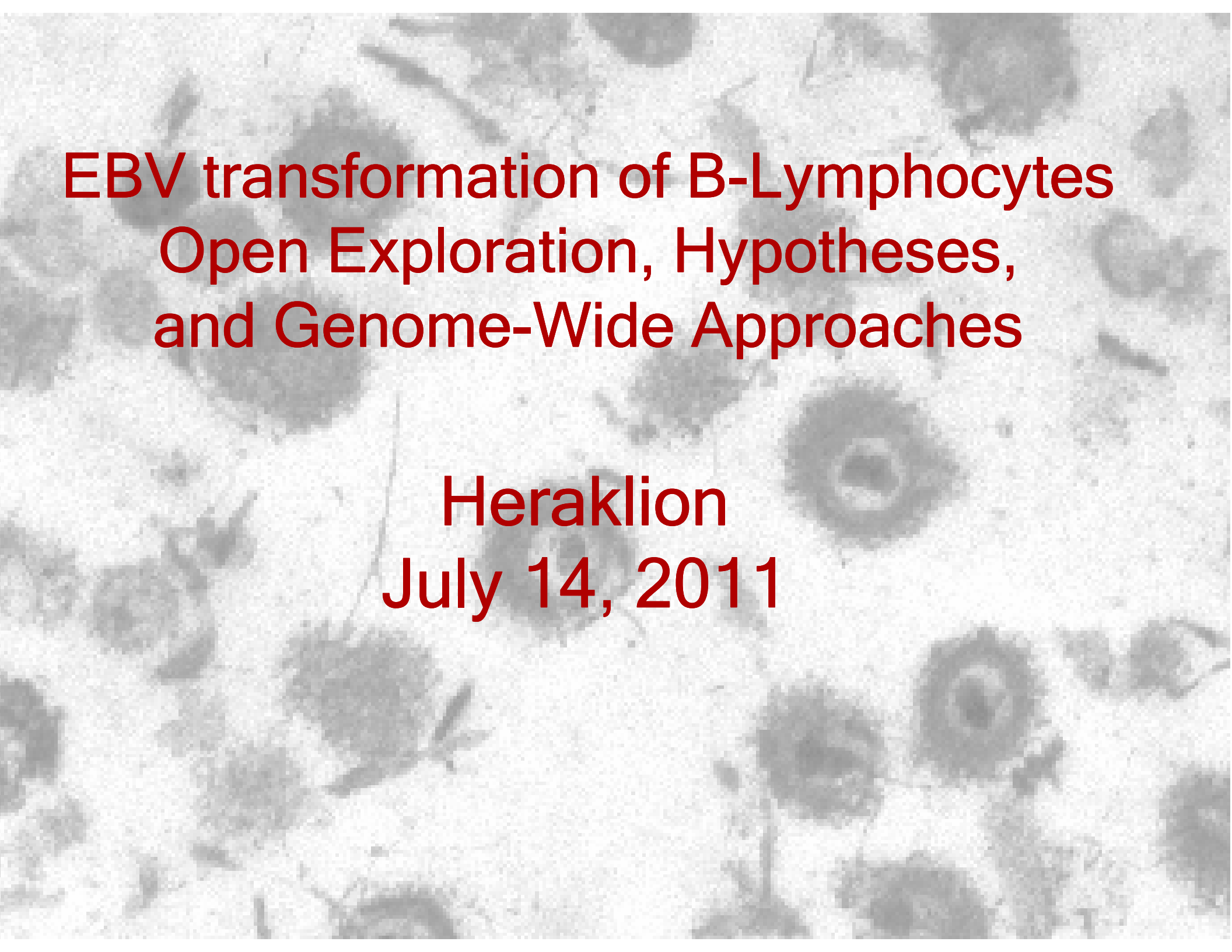
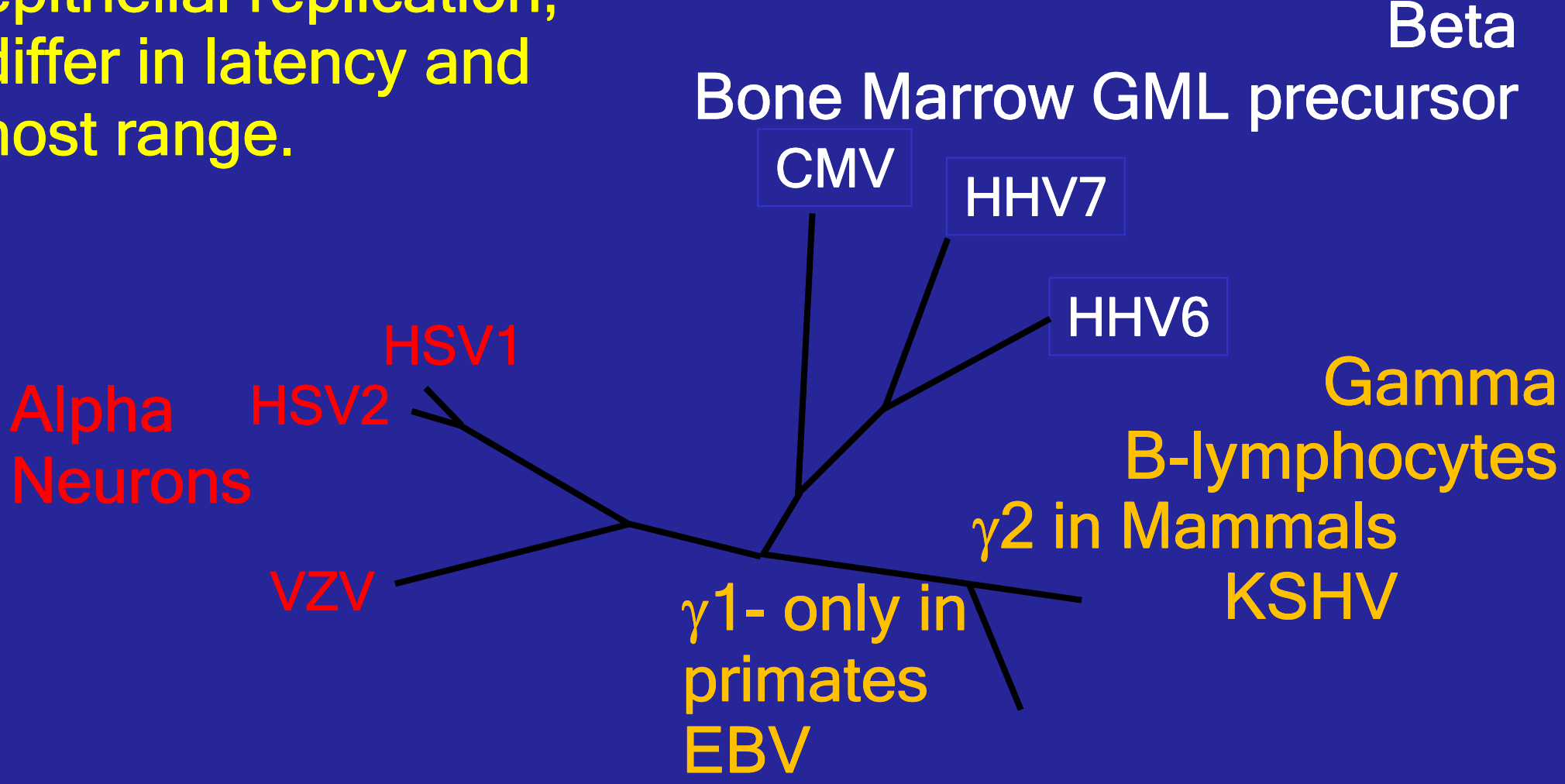

EBV transformation of B-Lymphocytes
Open Exploration, Hypotheses,
and Genome-Wide Approaches
Heraklion
July 14, 2011

8 Human Herpesviruses have 43 core genes for epithelial replication; differ in latency and host range.



In initial infection of peripheral blood B-cells, EBV Latency III infection of 6 nuclear and 2 integral membrane proteins causes B-cell proliferation, in vivo or in vitro. In lymph nodes expression restricted and immune T-cells eliminate Latency III infected cells. Without immune responses, LPD. In normal people, eBL, HD, NPC, GasCa

Primary EBV infection and Resting B-Lymphocytes (RBLs)

In primary human infection, EBV replicates in epithelia cells and infects RBLs in blood, tonsils and adenoids.

Initially, Latency III EBNA2, EBNALP, EBNA3C, EBNA3A, EBNA1, LMP1, and LMP2 are expressed and cause RBL proliferation.

In lymphatic organs, EBNAs and LMPs are down-regu ated and infected RBLs shift to a non-proliferative state. Infected RBLs become the sites for latency and, ultimately, re-activated virus replication.

Remaining Latency III proliferating RBLs are removed by NK and T-cell responses, which persist life-long .

RBLs

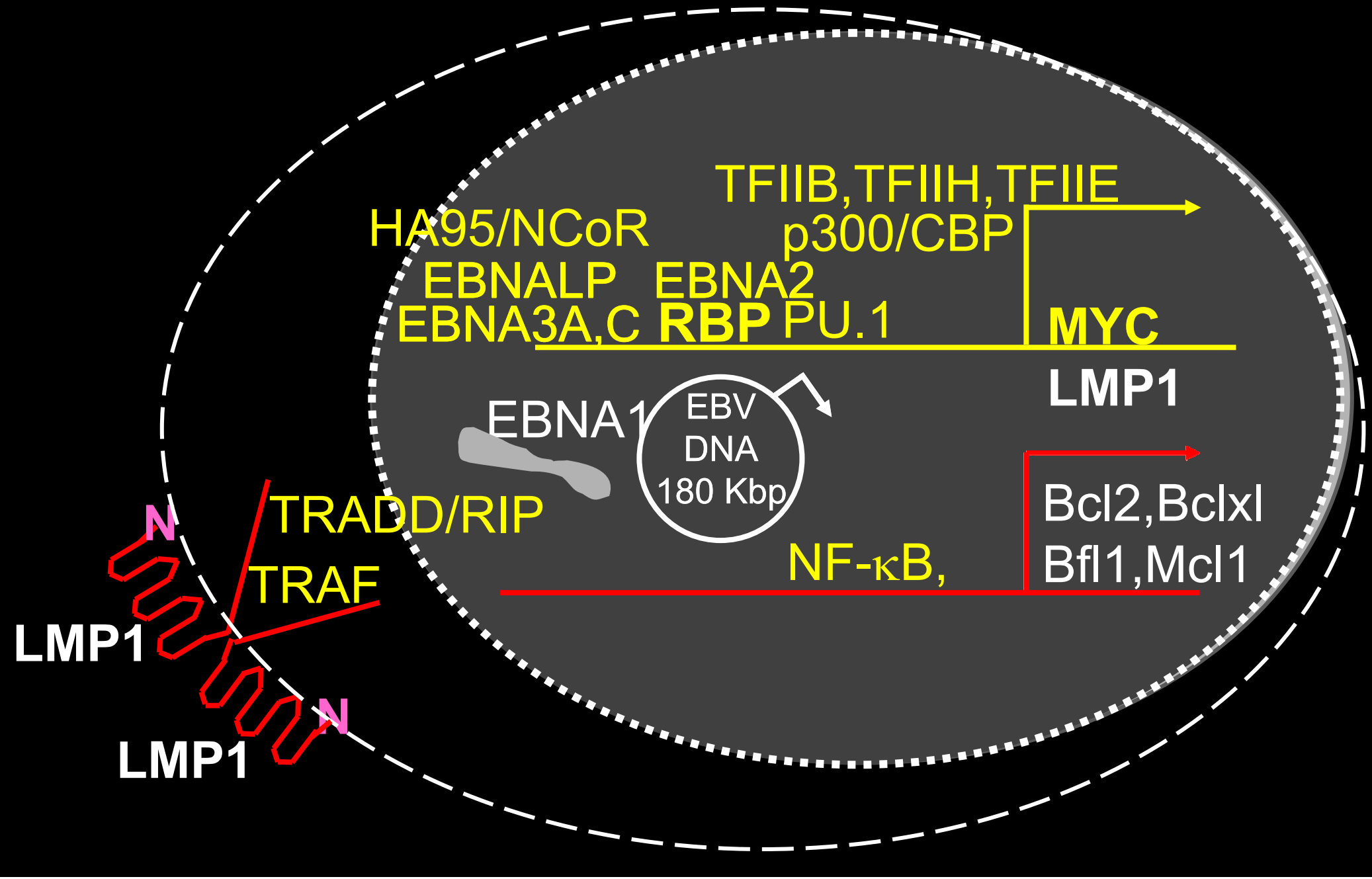
RBLs are mature sIg expressing B-cells.

RBLs are developmentally programmed to respond to antigen signaling through sIg in lymph node germinal centers, where antigen binding to RBL sIg induces *MYC*-driven hyper-proliferation.

Proliferating B-cells present antigen to CD4+ T-cells. In response, CD4+ T-cells to express CD40 ligand. CD40L binds to B-cell CD40R and up-regulates B-cell NF-kB and BCL2 expression, which prevent MYC-proliferation induced cell death.

Following the discovery in 1994 that EBNA2 and Notch regulate myc transcription through RBPJ, a cell transcription factor, the central hypothesis has been that EBNA2 and Notch directly regulate *MYC* to cause B-, and T- cell transformation . How?

EBNA2/RBPJ up-regulate *MYC* and LMP1. LMP1 up-regulates NFkB and Bcl2 family proteins, which are essential *MYC* driven cell survival and for RBL conversion to LCLs.

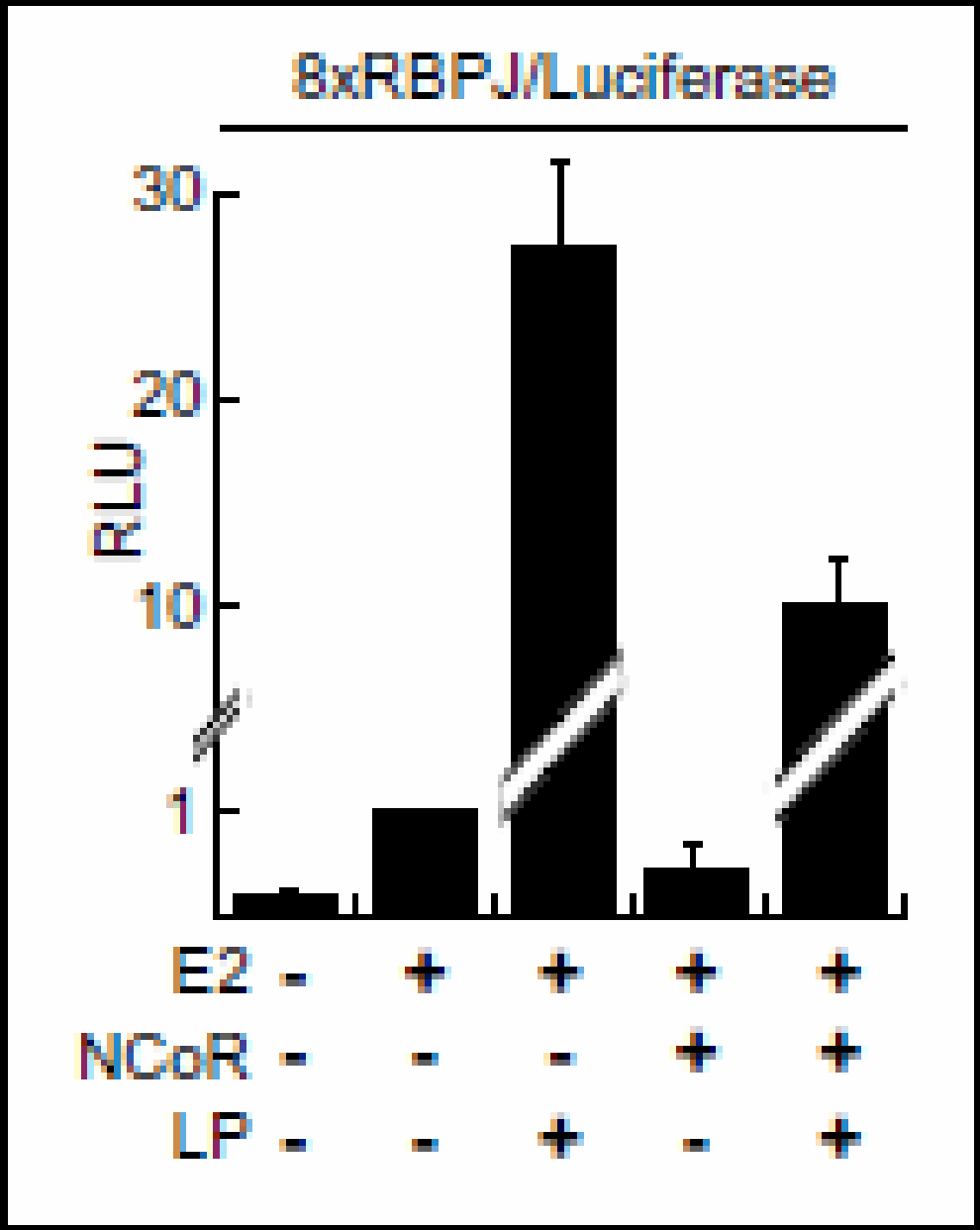


Recent Advances

EBV Nuclear Antigen EBNALP dismisses NCoR and RBPJ repressors from enhancer sites and EBNA2 increases NCoR-deficient RBPJ at enhancer sites. (Portal, et al, PNAS, 2011)

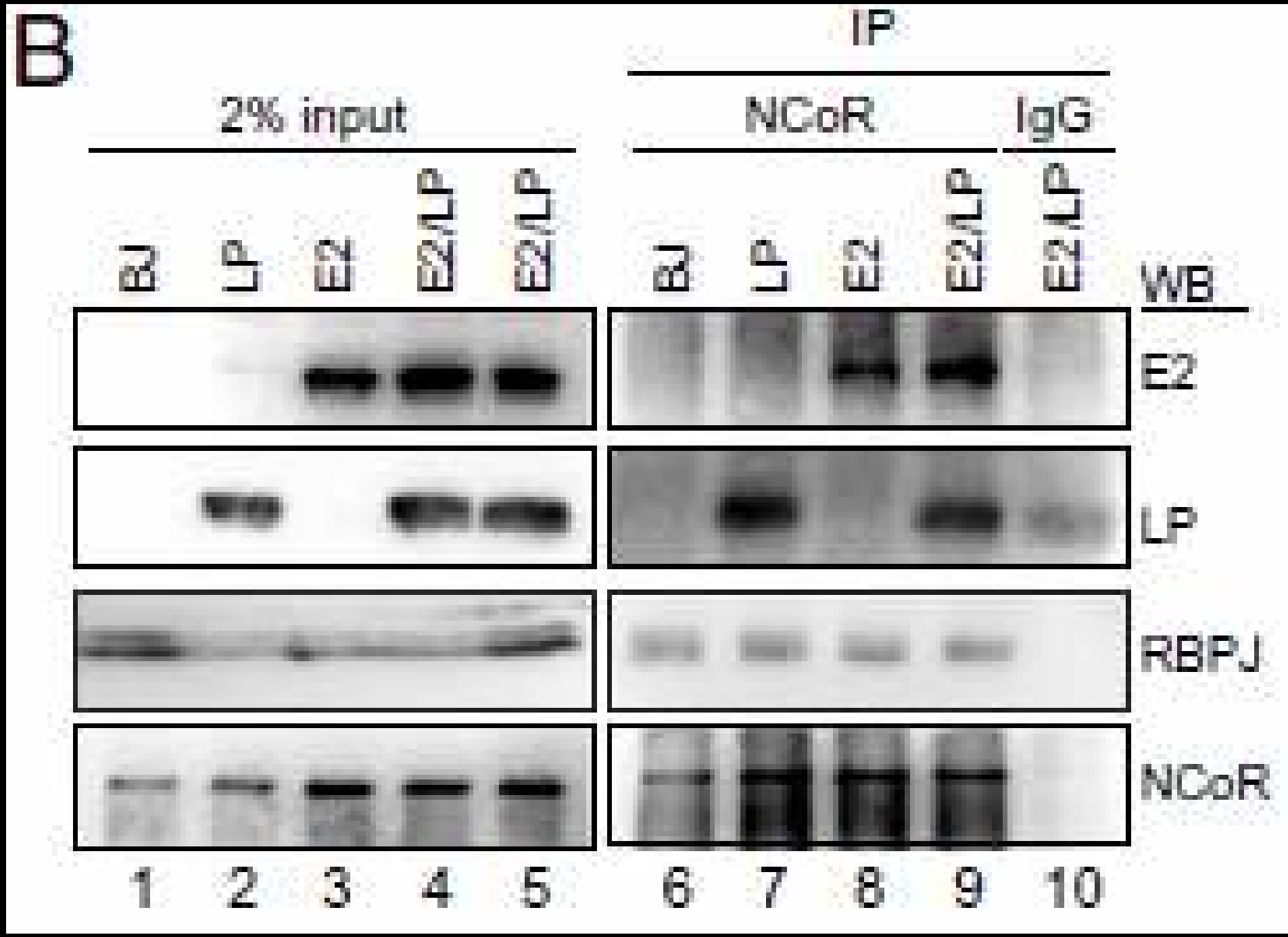
The transformation essential EBV Nuclear Proteins 3C and 3A maintain lymphoblastoid cell growth by repressing (MYC- induced) p16INK4A and p14 ARF expression. ShRNAs to p16 and p14 or HPV 16 E7 and E6 protein expression, which block p16 and p14 effects are required to restore LCL growth following conditional EBNA3A or EBNA3C inactivation.
Maruo S, et al.,2011

EBNALP inhibits NCoR repression RBPJ at enhancers.

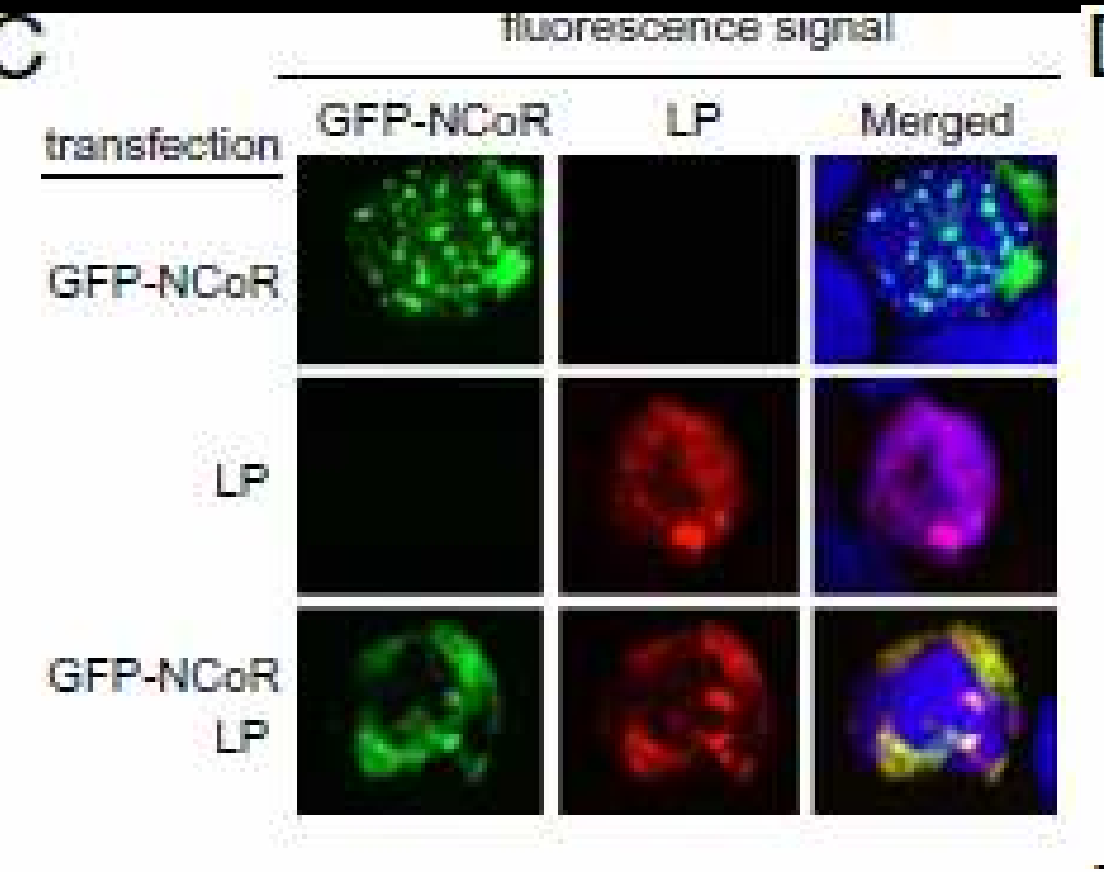


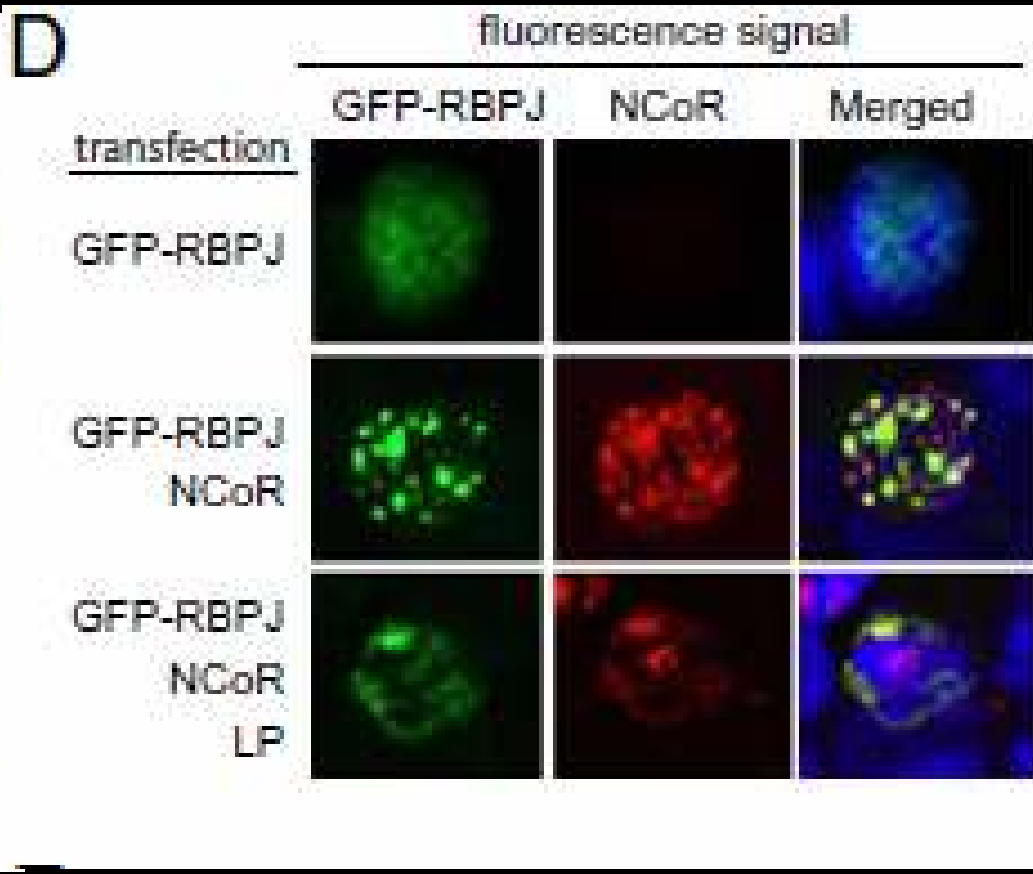
(Portal et all, 2011)

NCoR associates with EBNALP, RBPJ and EBNA2

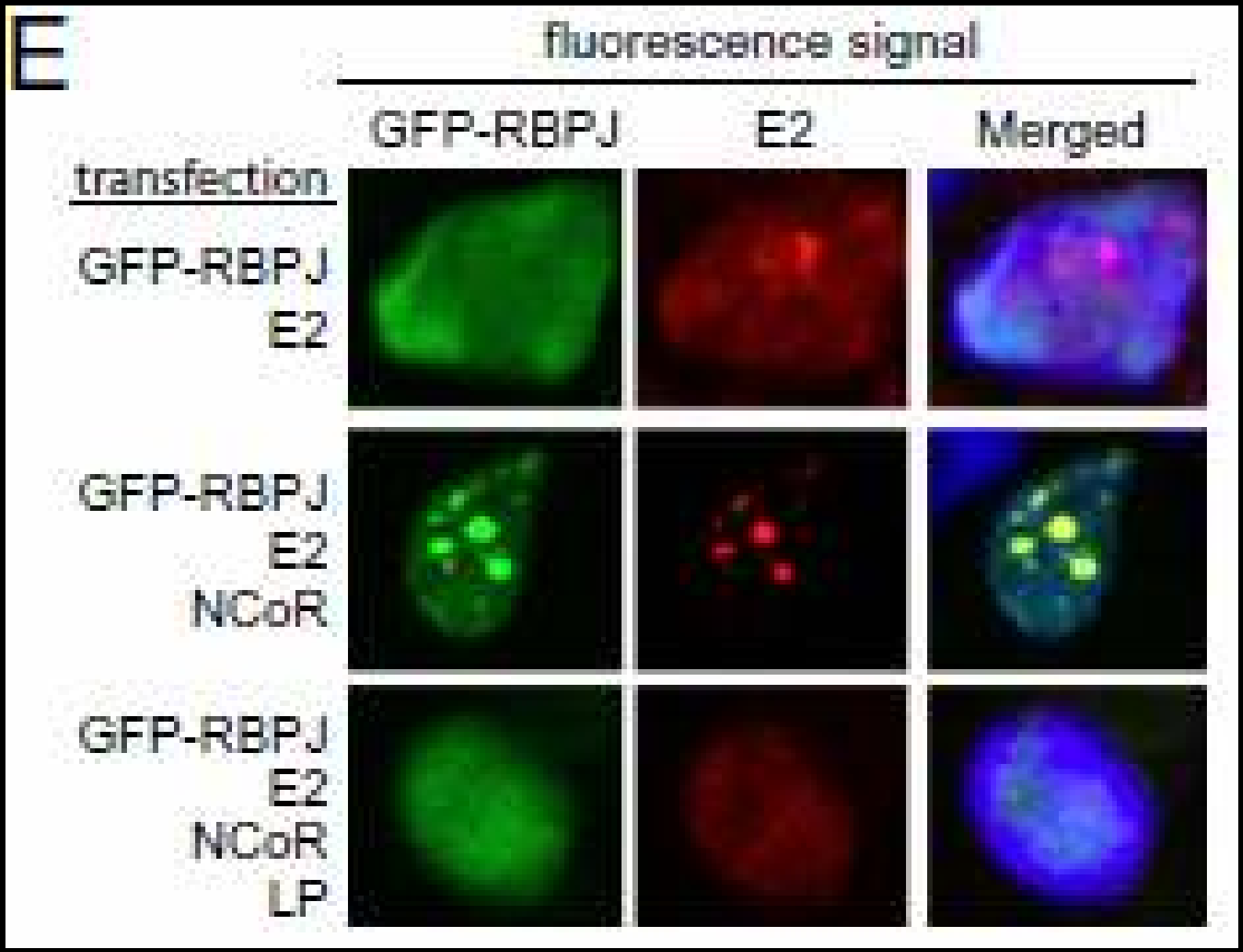


EBNALP dismisses NCoR from Matrix associated deacetylase bodies (sites of repressed chromatin)

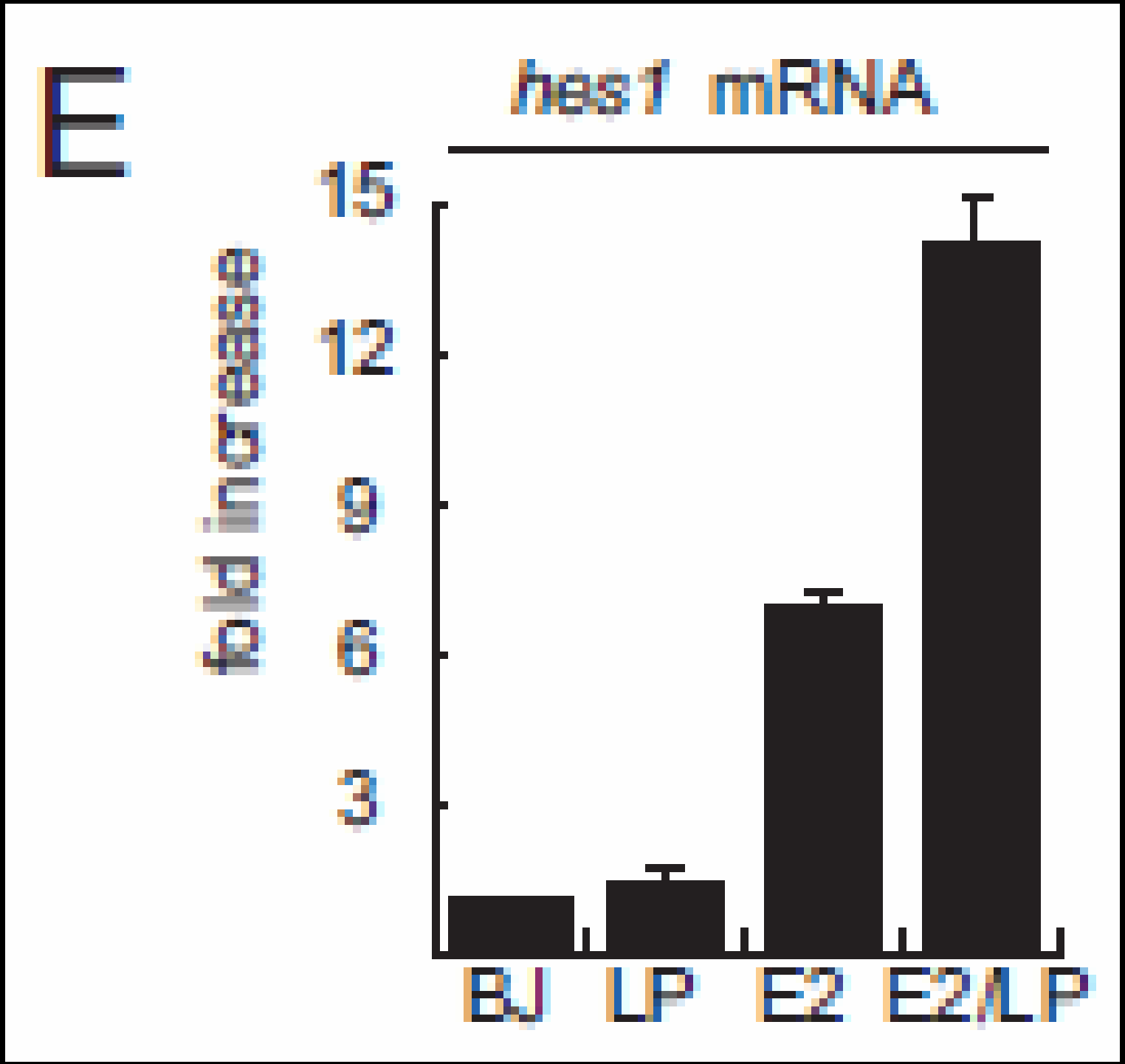




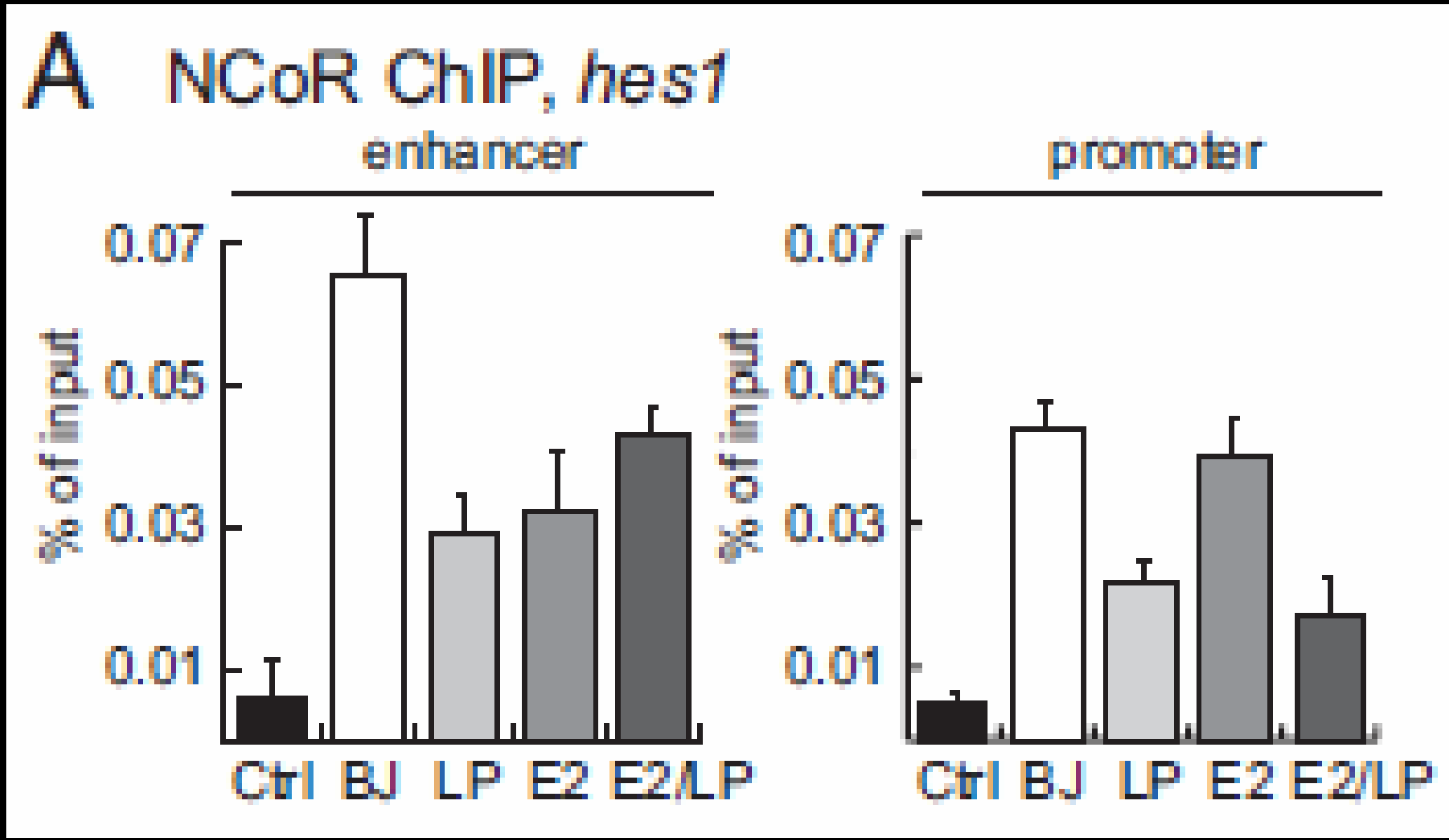
NCoR localizes RBPJ and EBNA2 to MAD bodies and EBNALP dismisses RBPJ and EBNA2 to nucleoplasm



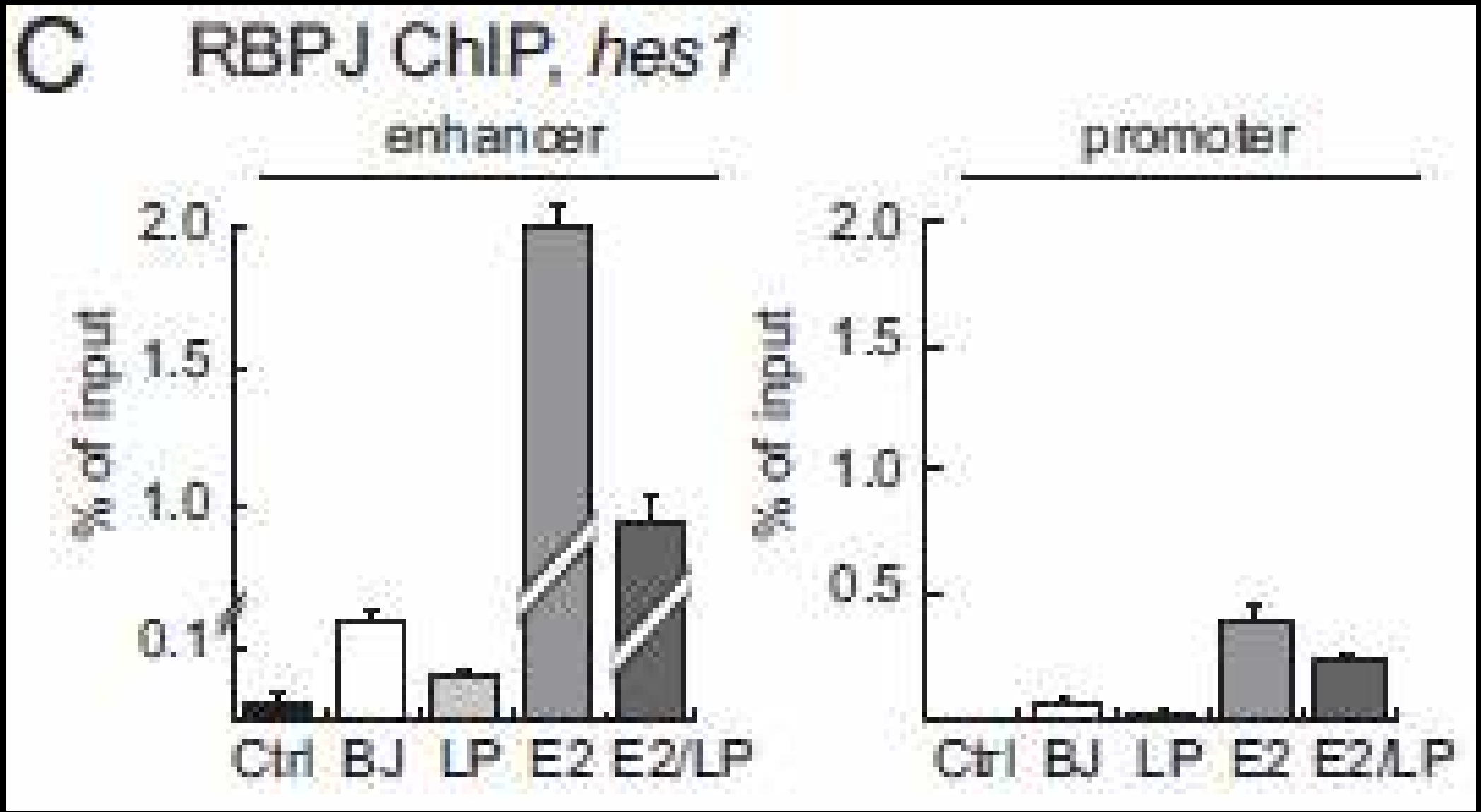
EBNALP co-activates the HES1 promoter with RBPJ.



EBNALP dismisses NCoR from enhancer sites

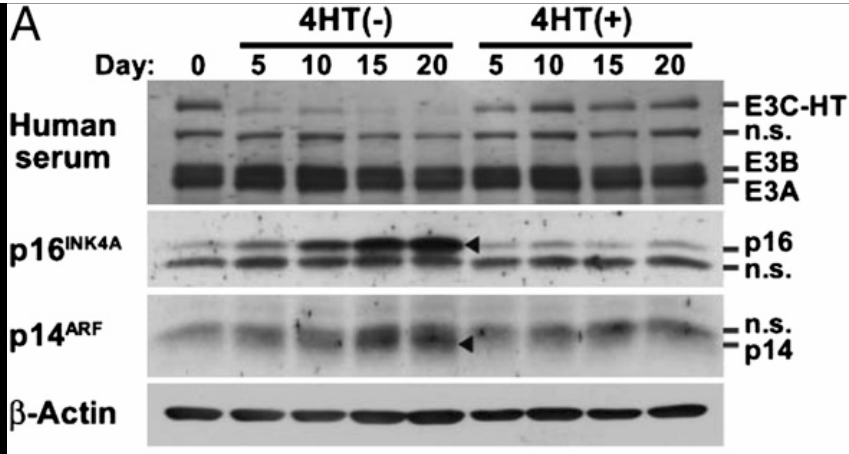


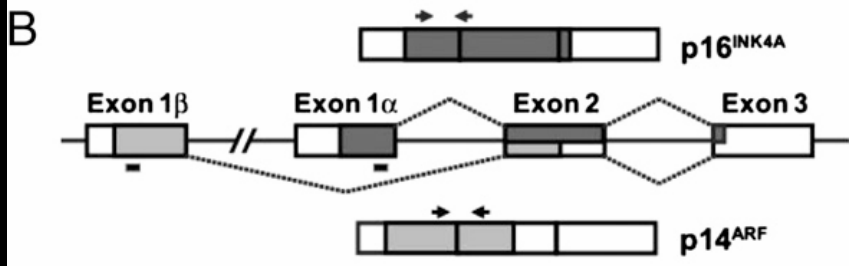
EBNALP dismisses RBPJ from enhancer sites and EBNA2 increases NCoR-deficient RBPJ at these sites.

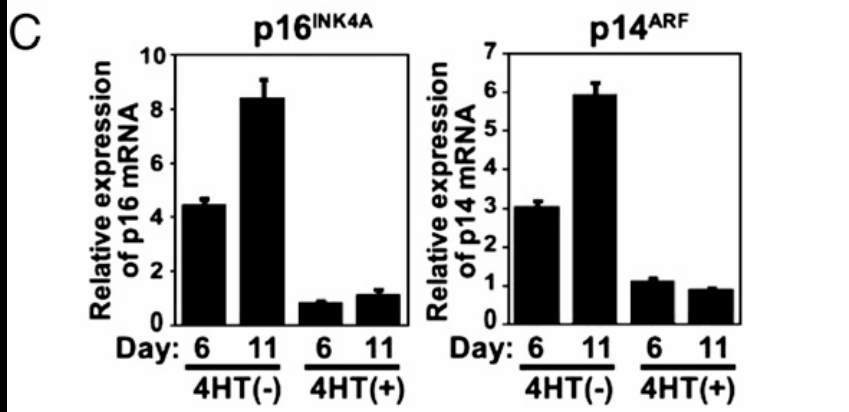


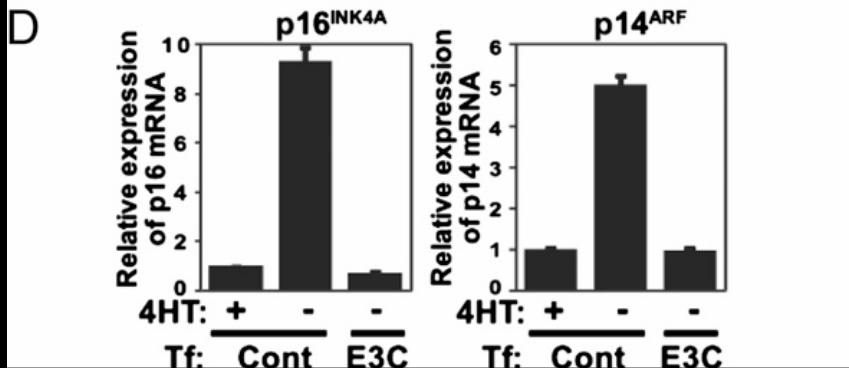
EBNA3A and EBNA3C are essential for repression of p14 and p16 responses to EBNA2-induced MYC.

Conditional inactivation of EBNA3C in an LCL results in p16 and p14 induction and cessation of cell growth.

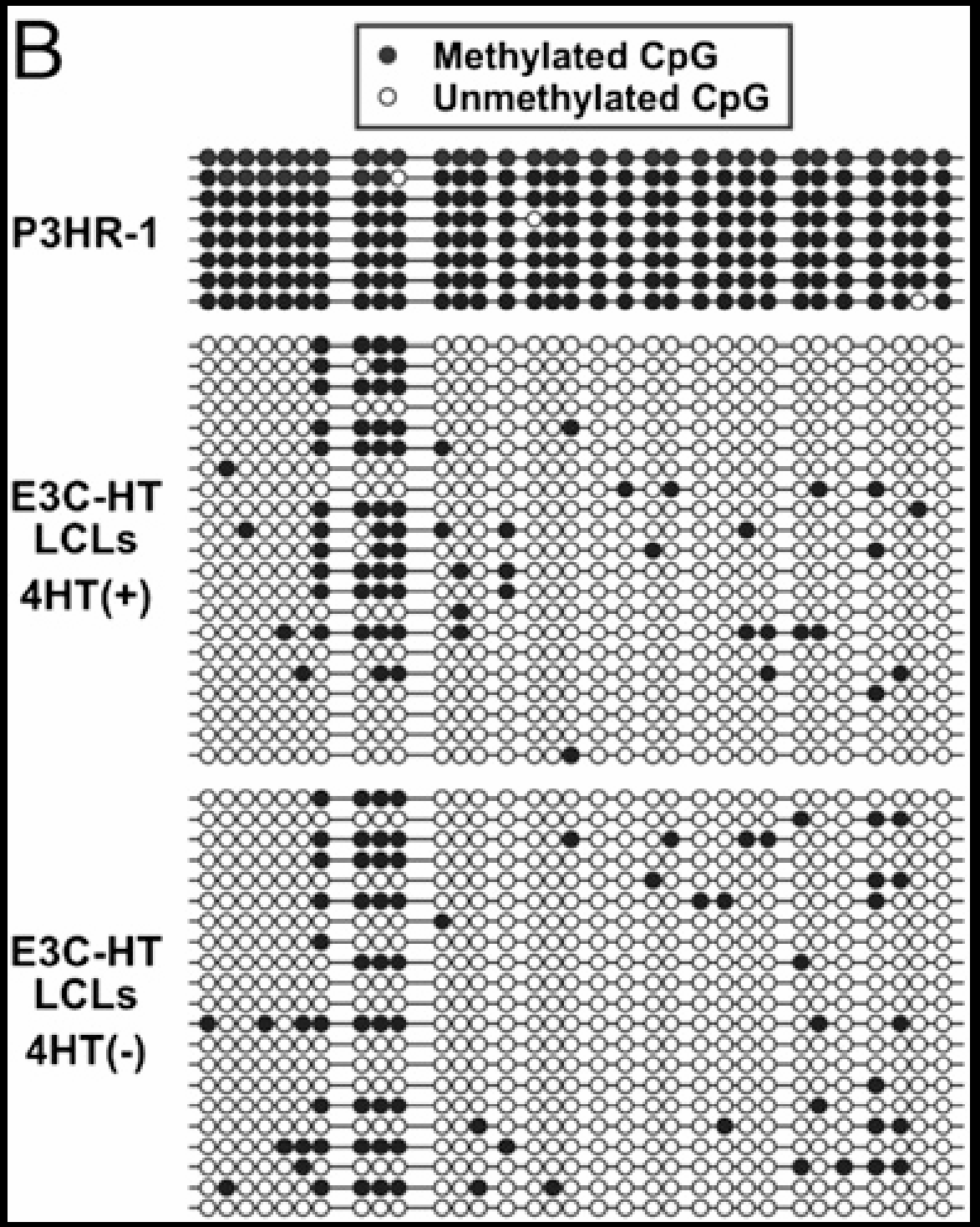






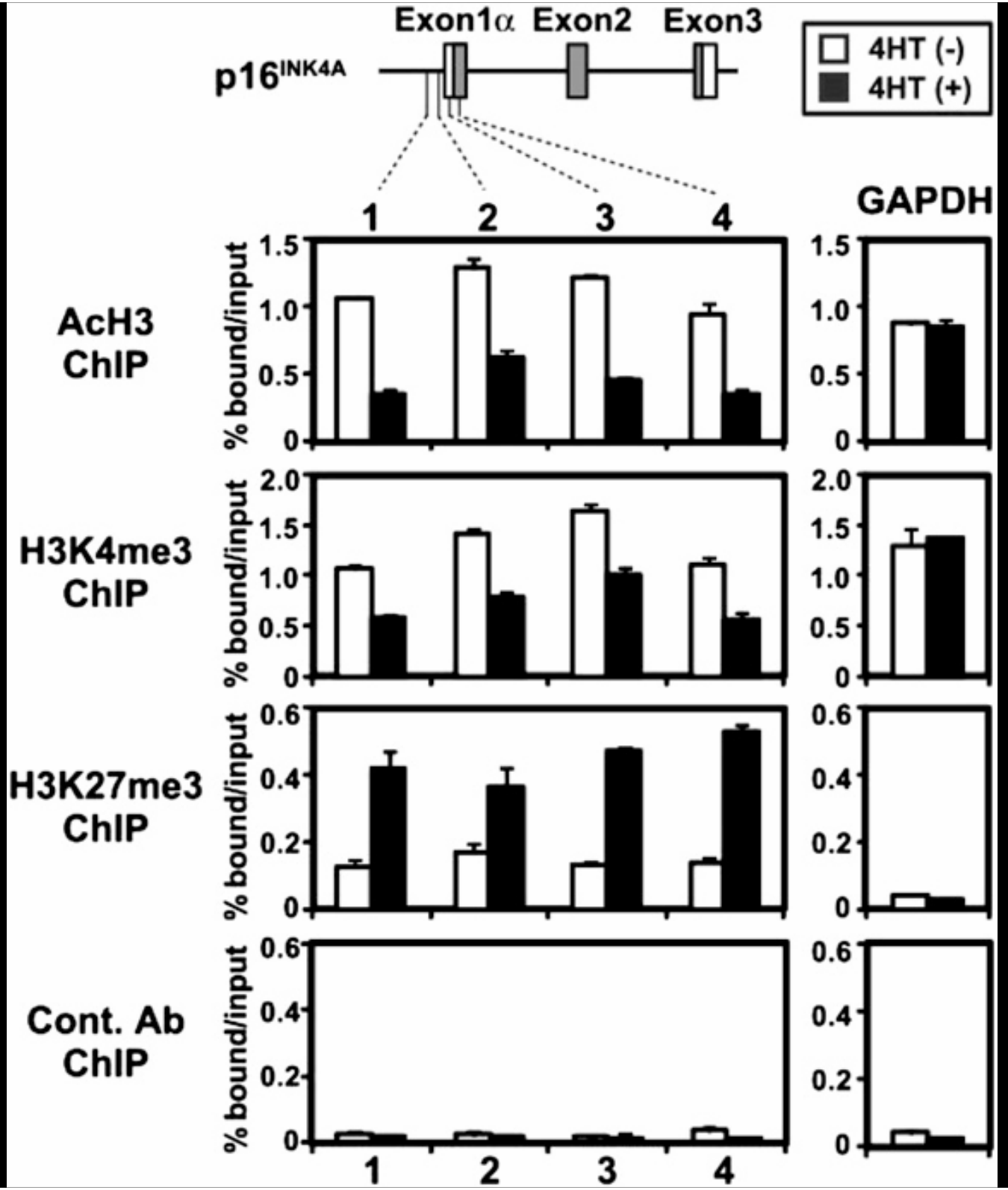


EBNA3C inactivation does not alter LCLsp14ARF or p16INK4A promoter CpG methylation

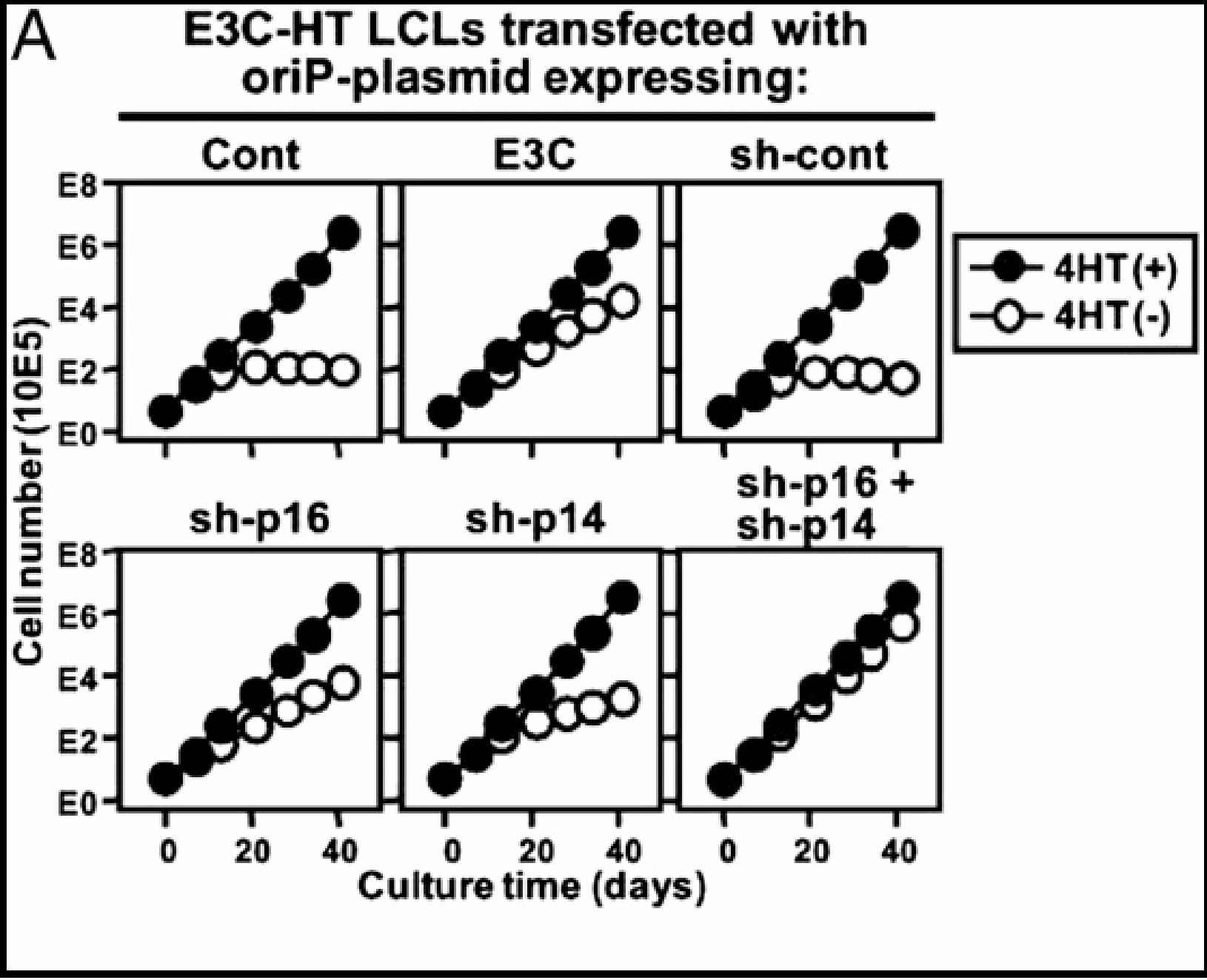


Maruo, PNAS 2011

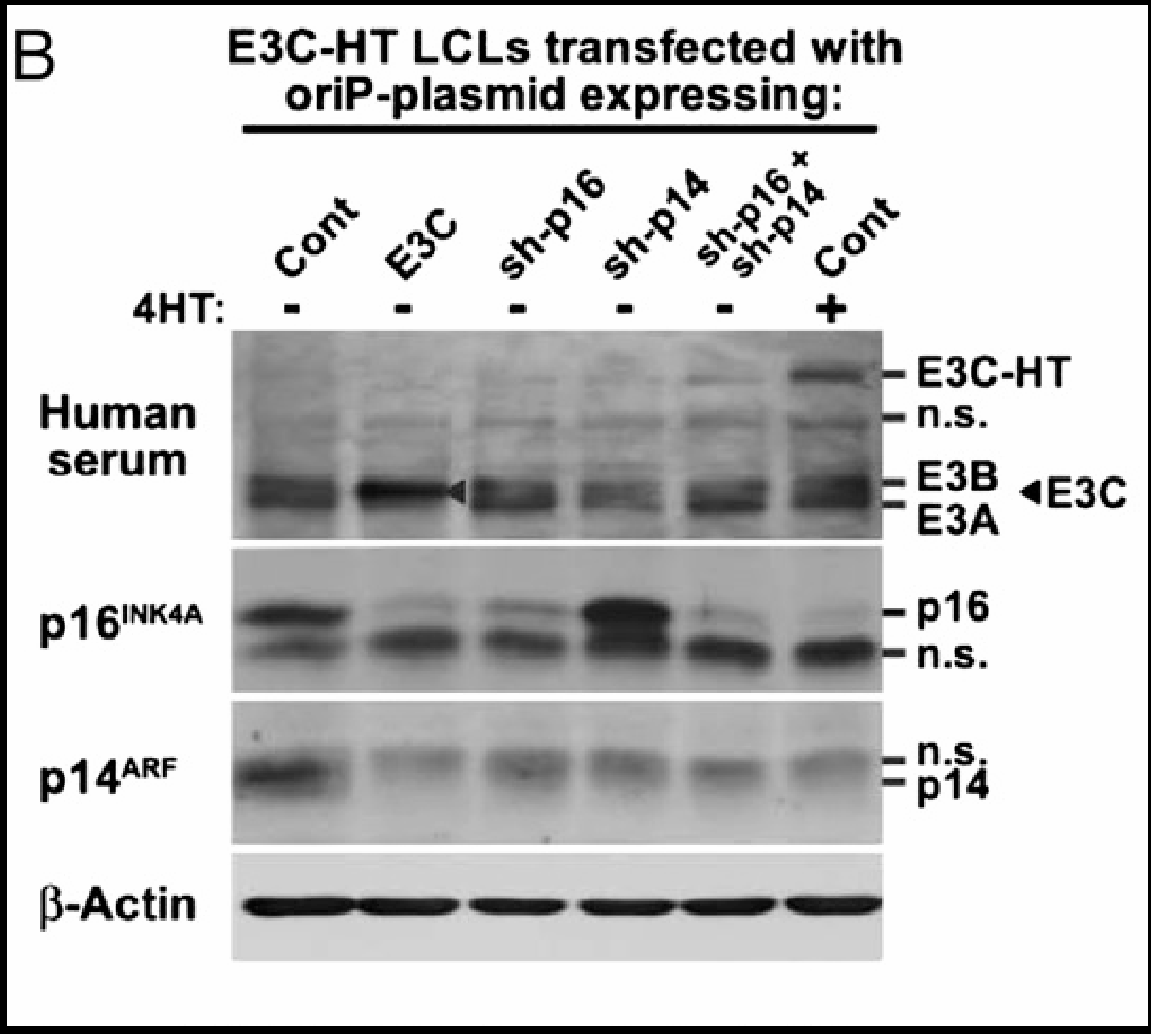
EBNA3C inactivation increases p16INK4A histone H3 acetylation and H3K4me3 and reduces of H3K27 trimethylation in LCLs. E3CHT LCLs cultured in the absence (−) or presence (+) of 4HT for 20 d were ChIP and qRT-PCR analyzed.



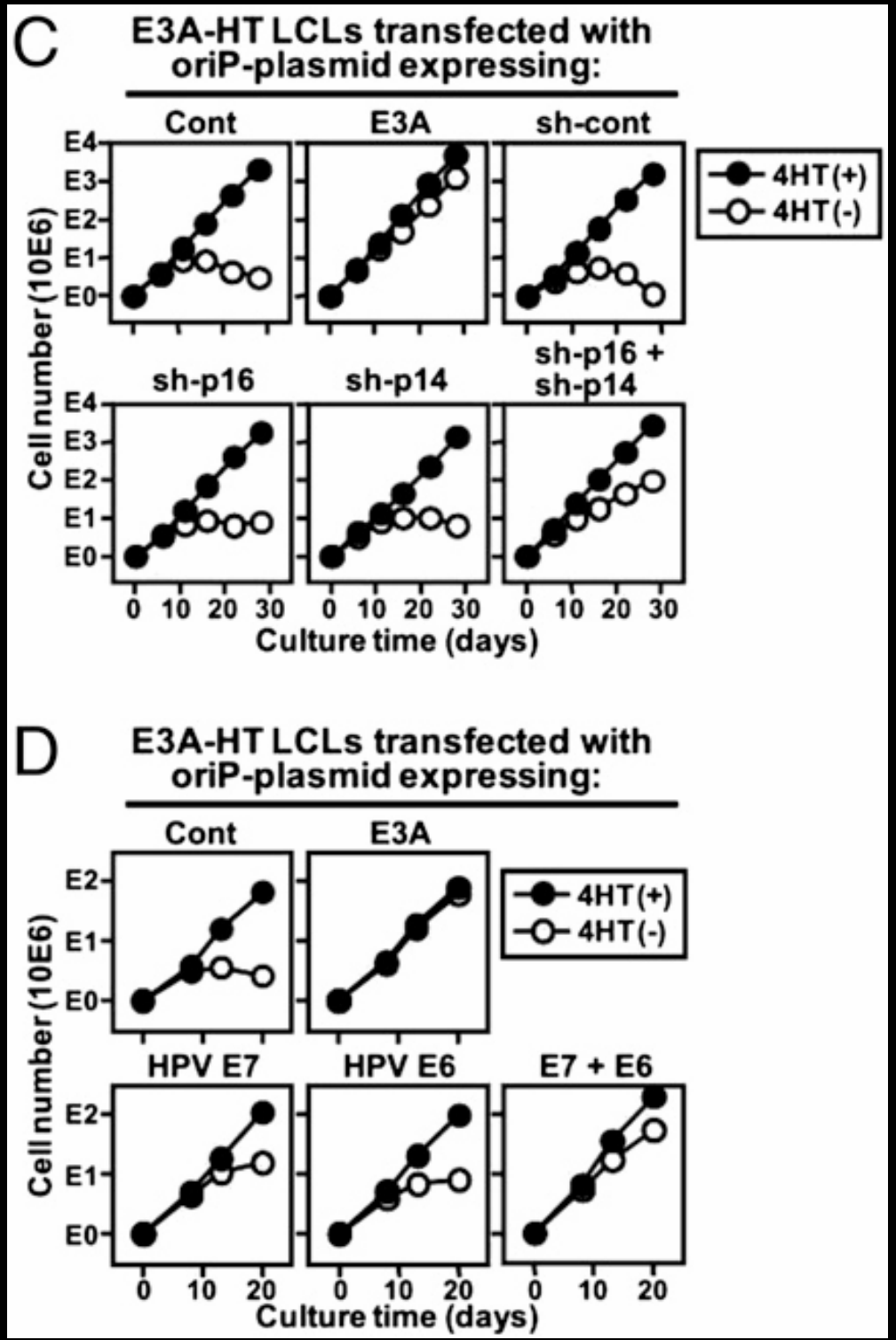
E3CHT LCLs transfected with an oriP plasmid expressing EBNA3C, an oriP plasmid expressing sh-cont, sh-p16, sh-p14), or sh-p16- and sh-p14- Five days later cells were put in medium with (+) or without (−)4HT.

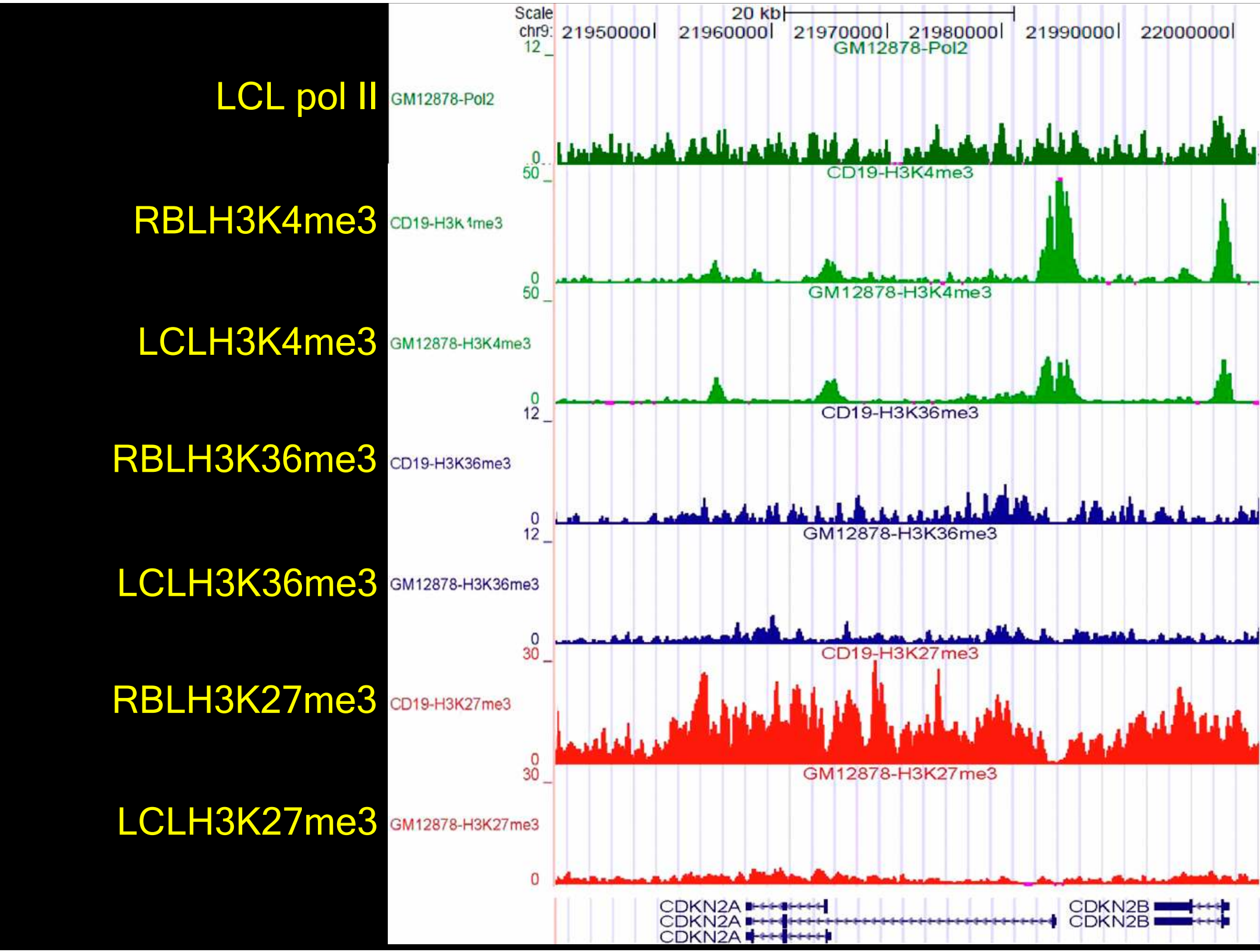


Day 41 cell lysates Western blotted with EBV-, p16INK4A-, p14ARF-, or actin-specific antibodies. Arrowhead indicates transfected E3C.



EBNA3A inactivation also induces p16INK4A and p14ARF expression and LCL growth arrest, substantially restores cell growth.



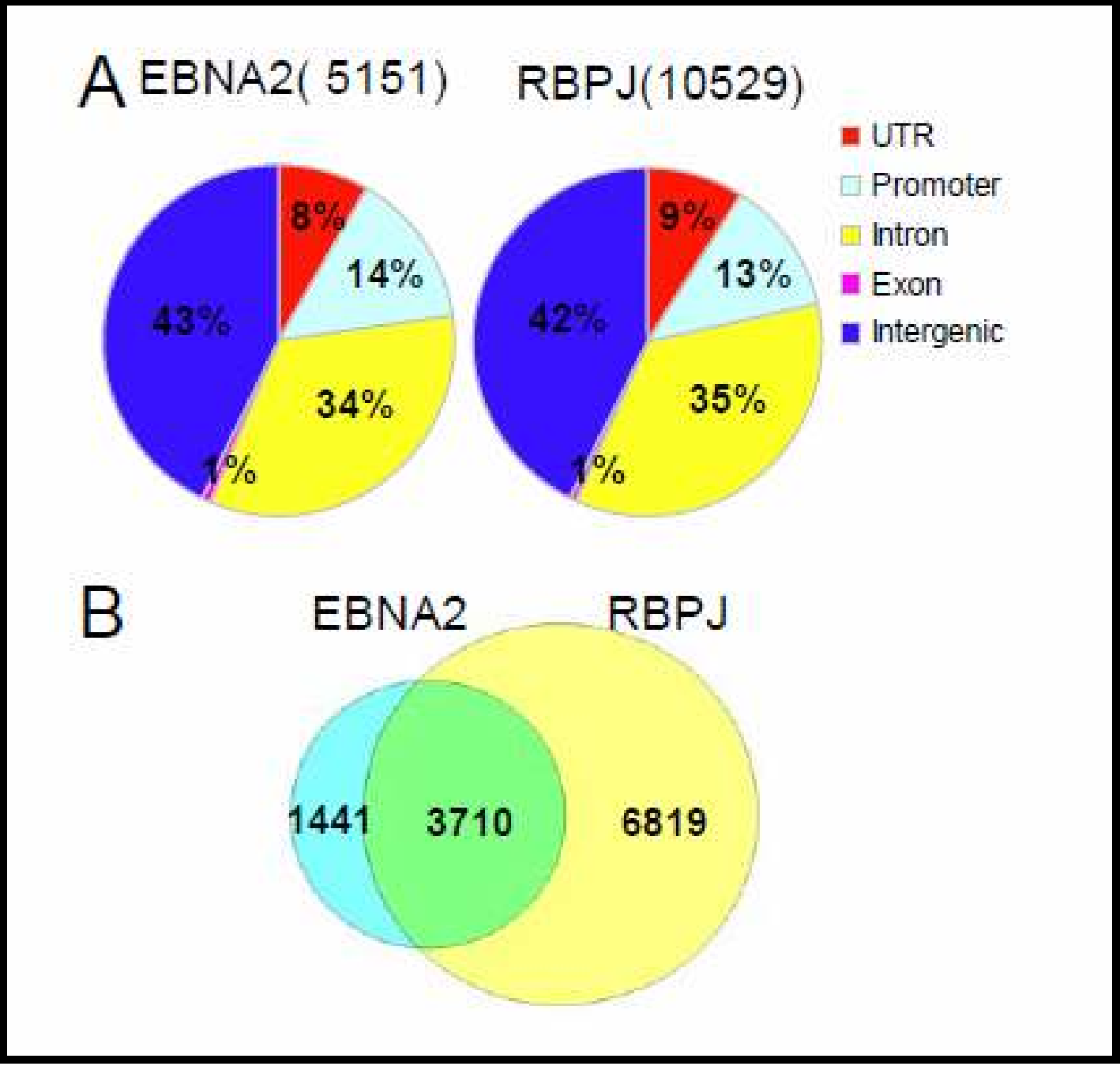

LCL pol II
RBLH3K4me3
LCLH3K4me3
RBLH3K36me3
LCLH3K36me3
RBLH3K27me3
LCLH3K27me3
Scale
chr9:
20 kb
21950000
21960000
21970000
21980000
21990000
22000000
GM12878-Pol2
CD19-H3K4me3
GM12878-H3K4me3
CD19-H3K36me3
GM12878-H3K36me3
CD19-H3K27me3
GM12878-H3K27me3
CDKN2A
CDKN2B

Comparison of active transcription-associated H3K4me3 and H3K36me3 histone modifications with Polycomb repression-associated H3K27me3 marks at the CDKN2A p16INK4A and p14ARF in LCLs relative to primary CD19 positive B cells using normalized (ENCODE) data revealed extensive H3K27me3 through the CDKN2Ap16INK4A and p14ARF locus in resting CD19+ primary B cells, except at the p16INK4A and p14ARF promoters versus very little H3K27me3 throughout the locus in LCLs. Whereas H3K36me3 was low through the locus in both resting B cells and LCLs,H3K4me3 levels were high at the p16INK4A and p14ARF promoters. The coupling of low overall H3K27me3 and highH3K4me3 at the p16INK4A and p14ARF promoters, with above background PolII through these loci in LCLs (Fig. S2) is consistent with p16INK4A and p14ARF being poised for transcription activation in LCLs.

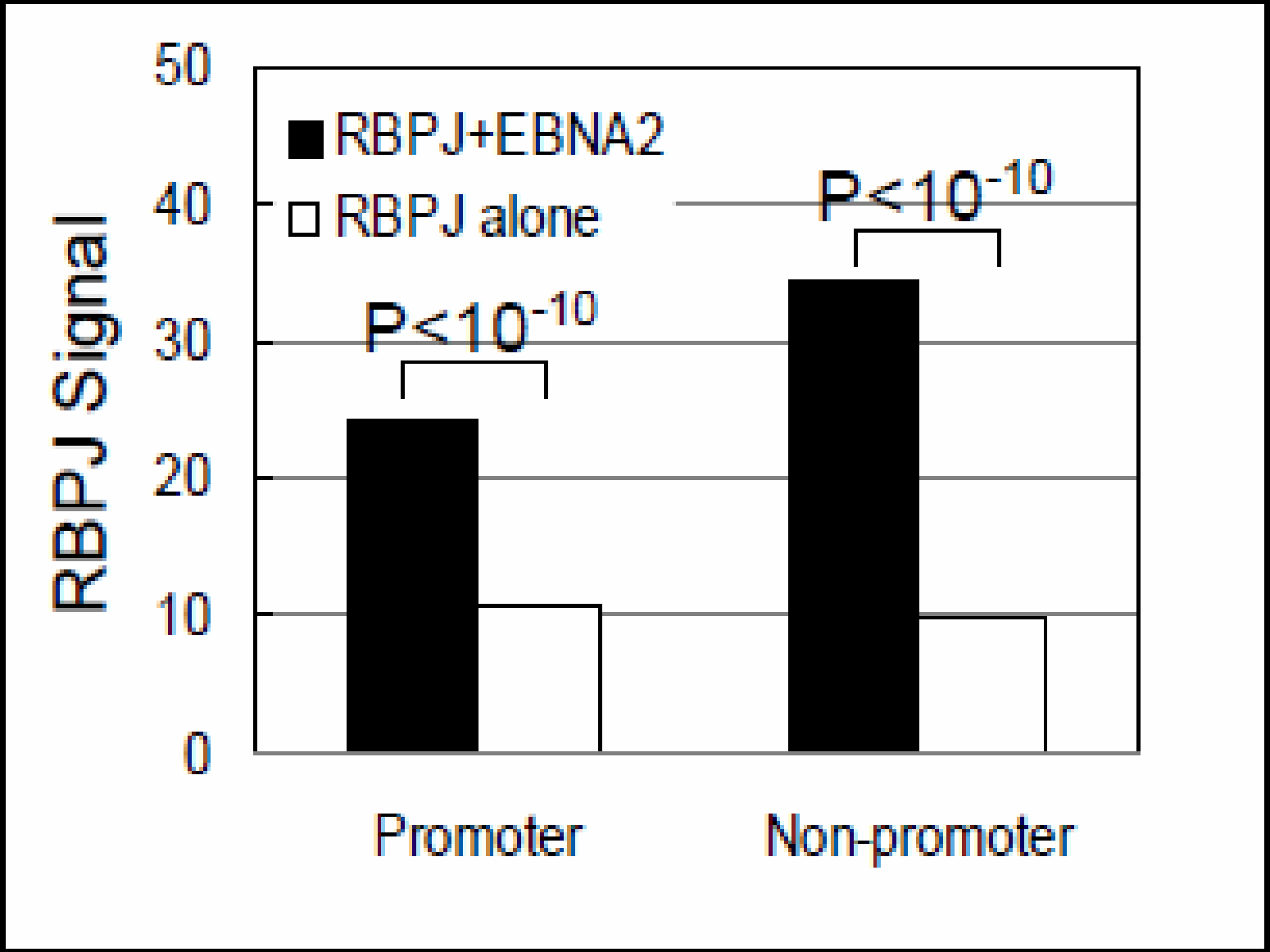
Epstein-Barr virus exploits intrinsic B-lymphocyte transcription programs to achieve immortal cell growth

Bo Zhao, James Zou, Hongfang Wang, Eric Johannsen, Chih-Wen Peng, John Quackenbush, Jessica C. Mar, Cynthia Morton, Matthew L. Freedman, Stephen C. Blacklow, Jon C. Aster, Bradley E. Bernstein, and Elliott Kieff

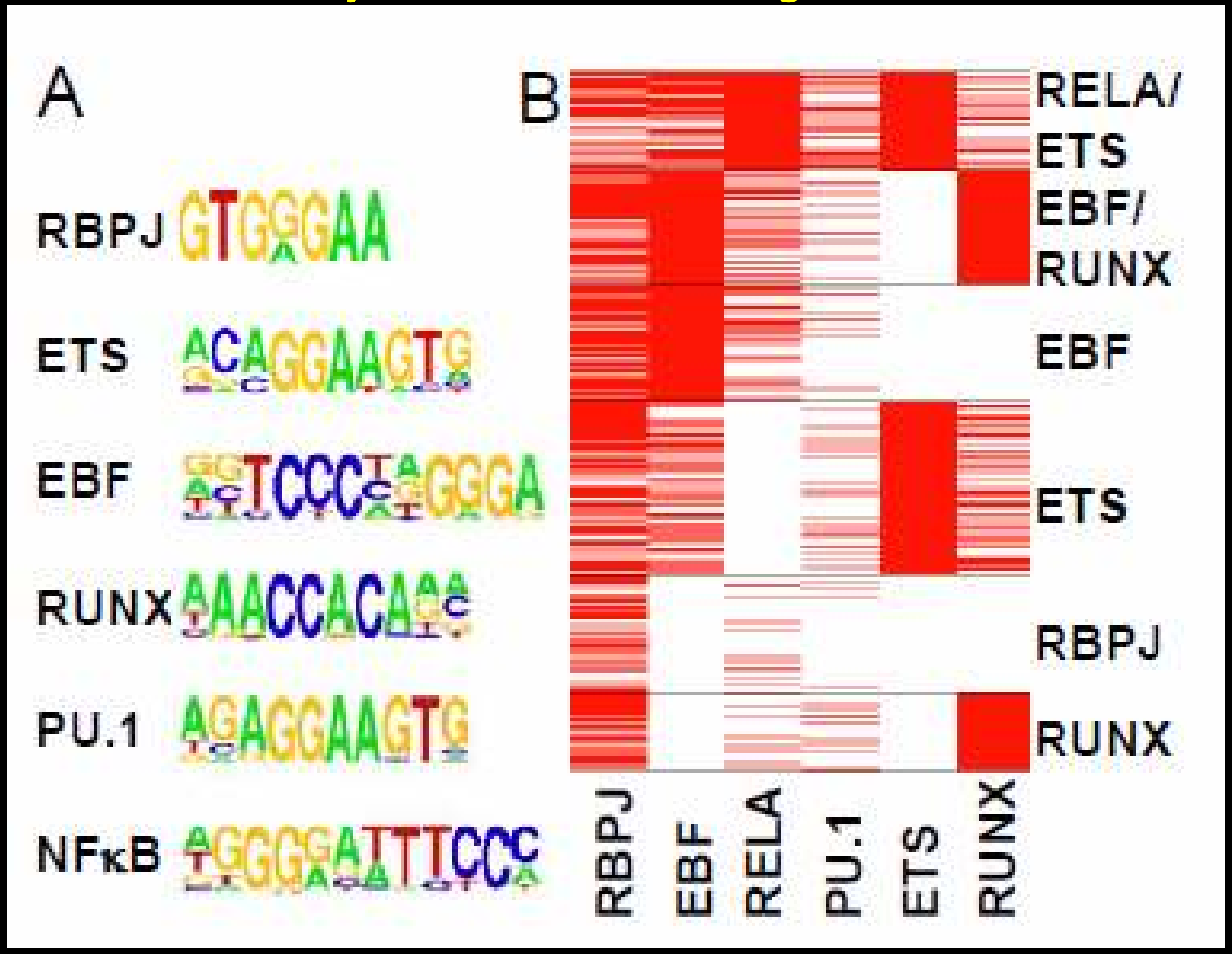
EBNA2 and RBPJ localize predominantly at Intergene and Intron sites, not at Promoter sites



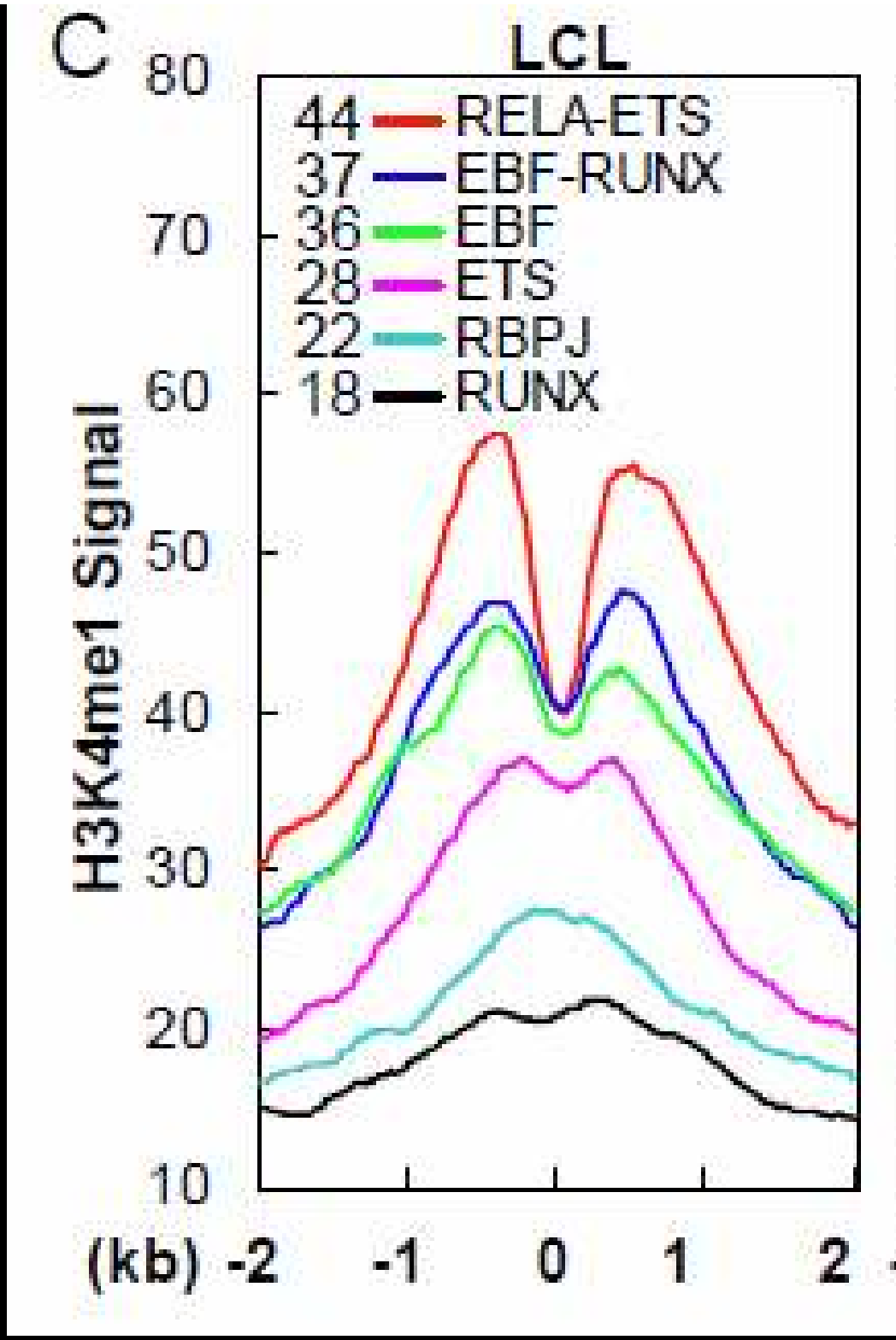
EBNA2/RBPJ has higher signal s than RBPJ at promoter and particularly at non-promoter sites



ChIP-Seq for EBNA2 and RBPJ reveals EBNA2 sites are enriched for RBPJ, ETS, EBF, RUNX , PU.1, and NF-kB RELA.
K-Means Clustering detects RELA-ETS, EBF, RUNX, EBF, ETS, RBPJ and RUNX clusters as associated factors at EBNA2 sites.
These stratify with H3K4Me1 signals at EBNA2 sites.



K-Means Clustering detected RELA-ETS, EBF, RUNX, EBF, ETS, RBPJ and RUNX clusters . These stratify with H3K4Me1 signals at EBNA2 sites.



RBLs have less, but highly similar H3K4me1 at sites for EBNA2 in LCLs.EBNA2 effects are superimposed on an RBL open chromatin state

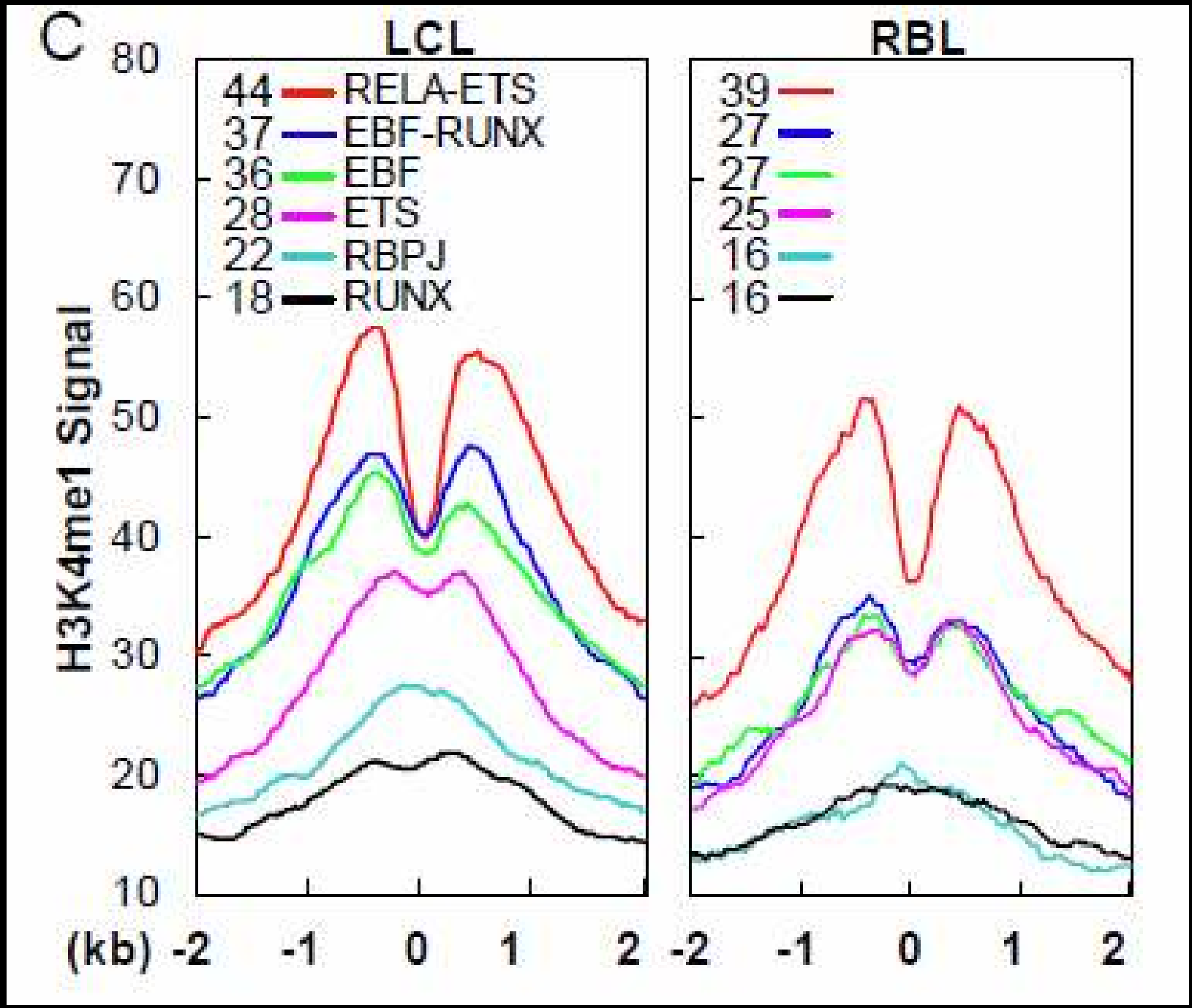


Table 2. Co-factors at EBNA2 and RBPJ sites

		EBF	PU.1	NF-kB	ETS	RUNX	CREB	P300
EBNA2/RBPJ								
(3710)	(#)	2240	620	1162	1300	1538	70	827
	(%)	60%	17%	31%	35%	41%	2%	22%
EBNA2 only								
(1441)	(#)	524	230	410	649	427	42	302
	(%)	36%	16%	28%	45%	39%	3%	21%
RBPJ only								
(6819)	(#)	2527	966	1638	2415	2513	339	1045
	(%)	37%	14%	24%	35%	37%	5%	15%

Table S1. TF motif prediction and actual TF binding comparison

TF motifs	Prediction (500 bp)	Actual binding (100 bp)
RBPJ	78%	72%
ETS	39%	
EBF	39%	54%
RUNX	43%	
PU.1	22%	17%
NF-κB	22%	31%

EBF is important for EBNA2 activation of the EBV LMP1 promoter.

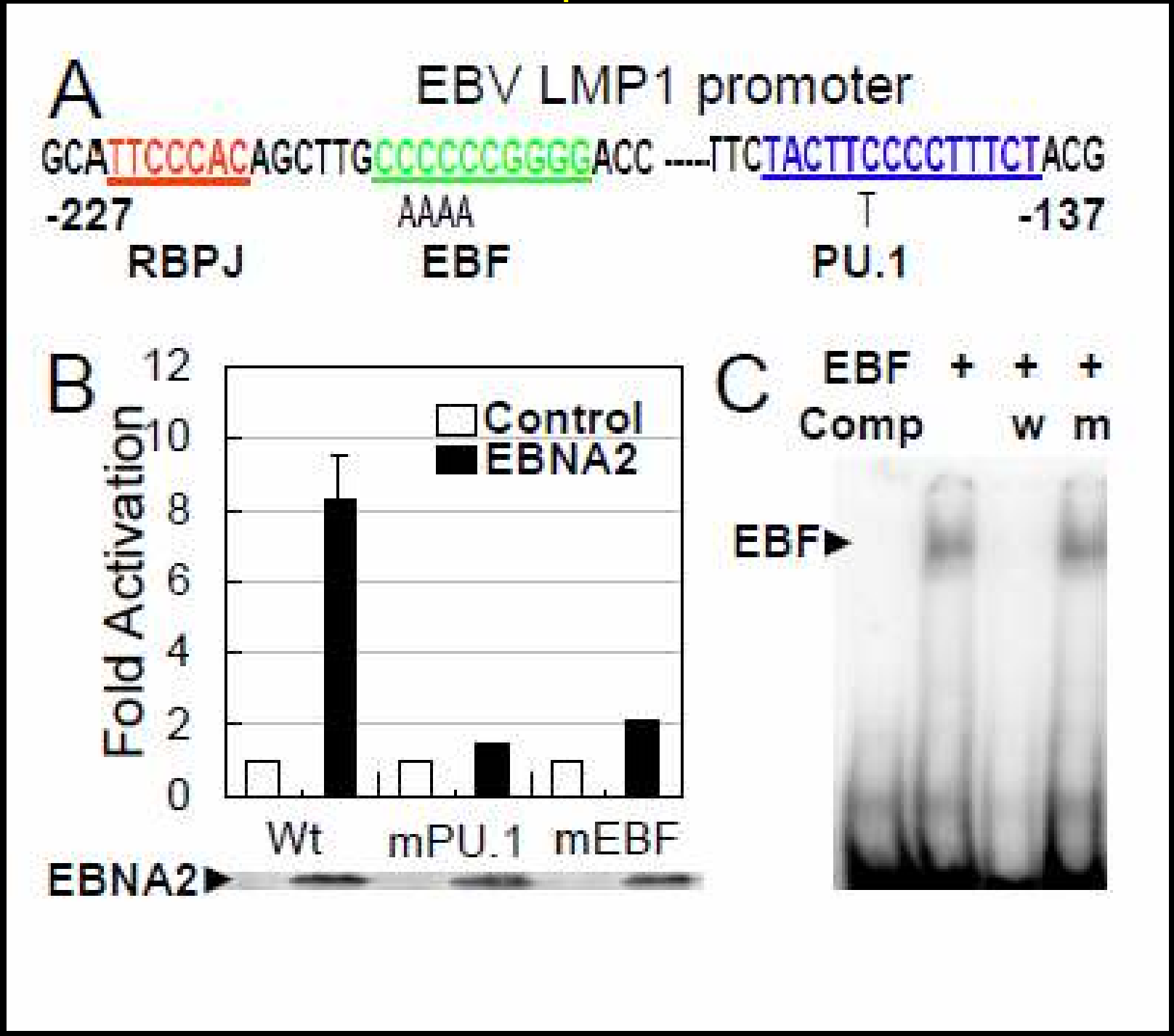


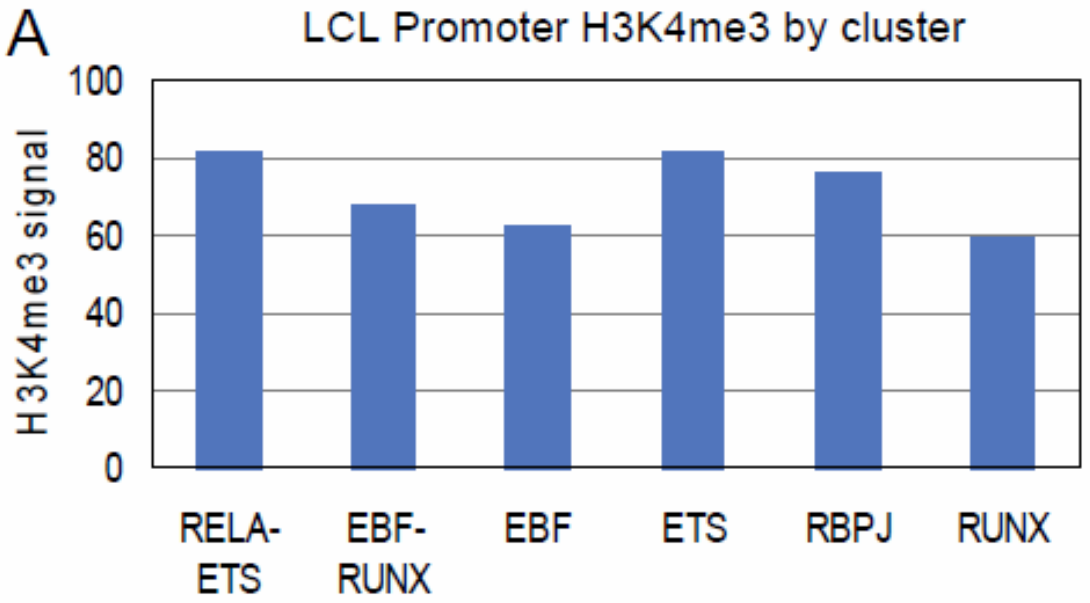
Table S1. EBNA2 and RBPJ DNA binding at different clusters

Clusters	Sites	Promoter (%)	P300 (%)	PolII (%)	EBNA2 Signal (RPKM)	RBPJ Signal (RPKM)
RELA-ETS	724	29	41	17	14	28
EBF-RUNX	841	8	37	3	20	42
EBF	859	9	30	4	17	36
ETS	1280	18	15	8	12	23
RBPJ	871	13	6	3	10	18
RUNX	576	7	5	1	11	23
RBPJ(No EBNA2)	6819	11	5	6	4	10

H3K4me1 signals at EBNA2 binding sites with different co-factors

	Fold change over baseline	P value	95% confidence interval
EBF	**1.68**	**$<10^{-4}$**	**(1.61-1.76)**
NFKB	**1.22**	**$<10^{-4}$**	**(1.14-1.30)**
Pu1	**1.21**	**$<10^{-4}$**	**(1.11-1.31)**
ETS	**1.08**	**$<10^{-4}$**	**(1.01-1.15)**
RUNX	0.99	ns	(0.92-1.06)

Figure S3. At LCL promoter sites, RELA-ETS, ETS, RBPJ, EBF-RUNX, EBF, and RUNX clusters correlate with progressively lower H3K4me3 and RELA-ETS, ETS, EBF, RBPJ EBF-RUNX and RUNX clusters correlate with progressively higher H3K27me3 signals (A and B).



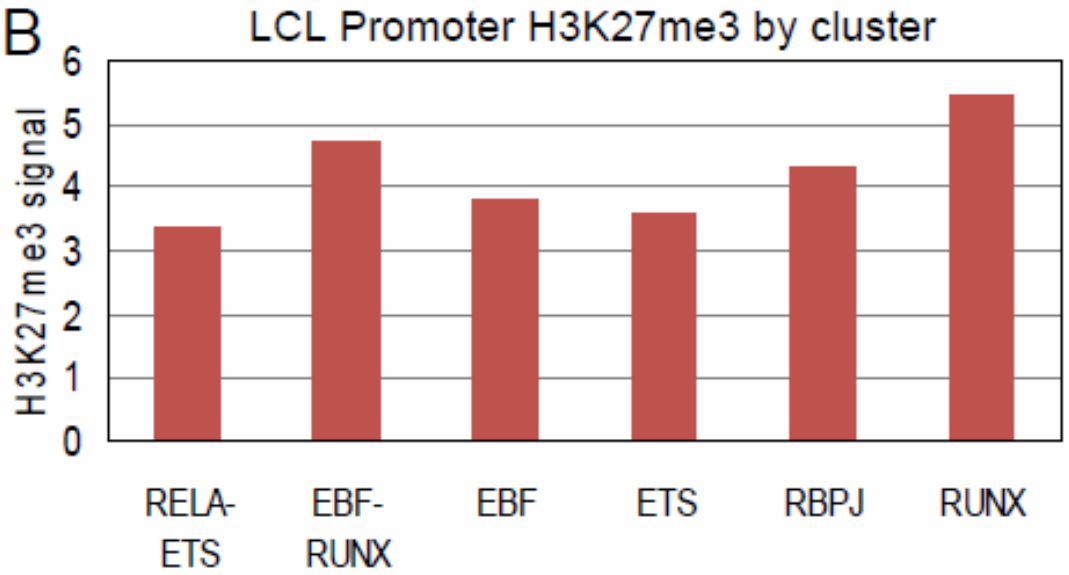
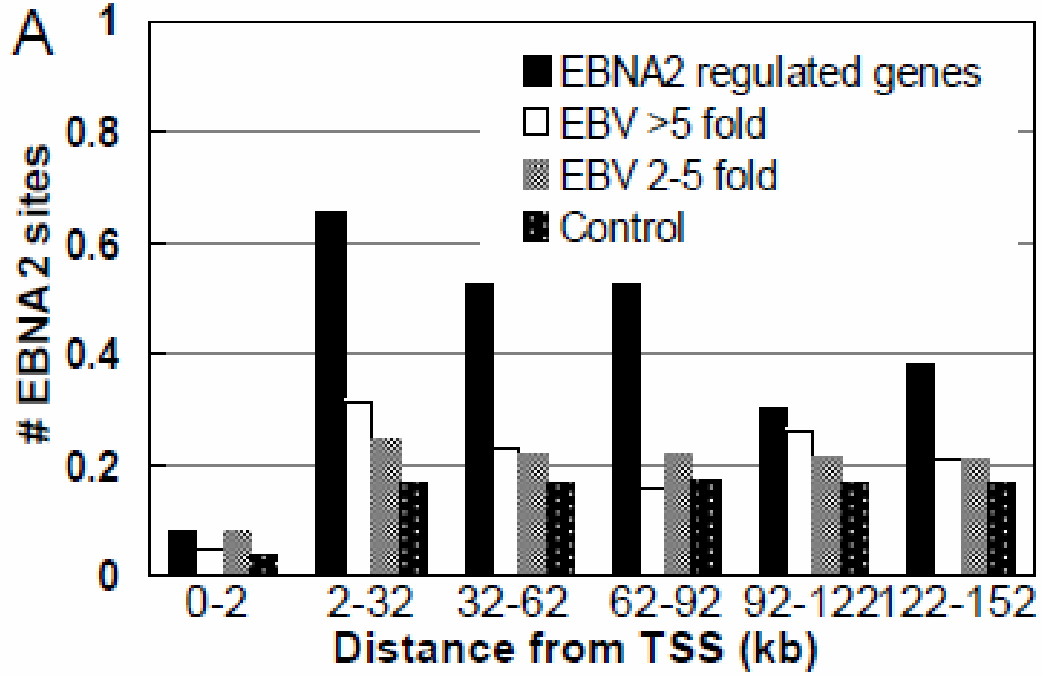
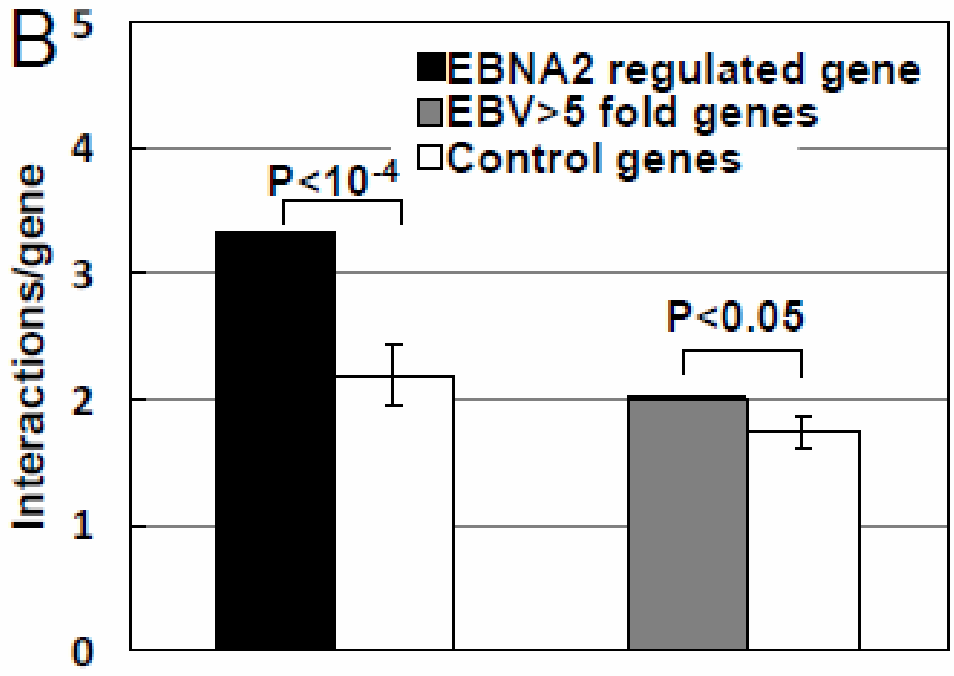
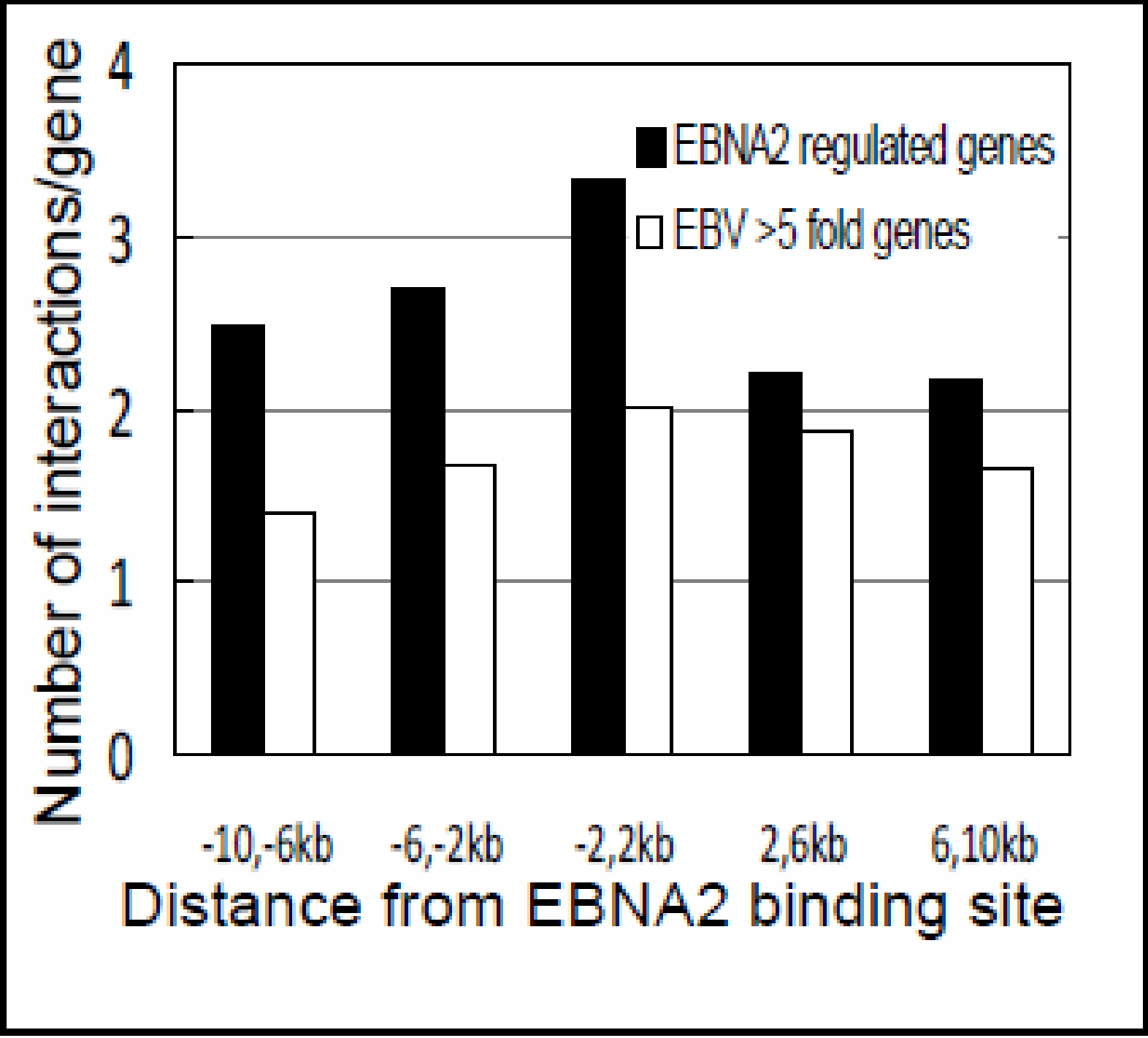


Figure 3. EBNA2 binding to non-promoter sites correlates with RNA up-regulation (A) and long-range putative enhancer and promoter interactions (B). 81 EBNA2 conditionally





HiC data based interactions



RBLs have less, but highly similar H3K4me1 at sites for EBNA2 in LCLs.EBNA2 effects are superimposed on an RBL open chromatin state

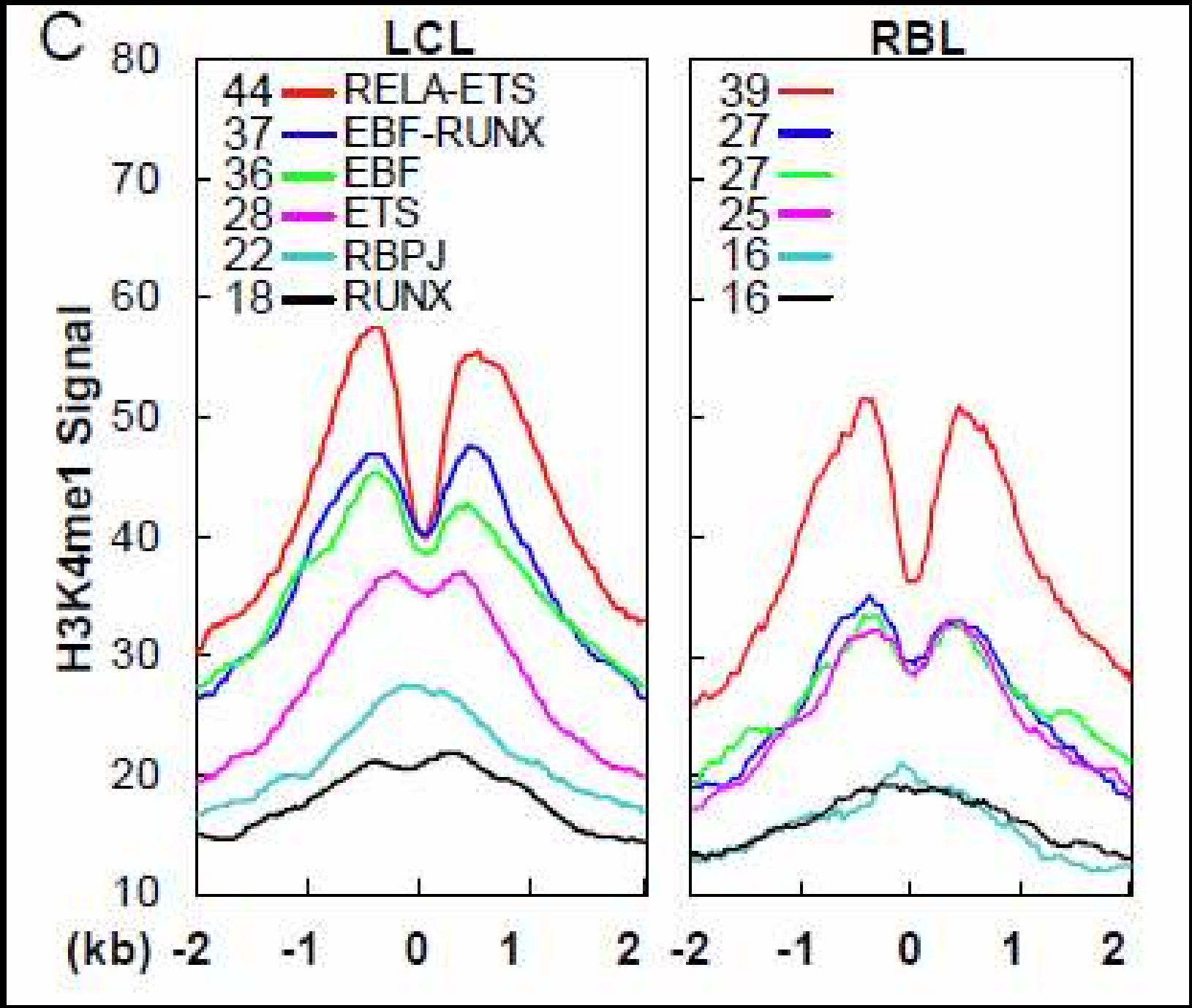
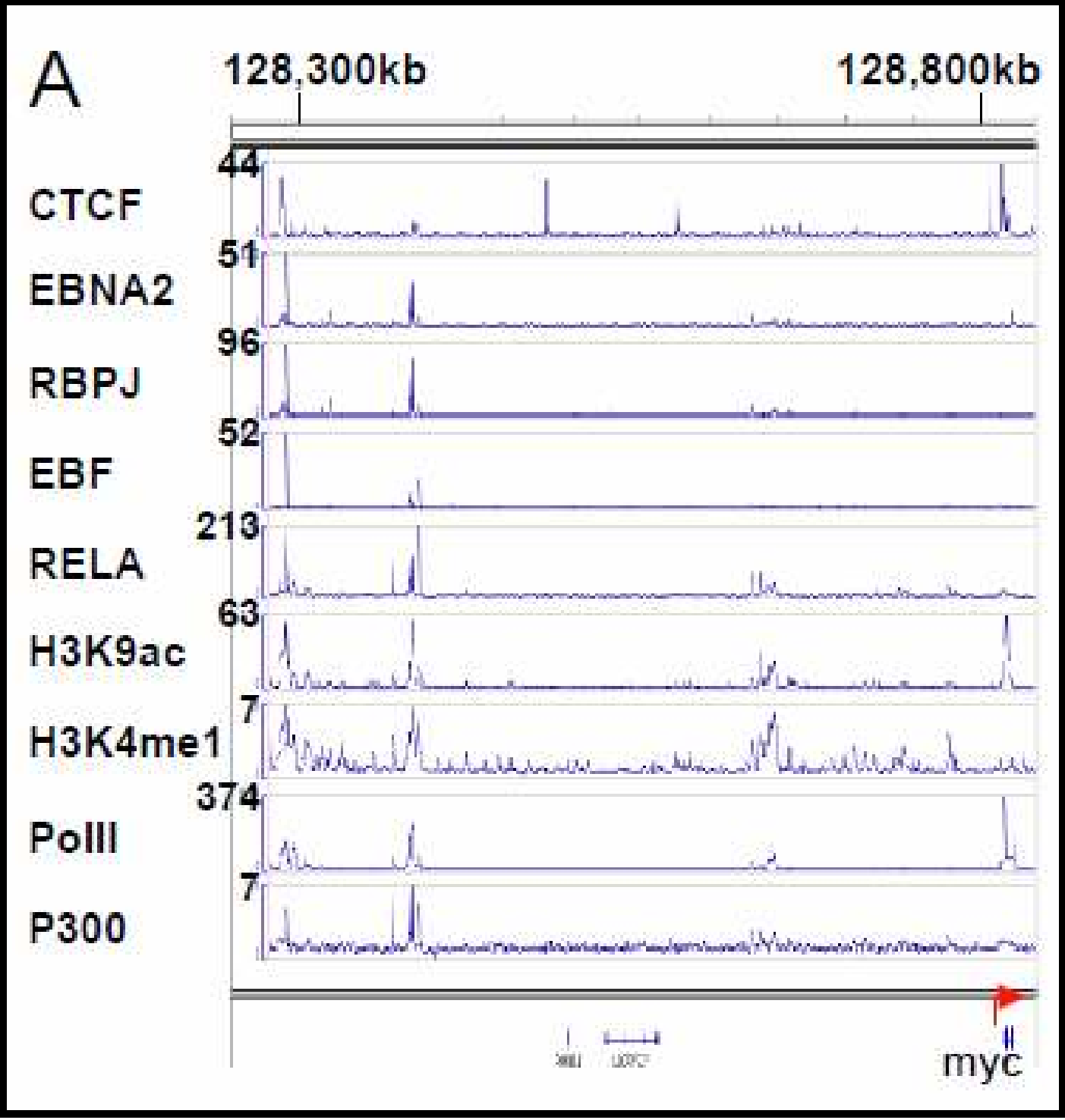


Table S3. EBNA2 DNA binding at 5,151 TF cluster sites, 72% with RBPJ, correlates specific TF clusters with promoter and nonpromoter p300, PolII, EBNA2, and RBPJ signals

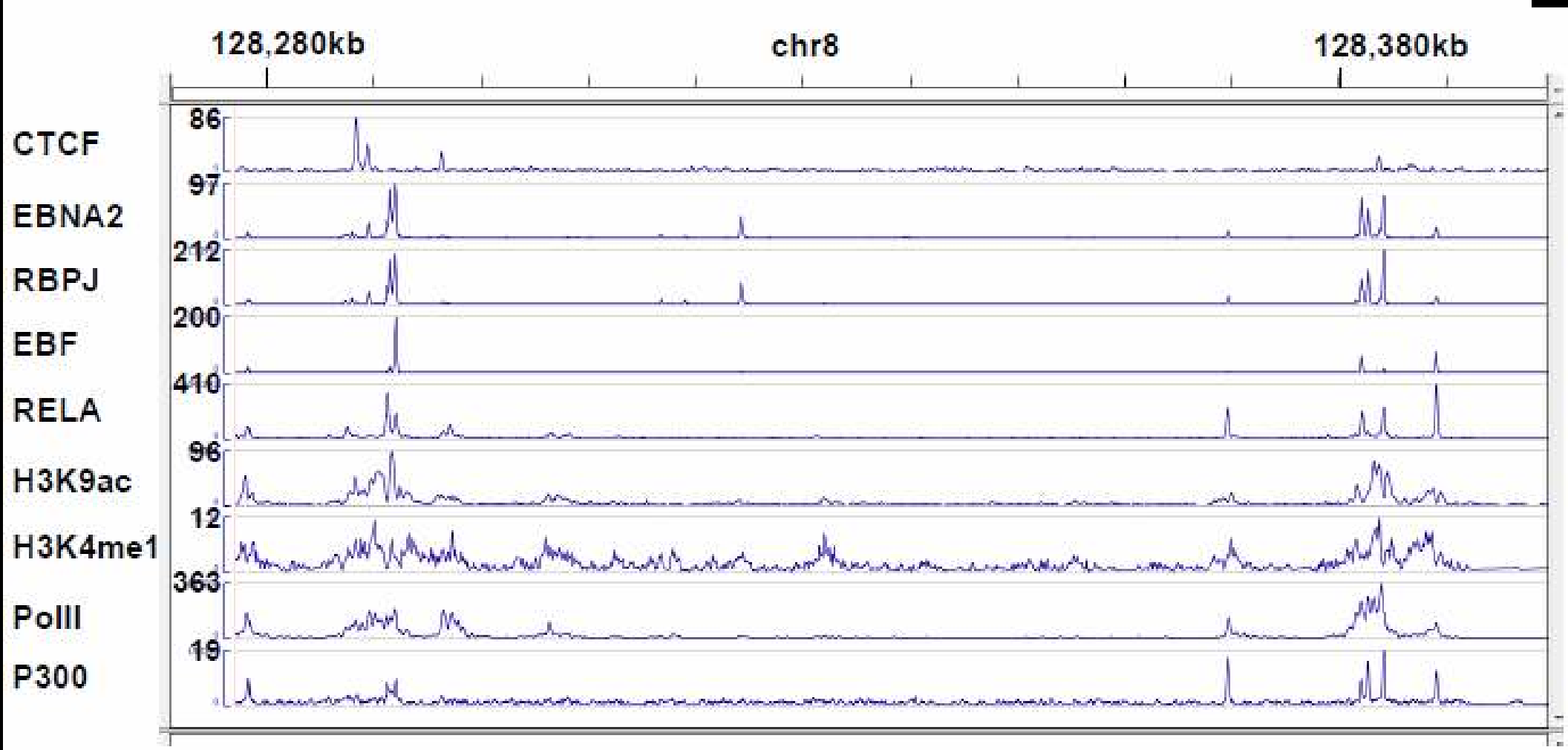
Clusters	Sites	Promoter (%)	P300 (%)	PolII (%)	EBNA2 signal (RPKM)	RBPJ signal (RPKM)
RELA-ETS	724	29	41	17	14	28
EBF-RUNX	841	8	37	3	20	42
EBF	859	9	30	4	17	36
ETS	1,280	18	15	8	12	23
RBPJ	871	13	6	3	10	18
RUNX	576	7	5	1	11	23
RBPJ(No EBNA2)	6,819	11	5	6	4	10

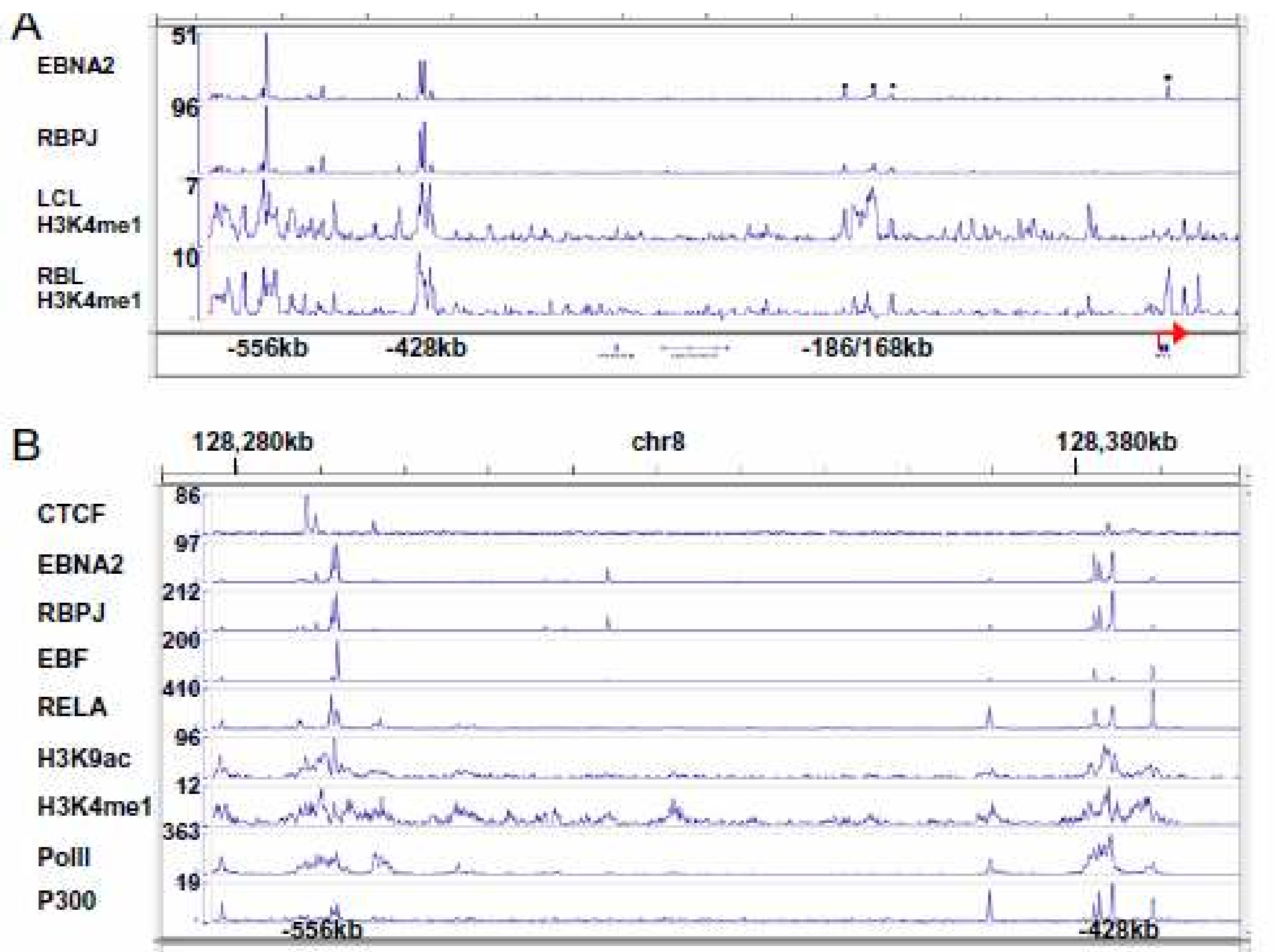
RPKM, reads per kilobase per million.

EBNA2 activates *MYC* through 428-556kb long range enhancers



Depiction of the 400-550kb MYC up-stream putative enhancers




A
EBNA2
RBPJ
LCL
H3K4me1
RBL
H3K4me1
51
96
7
10
-556kb
-428kb
-186/168kb
B
128,280kb
chr8
128,380kb
CTCF
EBNA2
RBPJ
EBF
RELA
H3K9ac
H3K4me1
PolII
P300
86
97
212
200
410
96
12
363
19
-556kb
-428kb

EBNA2 gene regulation through the RBPJ transcription factor is essential for RBLs conversion to Lymphoblast Cell Lines. ChIP-seq investigation of EBNA2 and RBPJ sites in LCL DNA found EBNA2 at 5151 and RBPJ at 10,529 sites.

EBNA2 localized with RBPJ, predominantly at intergenic and intron sites.

EBNA2/RBPJ sites were sites of EBF (60%) RUNX (41%), ETS(35%), NF-kB (31%), and PU.1(17%) motifs.

Using ENCODE LCL data, EBF, RELA, and PU.1 were found at 54%, 31%, and 17% of EBNA2 sites (open sites).

K-Means clustering of EBNA2 sites found RELA-ETS, EBF-RUNX, EBF, ETS, RBPJ, and repressive RUNX ranked highest to lowest in symmetrical H3K4me1 signals and EBNA2-centered H3K4me1 depletion, marks of nucleosome-depleted, transcription initiation.

Although less, high level symmetrical H3K4me1 modifications and nucleosome depletion at the same sites in RBLs were remarkably similar to LCLs, indicating that EBNA2 localized in a pre-established RBL chromatin pattern, which likely evolved to enable RBL antigen-induced proliferation.

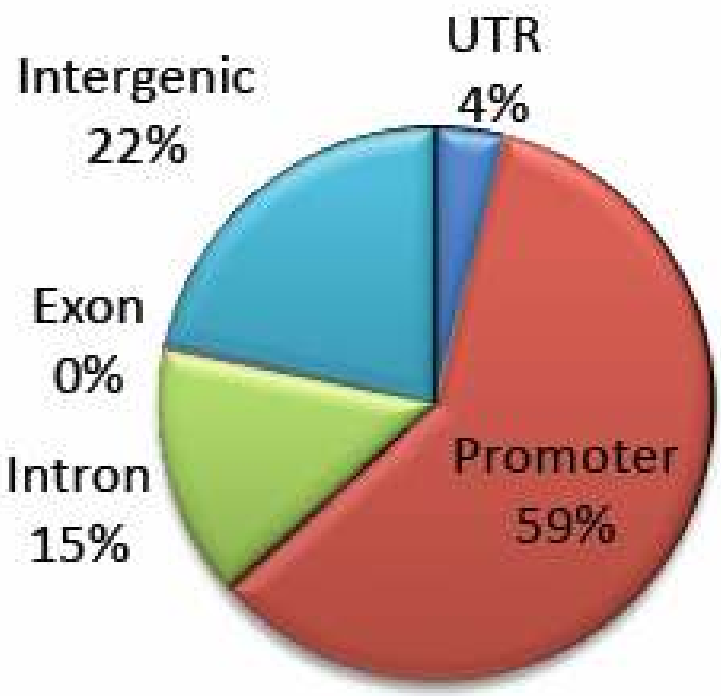
EBF was critical in EBNA2 activation of the EBV LMP1 promoter. LCL HiC data mapped >50 intergenic EBNA2 sites to EBNA2 up-regulated genes.

Fluorescence In Situ Hybridization (FISH), conditional EBNA2 FISH, Chromatin Conformation Capture (3C), and 3C q-PCR, linked EBNA2/RBPJ enhancers 428 kb 5' of *MYC to MYC.*

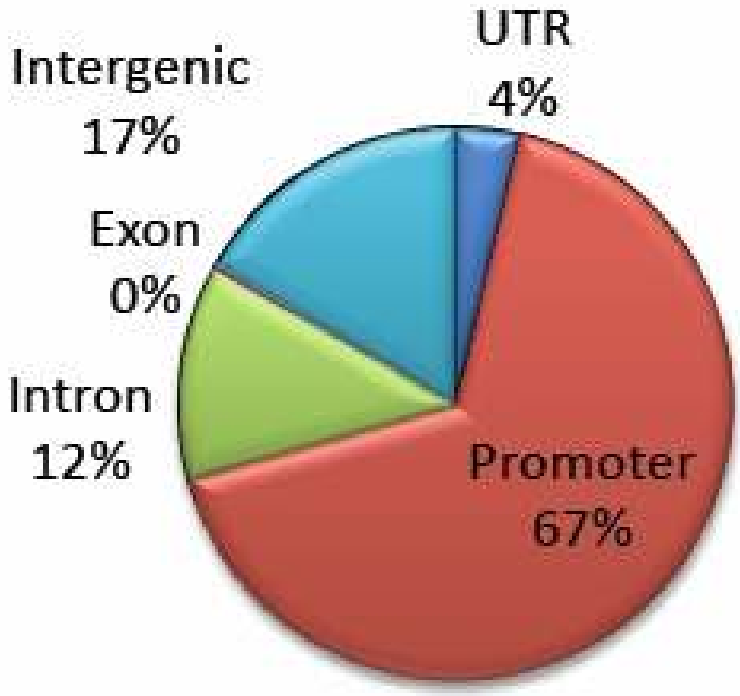
These data support the hypothesis that EBNA2 evolved to exploit the RBL transcription framework.

EBNA2 and RBPJ sites were 13% at promoters, whereas Notch1 and RBPJ sites were 59 and 67% at promoters

CUTLL1 Notch1: 3846 sites



CUTLL1 RBPJ: 2112 sites

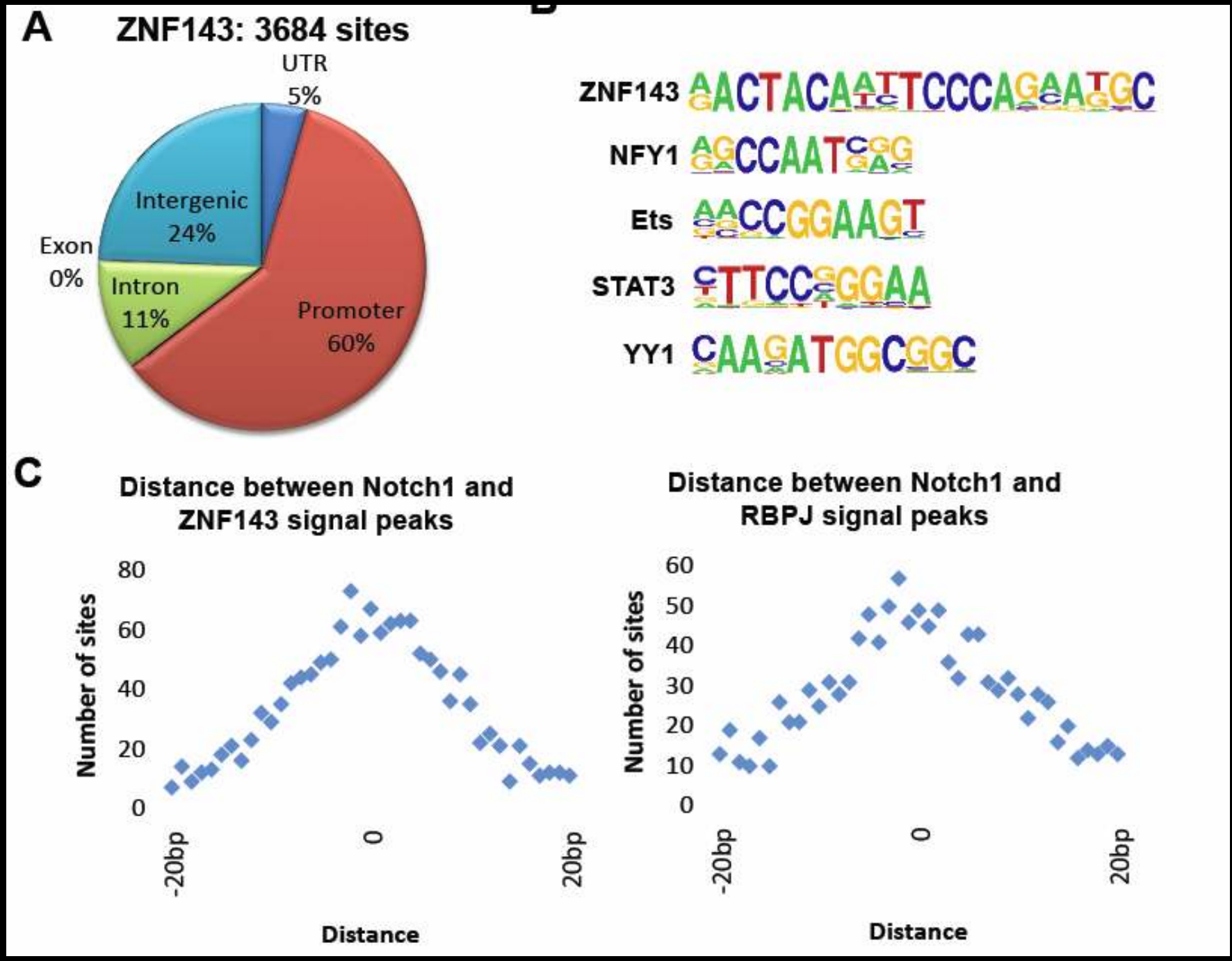


Notch1 sites

1. ZNF143 
2. Ets 
3. Runx 

RBPJ sites

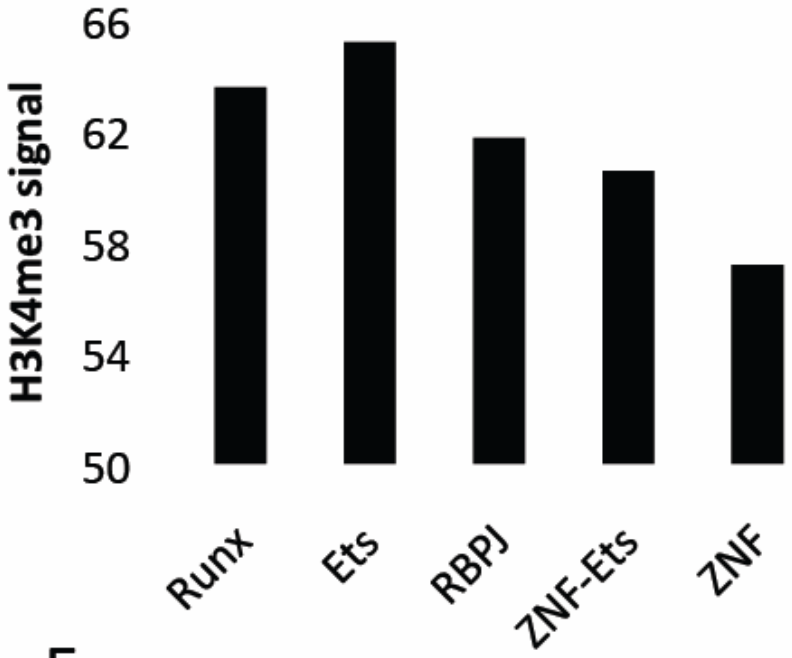
1. ZNF143 
2. CREB 
3. Ets 
4. Runx 


A
ZNF143: 3684 sites
UTR
5%
Intergenic
24%
Exon
0%
Intron
11%
Promoter
60%
ZNF143
NFY1
Ets
STAT3
YY1
C
Distance between Notch1 and
ZNF143 signal peaks
Number of sites
80
60
40
20
0
-20bp
0
20bp
Distance
Distance between Notch1 and
RBPJ signal peaks
Number of sites
60
50
40
30
20
10
0
-20bp
0
20bp
Distance

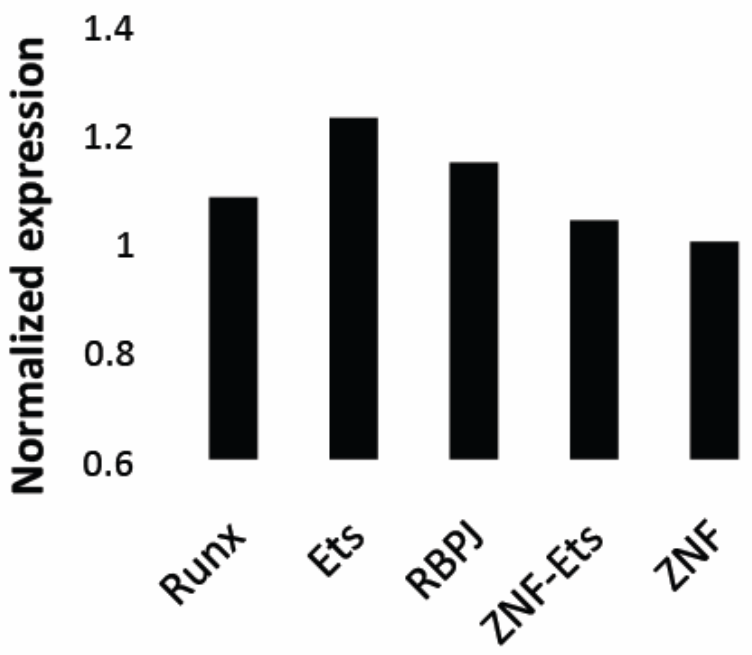
K means clusters of Notch Sites

	Fraction Promoter	Notch1 signal	RBPJ signal
Runx	0.21	11	7.6
Ets	0.62	8.6	8.7
RBPJ	0.54	9.2	8.3
ZNF-Ets	0.87	32	6.8
ZNF	0.84	39	7

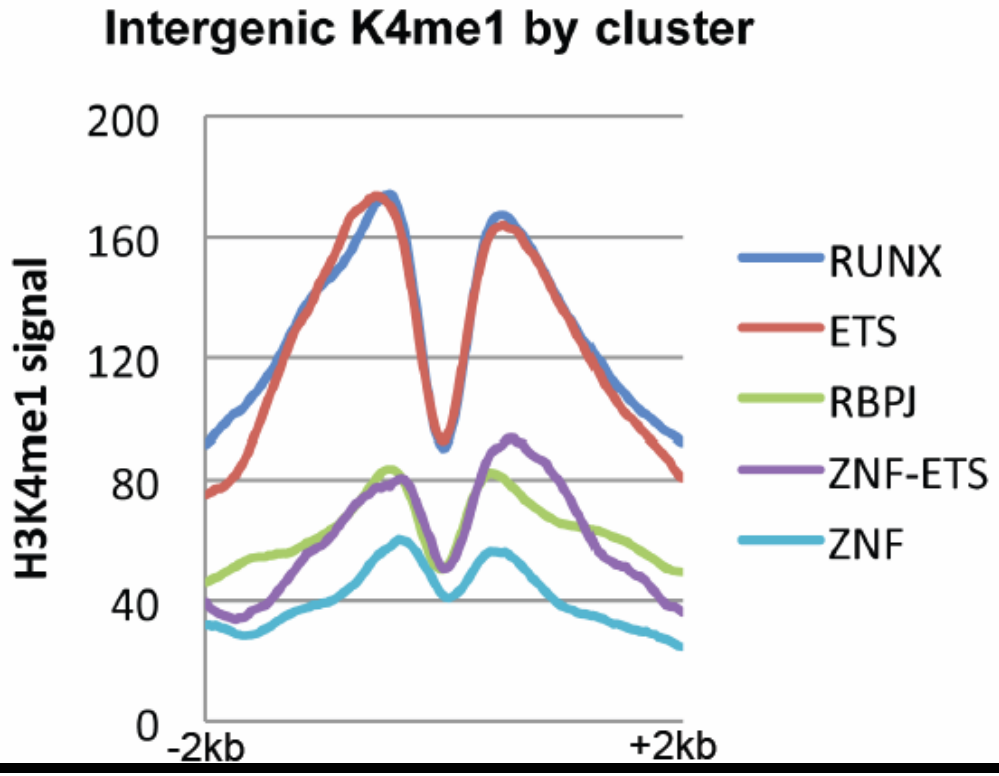
C


Promoter K4me3 by cluster
H3K4me3 signal
66
62
58
54
50
Runx
Ets
RBPJ
ZNF-Ets
ZNF

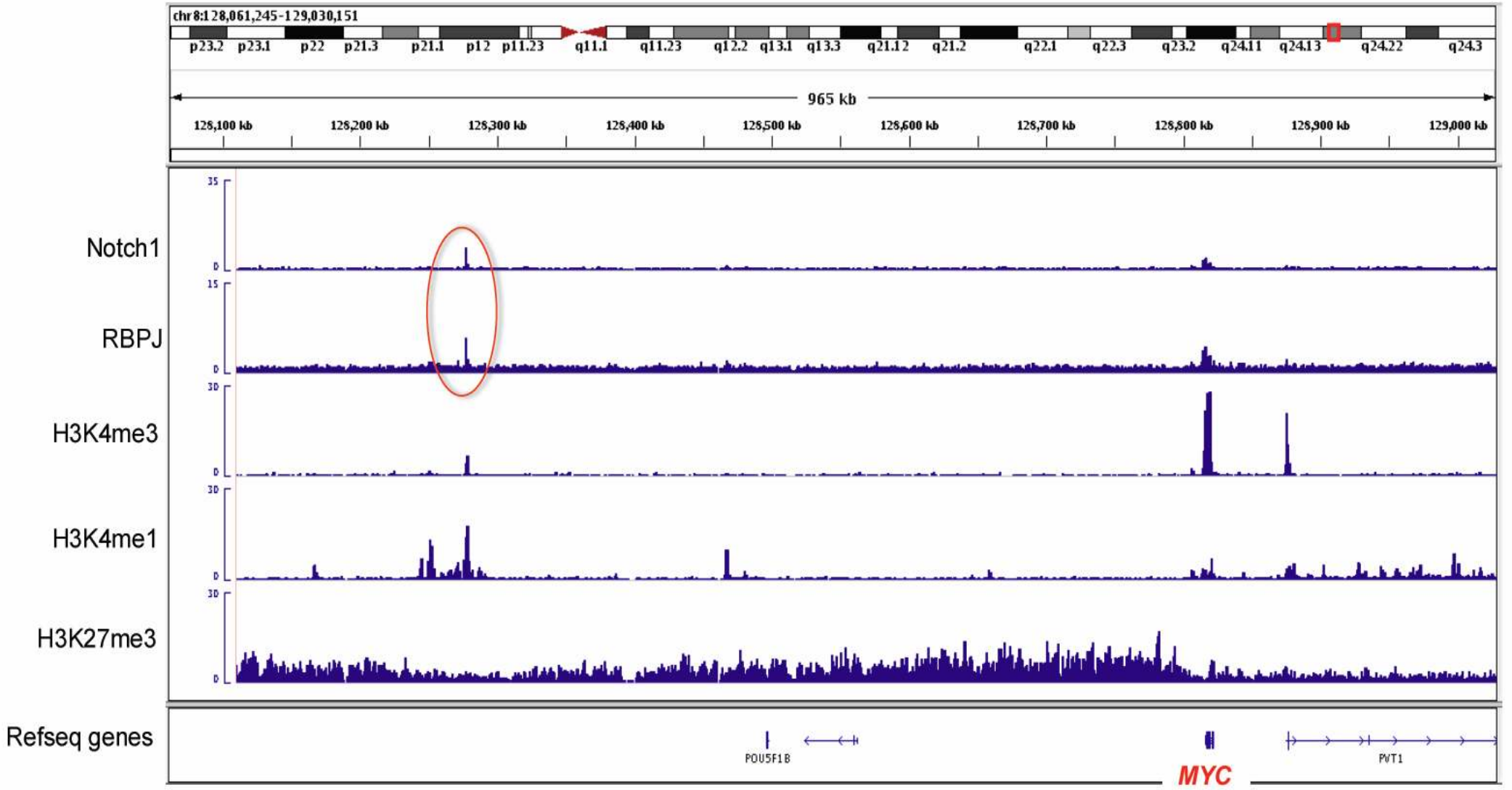
D


Gene expression by cluster
Normalized expression
1.4
1.2
1
0.8
0.6
Runx
Ets
RBPJ
ZNF-Ets
ZNF

E


Intergenic K4me1 by cluster
H3K4me1 signal
200
160
120
80
40
0
-2kb
+2kb
RUNX
ETS
RBPJ
ZNF-ETS
ZNF

E


chr8:128,061,245-129,030,151
p23.2 p23.1 p22 p21.3 p21.1 p12 p11.23 q11.1 q11.23 q12.2 q13.1 q13.3 q21.12 q21.2 q22.1 q22.3 q23.2 q24.11 q24.13 q24.22 q24.3
965 kb
128,100 kb
128,200 kb
128,300 kb
128,400 kb
128,500 kb
128,600 kb
128,700 kb
128,800 kb
128,900 kb
129,000 kb
Notch1
RBPJ
H3K4me3
H3K4me1
H3K27me3
Refseq genes
POU5F1B
MYC
PVT1

Gain-of-function mutations in Notch1 are common in human and murine T lymphoblastic leukemia (T-LL). Notch1 regulates gene expression by forming transcription activation complexes with the DNA-binding factor RBPJ. ChIP-Seq was therefore used to identify Notch1 and RBPJ binding sites in human and murine T-LL genomes. In both species, Notch1 bound preferentially to promoters, to RBPJ binding sites, and near imputed ZNF143, Ets and Runx sites. Human ZNF143 ChIP-Seq confirmed ZNF143 at ~40% of Notch1 sites. Notch1/ZNF143 sites often lacked detectable RBPJ and had high Notch1 and ZNF143 ChIP-Seq signals, yet were associated with less nucleosome depletion and lower promoter H3K4me3 or intergenic H3K4me1 signals than other Notch1 sites. Notch1 bound at ZNF143 sites was also resistant to gamma-secretase inhibitors, suggesting that these complexes are unusually stable. Notch1 binding site motif analysis identified imputed Runx, Ets, RBPJ, ZNF143-Ets, and ZNF143 motif clusters, with differing genomic distributions and chromatin marks. Notch1 binds ~25% of robustly affected target gene promoters in human T-LL cells and to candidate enhancers for other target genes, including *MYC*, *DTX1*, *IGF1R*, and *IL7R*. Human and murine T-LL genomes also had many sites that bind only RBPJ. Murine RBPJ "only" sites were highly enriched for imputed REST sites, whereas human RPBJ "only" sites lacked REST motifs and had imputed CREB sites. Thus, there is a conserved network of cis-regulatory factors that interacts with Notch1 to regulate gene expression in T-LL cells, as well as novel classes of divergent RBPJ "only" sites also likely to have roles in transcription regulation.

Hongfang Wang[1]*, James Y. Zou[2*+], Bo Zhao[3], Eric Johannsen[3], Todd Ashworth[1], Hoifung Wong[1], Stephen C. Blacklow[4], Kelly Arnett[4], Bradley E. Bernstein[5], Elliott Kieff[3+], Jon C. Aster[1]

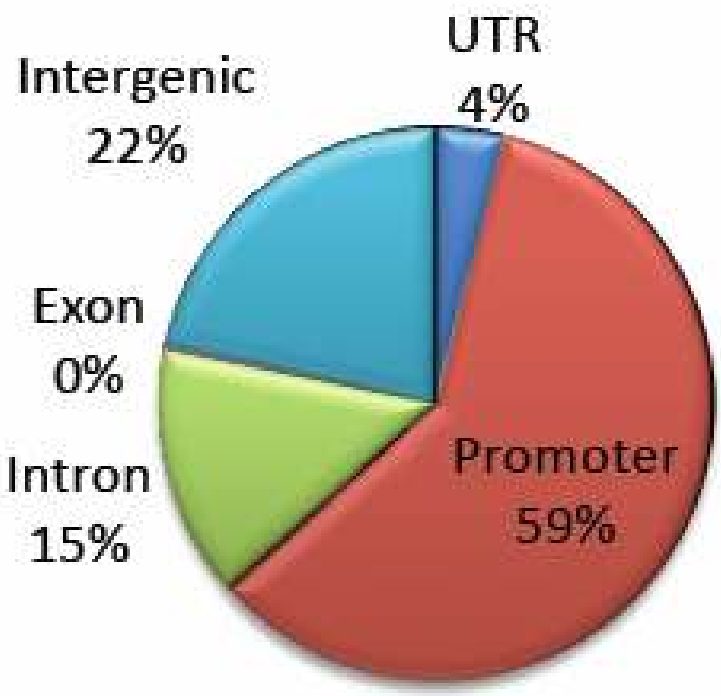
[1]Department of Pathology and [3]Departments of Medicine and Microbiology and Molecular Genetics, Brigham and Women's Hospital, Boston, MA, 02115
[2]School of Engineering and Applied Sciences, Harvard University, Cambridge, MA 02138
[4]Department of Cancer Biology, Dana Farber Cancer Institute, Boston, MA 02115
[5]Howard Hughes Medical Institute, Department of Pathology, Massachusetts General Hospital, Boston, Massachusetts 02114

Bo Zhao[1]*, James Y Zou[2, 3]*, Hongfang Wang[4], Eric Johannsen[1], Chih-wen Peng[1], John Quackenbush[5], Jess Mar[5], Cynthia Morton[4], Mathew Freedman[6], Stephen Blacklow[4] Jon Aster[4], Bradley E Bernstein[3], and Elliott Kieff[1]

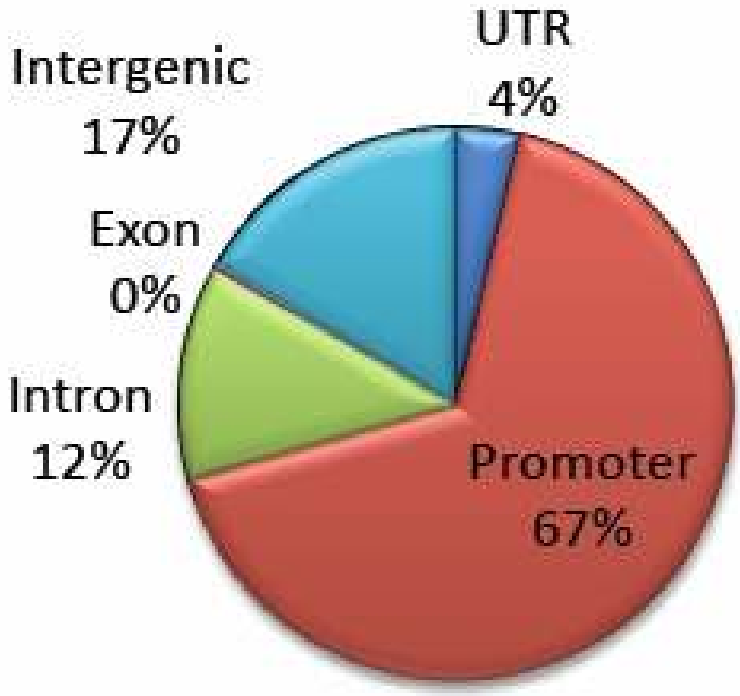
[1] Departments of Medicine and Microbiology and Molecular Genetics
Channing Laboratory
181 Longwood Avenue
Brigham and Women's Hospital
Harvard Medical School
Boston, MA 02115
[2] School of Engineering and Applied Sciences
Harvard University
[3] Broad Institute
Department of Pathology
Massachusetts General Hospital and Harvard Medical School
Boston, Massachusetts 02114
[4]Department of Pathology
Brigham and Women's Hospital
Harvard Medical School
Boston, MA, 02115
[5] Department of Biostatistics, Harvard School of Public Health, Boston, MA 02115, Department of Biostatistics and Computational Biology and Department of Cancer Biology, Dana-Farber Cancer Institute, Boston, MA 02115
[6] Department of Medical Oncology
Dana Farber Cancer Institute
Boston MA 02115, USA.
*These authors contributed equally to this work
Corresponding authors: Elliott Kieff, Channing laboratory, Brigham and Women's Hospital and Harvard Medical School, 181 Longwood Avenue, Boston, MA 02115
Phone: 617-525-4252. Fax: 617-525-4257. E-mail: ekieff@rics.bwh.harvard.edu

EBNA2 and RBPJ sites were 13% at promoters, whereas Notch1 and RBPJ sites were 59 and 67% at promoters

CUTLL1 Notch1: 3846 sites



CUTLL1 RBPJ: 2112 sites



Notch1 sites

1. ZNF143 
2. Ets 
3. Runx 

RBPJ sites

1. ZNF143 
2. CREB 
3. Ets 
4. Runx