**Supplementary Information**

# Dynamic long-range chromatin interactions control *Myb* proto-oncogene transcription during erythroid development

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## Contents

This section includes eight Supplementary Figures, Supplementary Materials and Methods, and Supplementary References.

## Supplementary Figure Legends

**Supplementary Figure 1** Transcription factor occupancy at the *Myb-Hbs1l* locus. (Related to Figure 1) **(A)** ChIP-Seq profiles of LDB1, GATA1, TAL1, ETO2 and associated controls. The *y*-axis represents ChIP-Seq tag densities. ChIP-Seq was performed in MEL cells (MEL) and in E13.5 primary fetal liver cells (FL E13.5). Genomic coordinates are shown at the top (UCSC Mouse July 2007 Assembly (mm9)). **(B)** close-up view of the -36kb, -61kb, -68kb, -81kb and -109kb binding sites showing perfect co-localization of the LDB1-complex components. **(C)** RNA-Seq profiles of *Myb* and *Hbs1l* in MEL cells. RNA-Seq signals covering *Myb* and *Hbs1l* exons are shown. The *y*-axis represents tag densities.

**Supplementary Figure 2** p300 does not occupy the *Myb-Hbs1l* intergenic region in fetal brain tissue and KLF1 occupies the -81kb enhancer *in vivo*. (Related to Figure1) **(A)** p300 ChIP-Seq tracks from published datasets showing no occupancy of the *Myb-Hbs1l* locus in the mouse developing forebrain and midbrain (Visel et al., 2009). **(B)** ChIP-Seq data showing

the binding of LDB1 and KLF1 at the *Myb-Hbs1l* locus in E13.5 and E14.5 fetal liver cells, respectively. **(C)** close-up view of the KLF1 -81kb binding site. KLF1 and associated control tracks were obtained from Tallack et al. (Tallack et al., 2010).

**Supplementary Figure 3** 3C-Seq experimental procedure. (Related to Figures 2 and 5) Cells are crosslinked with formaldehyde to preserve three-dimensional nuclear architecture. Crosslinked chromatin is subjected to restriction endonuclease digestion using an enzyme recognizing a 6-bp sequence (e.g. Hind III), diluted and ligated in conditions favoring intra-molecular ligation events. The digested and ligated chromatin is then de-crosslinked and submitted to a second restriction digest using an endonuclease recognizing a 4-bp sequence (e.g. Nla III) to reduce fragments sizes. The resulting digested DNA is then diluted and ligated in conditions favoring self-ligation events (resulting in circular DNA molecules). Viewpoint-specific primers harbouring Illumina adapter sequences are then used to amplify the (unknown) genomic DNA fragments that were ligated to the fragment of interest (viewpoint). PCR products are sequenced on an Illumina GAII platform generating 76 bp reads, which are then trimmed (viewpoint primer sequence removed) and aligned to the reference genome for visualization.

**Supplementary Figure 4** Long-range promoter-enhancer interactions within the *Myb-Hbs1l* locus are erythroid-specific. (Related to Figure 2) The *Myb* promoter 3C-Seq data obtained from FL E12.5 and FB E12.5 are shown (black bars) as in Figure 2, and the FL-specific interactions as compared to FB are shown in red ($p \leq 0.001$). The viewpoint fragment is indicated in blue and by the eye symbol.

**Supplementary Figure 5** Histone H3K36me3 profiles in mouse fetal liver cells and human K562 cell line. (Related to Figure 3) **(A)** The top track shows CTCF binding in K562 cells, the lower track depicts the K562 H3K36me3 ChIP-Seq profile. Data were obtained from the ENCODE database. **(B)** The top track shows CTCF binding in MEL cells, the lower track

depicts the H3K36me3 ChIP-Seq profile obtained from mouse Ter119-negative fetal liver cells (Wong et al., 2011).

**Supplementary Figure 6** Erythroid markers and cell size characteristics measured during differentiation show proper maturation of differentiating MEL and primary cells. (Related to Figure 5) **(A)** Messenger RNA (mRNA) levels of *Gypa* and *Hbb-b1* were measured during differentiation of MEL cells (left panel) and primary cells (Mouse E13.5 fetal liver cells cultured *ex vivo*, right panel) by qRT-PCR. Signals were normalized to *Rnh1* or *Calr* expression, and day 0 (MEL) or 0h (primary cells) values were set to 1. Bars represent mean values of 3 independent experiments; error bars denote SEM. **(B)** Cell size measurements performed during the differentiation of murine primary erythroid progenitors. Cell numbers are depicted on the y-axis and cell size on the x-axis.

**Supplementary Figure 7** Enhancer-associated proteins, histone modifications and polII occupancy during erythroid differentiation. (Related to Figures 3 and 6) **(A)** ChIP experiments performed in MEL cells before (Day 0; D0) and after (Day 4; D4) differentiation using an antibody (Ab) against the designated protein or histone modification. Control ChIP experiments are also shown (IgG). Bars represent mean values of at least 2 independent experiments, error bars denote SEM. **(B)** CTCF and total polII ChIP-Seq tracks are shown. The polII signal covers the whole *Myb* gene in undifferentiated MEL cells (Day 0), whereas in differentiated cells (Day 4) it is restricted to the first intron up to the CTCF site.

**Supplementary Figure 8** Knockdowns of *Ldb1* and *Klf1* in MEL cells and *Ctcf* in primary erythroid cells. (Related to Figure 7) Gene expression measurement of *Tal1*, *Gata1*, *Gypa*, and *Hbb-b1* after **(A)** Ldb1 or **(B)** Klf1 knockdown using two independent shRNAs. Note that the reduction in *Hbb-b1* gene expression after Klf1 knockdown is in accordance with previous reports (Nuez et al., 1995; Perkins et al., 1995; Pilon et al., 2008). **(C)** Ctcf knockdown in

primary erythroid cells (E13.5 fetal liver) and gene expression analysis of *Myb*, *Hbs1l*, *Ahi1* (primary transcripts), and *Gypa*, *Hbb-b1*, *Gata1*, *Klf1*, *Ldb1* (mRNA).

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## Cell Culture

Mouse erythroleukemia (MEL) cells and primary erythroid progenitors (obtained from fetal liver of E13.5 mouse embryos) were cultured as previously described(Soler et al., 2010). Primary mouse erythrocytes were differentiated at day 3 or 4 of *ex vivo* culturing by transferring them to StemPro-34 Serum-Free medium supplemented with 2 mM L-Glutamine, penicillin/streptomycin, 10U/ml EPO and 1 mg/ml Transferrin for 16 hours. Mouse dermal fibroblasts (MDF) were maintained under low oxygen (3%) conditions in DMEM/Ham's F-10 medium (50%/50%, Lonza). All animal experiments were carried out according to institutional and national guidelines. CDK9 inhibition was achieved by treating MEL cells with 100 μM of 5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) or 500 nM Flavopiridol. Cells were harvested at 1, 2 and 3 hours of treatment. RNA, protein and 3C material were prepared and analyzed as described below.

## Quantitative reverse transcriptase PCR

Total RNA was extracted from MEL cells or primary erythrocytes using Trizol extraction (Invitrogen) or the Qiagen RNeasy Micro Kit. First-strand cDNA was synthesized using the SuperScript® III First Strand Synthesis System and random hexamer primers (Invitrogen). Real-time PCR was performed using Platinum Taq Polymerase and SYBR Green (Invitrogen) on a Bio-Rad CFX96 PCR system. Ribonuclease/angiogenin inhibitor 1 (*Rnh1*) or Calreticulin (*Calr*) mRNA was amplified in parallel for normalization. Primer sequences are listed below.

## Luciferase assays

MEL cells were transfected with 0.5 μg pGL3 (Promega) constructs (containing the intergenic enhancer regions cloned upstream of the endogenous mouse *Myb* promoter driving luciferase expression) using GeneCellin (BioCellChallenge) according to the manufacturer's

instructions. Luciferase expression was measured 48 hours after transfection and normalized to Renilla signals.

**Antibodies**

The following antibodies were used in this study: anti-LDB1 (Santa Cruz, N-18), anti-VCP (Abcam, ab11433), anti-GATA1 (Santa Cruz, M-20), anti-TAL1 (Santa Cruz, H-60), anti-CDK9 (Santa Cruz, C-20), anti-TIF1γ (Bethyl Laboratories, A301-059A), anti-ETO2 (Santa Cruz, C-20), anti-CTCF (Millipore, 17-10044), anti-p300 (Santa Cruz, N-15), anti-polII (Santa Cruz, N-20), anti-H3K4Me1 (Abcam, ab8895), anti-H3K27Ac (Abcam, ab4729), anti-H3K36Me3 (Abcam, ab9050). KLF1 antibody (5-V) was kindly provided by Dr. Sjaak Philipsen. Ser2-P (3E10) and Ser5-P (3E8) polII antibodies were a kind gift of Dr. Dirk Eick.

**Western Blot**

Cells were lysed in RIPA-buffer (1× PBS containing 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS and protease inhibitors), boiled in Laemmli buffer and loaded onto NuPAGE precast 4-12% gradient Bis-Tris acrylamide gels (Invitrogen). Proteins were transferred to nitrocellulose membranes and probed for the protein of interest using the antibodies mentioned above. Fluorescent secondary antibodies were used for visualization. Pixel density measurements were performed using ImageQuant 5.2 software (Amersham Biosciences).

**RNA interference**

Lentivirus particles were produced as described (Addgene)(Soler et al., 2010). ShRNA sequences were cloned into the pLL3.7 backbone using standard methods, and cloning procedures were verified by sequencing. shRNA sequences used are as follows: *Ldb1* sh2 5'-GGACCAAAGAGATATACCA-3' and *Ldb1* sh3 5'-GACTCTGTGTGATACTAGA-3'. *Klf1* and *Ctcf* shRNAs were obtained from Sigma (TRC shRNA library): sh*Ctcf*: 5'-GCAGAGAAAGTAGTTGGTAAT-3'; *Klf1* sh3 5'-TGAGACTGTCTTACCCTCCAT-3' and *Klf1*

sh4 5'-CCACTTAGCTCTGCACATGAA-3'. Non-targeting shRNAs were used as a control. Cells were harvested 48 or 72 hours after transduction and processed for RNA/protein extraction as described above.

**Chromosome conformation capture (3C) and 3C-sequencing procedure**

HindIII was used as the primary restriction endonuclease. Purified 3C DNA was either used for quantitative Taqman PCR to detect individual interaction events (Splinter et al., 2006) or used for 3C-Seq library preparation. The PCR signals were normalized as described (Palstra et al., 2003), with the highest crosslinking frequency per graph set to 1. Due to high variability in overall crosslinking frequencies using *ex vivo* cultured FL cells, a locus-internal control fragment (the 4$^{th}$ HindIII fragment downstream of the -81kb binding site, which showed consistently low crosslinking frequencies) was set to 1. The 3C-Seq library was prepared as described (Supplementary Figure 3) (Soler et al., 2010), using either NlaIII (*Myb* prom and -36kb viewpoints) or DpnII (-81kb viewpoint) as secondary restriction endonucleases. Viewpoint-interacting DNA fragments were PCR amplified using viewpoint-specific divergent primers (sequences listed below) linked to standard Illumina adapter sequences. The resulting 3C library was single-read sequenced on the Illumina Genome Analyzer II platform generating 76 bp reads. Images were recorded and analyzed by the GAP pipeline. The resulting reads were trimmed to remove viewpoint-specific primer sequences and mapped against NCBI build 37.1 of the mouse genome using ELAND alignment software. To calculate the coverage, aligned reads were extended to 56 bp in the 3' direction using the r3C-Seq pipeline (Thongjuea, Stadhouders et al., in preparation). The interaction-enriched regions per HindIII fragment were measured by calculating the number of reads per million (RPM) per restriction fragment, requiring at least 1 or more RPM. Comparisons of interactions per HindIII fragment between datasets were tested for significance using a combined running mean/Poisson distribution method at a cut-off of P≤0,001. In brief, Interactions were defined as "true" when they satisfied the following two criteria:

(1) An adjusted p-value ≤0.001

(2) A fold change (interaction signal of fetal liver/interaction signal of fetal brain) ≥2.

P-values were calculated based on a combination of the Poisson distribution and running mean. In order to obtain p-values of interactions per restriction fragment from the Poisson distribution, we calculated λ (the mean interaction signal per restriction fragment) for the Poisson probability function. We generated λ as follows: (1) in the same restriction fragment from both fetal liver and brain, we defined λ(local) as the mean interaction signal from fetal brain and (2) we defined λ(global) by calculating the mean interaction signal in each 100 KB overlapping window chromosome-wide (running mean) from fetal liver and taking the mean of these. The final λ was defined as the maximum value of λ(local) and λ(global). This λ was used in the Poisson probability function described below:

$$p(x) = \frac{e^{-\lambda}\lambda^{x}}{x!}$$

Where *x* is the interaction signal per restriction fragment, *p*(*x*) is the probability of each interaction signal per restriction fragment and λ is λ(final). Finally, p-values were corrected using the Bonferroni correction method. Data were visualized using a local mirror of the UCSC genome browser.

**Primer Sequences**

ChIP

Myb prom F:TCTTTGTTTGATGGCATCTGTT, Myb prom R:AAAGGGGAGGAGAAGGAGGT

-36kb F:TCACTTCCTTCCTGTCTCG, -36kb R:GTCTGGTGGCGATGACTTA

-61kb F:GTTGGGCAAAGATACTGGAT, -61kb R:GCTCTAACCTCACTGTTTCA

-68kb F:ATCCAAGTGACGGTGACA, -68kb R:GCATCCTGATTGTGCTAACT

-81kb F:CCCAAGTGAGAGAAATGTTGAA, -81kb R:TGTTATCAGGGCGGTTCC

-109kb F:CTCCAACATCAGCCGACT, -109kb R:ATTGTGAGGTGAGTGCCC

Gata1 enh. F:TCAGGGAAGGATCCAAGGAA, Gata1 enh. R:CCGGGTTGAAGCGTCTTCT

Amylase F:CTCCTTGTACGGGTTGGT, Amylase R:AATGATGTGCACAGCTGAA

Myb -23kb F:GTGGTTCATTTCAGTGGCA, Myb -23kb R:CCGTGTCCTCTAACTCTTGA

Myb +1.5kb F:AAAGCGAAAGGCGAAACC, Myb +1.5kb R:GCATCGGAACAAGAAGGG

CTCF intr.1 F:CAATAGTCAGGTGAGGGAGA, CTCF intr.1 R:ACTAGGTGCGTGGACAAC

Myb intr.3 F:CAGAAATGAACACAGCCAAA, Myb intr.3 R:TCTCAACTTACAGTCCCACT

Myb intr.14 F:GATGAGTTTCAGGGAGTGTC, Myb intr.14 R:CTTTCTGTGTATGTGTTTGGG

Expression analysis

Myb PT F:AAGCAGGAATCGGATGAATC, Myb PT R:AGCAGTGATGTCAAACAGTT

Myb-5' F: CGCATCTGTTGCTCTCTG. Myb-5' R:TCCTCTCGGAACTGTCAG

Hbs1l PT F:AACCCTGAAGAAGAATATGGC, Hbs1l PT R:TCAGCCAAAGAAGACATGC

Ahi1 PT F:AGAACGCAAAGCCCAAAC, Ahi1 PT R:ATCCCACAAAGGCACATAAT

Rnh1 F:TGCAGGCACTGAAGCACCA, Rnh1 R:TCCAGTGTGAGCAGCTGAG

CalR F:GACTTTCTGCCACCCAAG, CalR R:GTTCCCACTCTCCATCCA

Ldb1 F:GTGACAATCTCTGGTGGGA, Ldb1 R:GGAAGTAGCGTGGTATCAG

Klf1 F:CTGTCTTACCCTCCATCAGT, Klf1 R:AAATCCTGCGTCTCCTCA

Gata1 F:TGCCTGTGGCTTGTATCA, Gata1 R:TGTTGTAGTGGTCGTTTGAC

Tal1 F:GTTCACCAACAACAACCG, Tal1 R:ATTCACATTCTGCTGCCG

Gypa F:TGAAGTGTCTGCTGCGTT, Gypa R:CCGATAATCCCTGCCATCA

Hbb-b1 F:ACTGATGCTGAGAAGGCT, Hbb-b1 R:CAGAGGCAGAGGATAGGTC

3C Taqman qPCR

Myb prom universal F:AGTTCAAGACTTGTGCTGACTG

Myb prom universal Probe:5'-FAM-AACCCCTAAAGCACTTGGGACACTCACAC-3'-BHQ1

20898686 R:CCTAGGTTCTCCTCTCCTACCA,

20904395 R:GACAATTTGACATGAATTGCAAGC

20909424 R:CAAGAACCAAGACGCCTCAG

20915439 R:AGTAAATCTTGCTGCCCTCAAG

20923231 R:TGCCTTGGGCAGTTCTATAGAG

20939397 R:TCCTTACTTCTGCTCTCAAACAC

20944478 R:CTTTGTAGGTCACTTTCTCCAGC

20947418 R:AGCAGGCTATTGTGAAAAGAGG

20950525 R:ACACATAGGCACAGAGGAAAGTT

20957119 R:CAACCTTTTCACTGGCAGAAATG

20974886 R:GAAGACCACTTAGGTAAACACTTTG

20978637 R:TGACACATTTGCTGCGAACAG

20986398 R:TGGAAAAGATCAGTAAGGCCAGA

21006046 R:ACTACATAACTTGGGTGTGTTGG

21013311 R:TGCTTCTTGGTCCCGGTGT

3C-Seq

Myb prom viewpoint F (HindIII):TCAAACATAGCTCAGAAAGCTT

Myb prom viewpoint R (NlaIII):ACCCTTTAATACAAGTTTCTCATG

-36kb viewpoint F (NlaIII):GGAAGGGACCACAGCATG

-36kb viewpoint R (HindIII):CTGCCCTCAAGCAAAGCTT

-81kb viewpoint F (HindIII):AGATTGTTTTTGGAAATAAGAAAAGCTT

-81kb viewpoint R (DpnII):CCACTTCAGCTCTTCTCACTCTATTAGATC

**Supplementary References**


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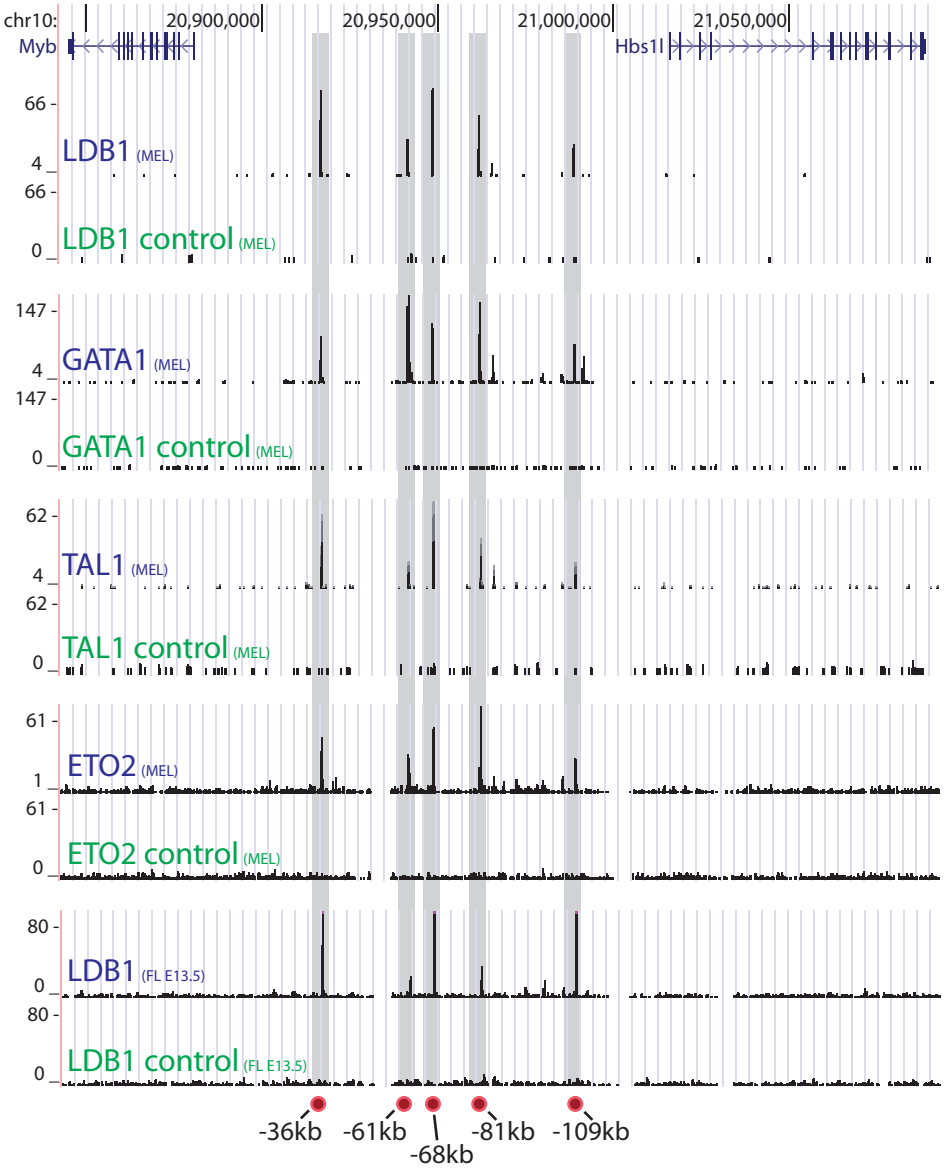
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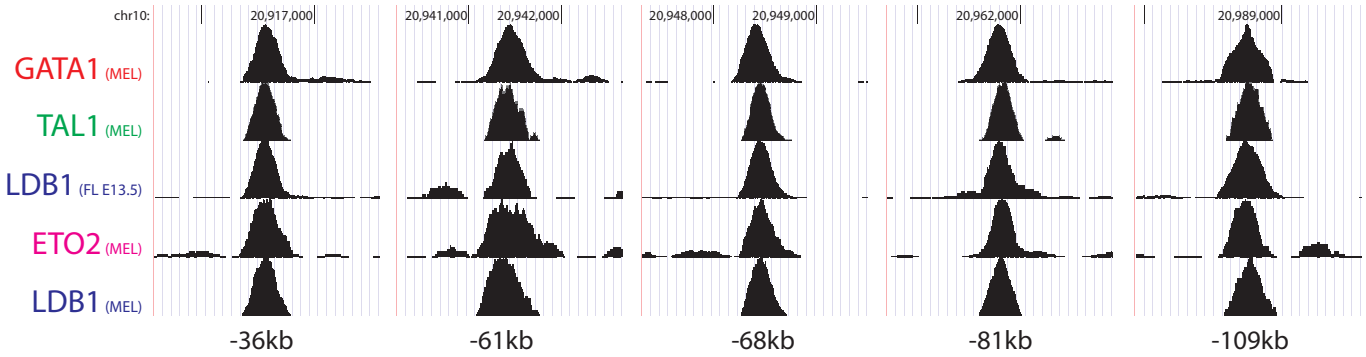
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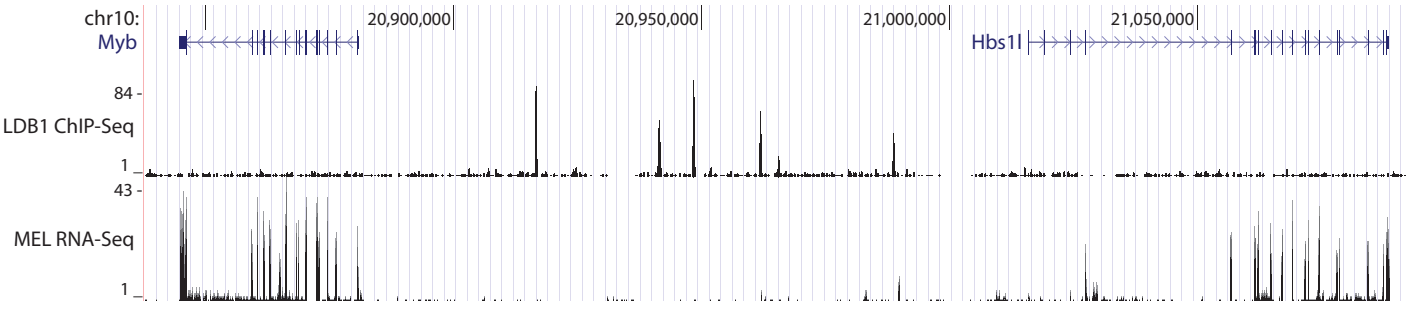
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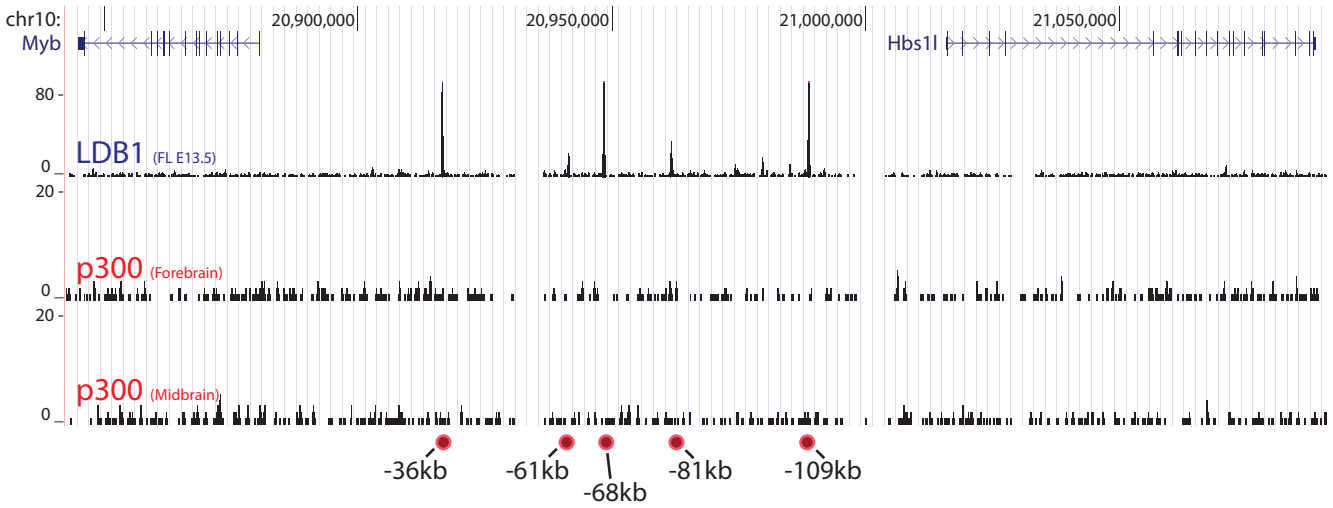
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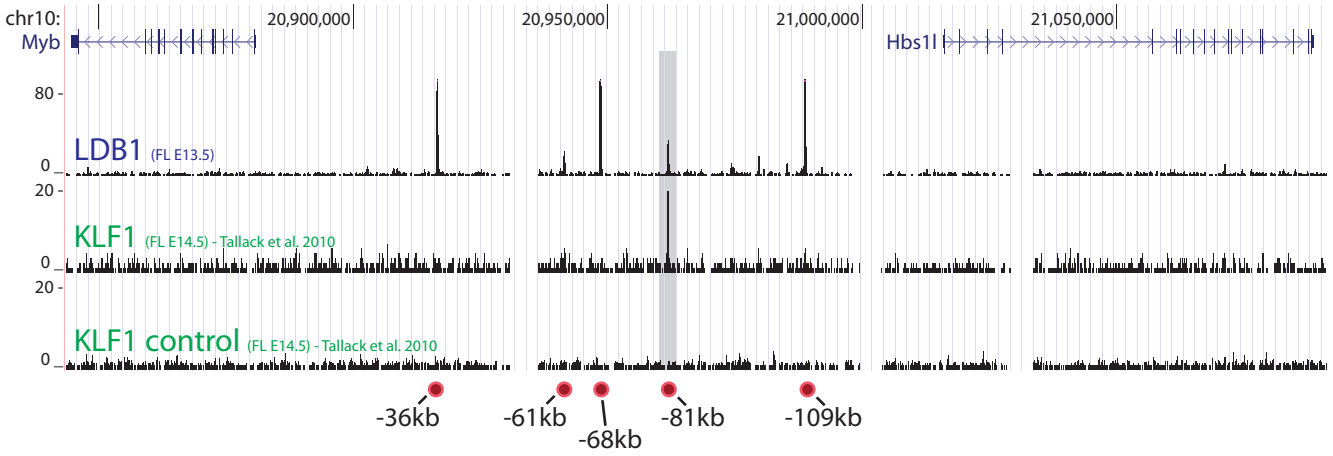
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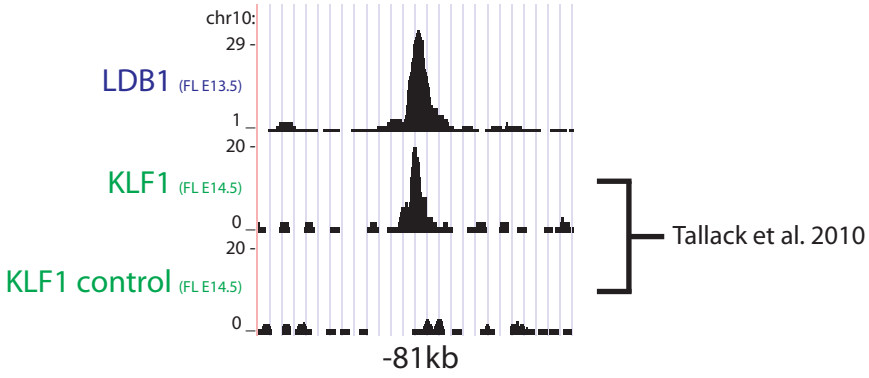
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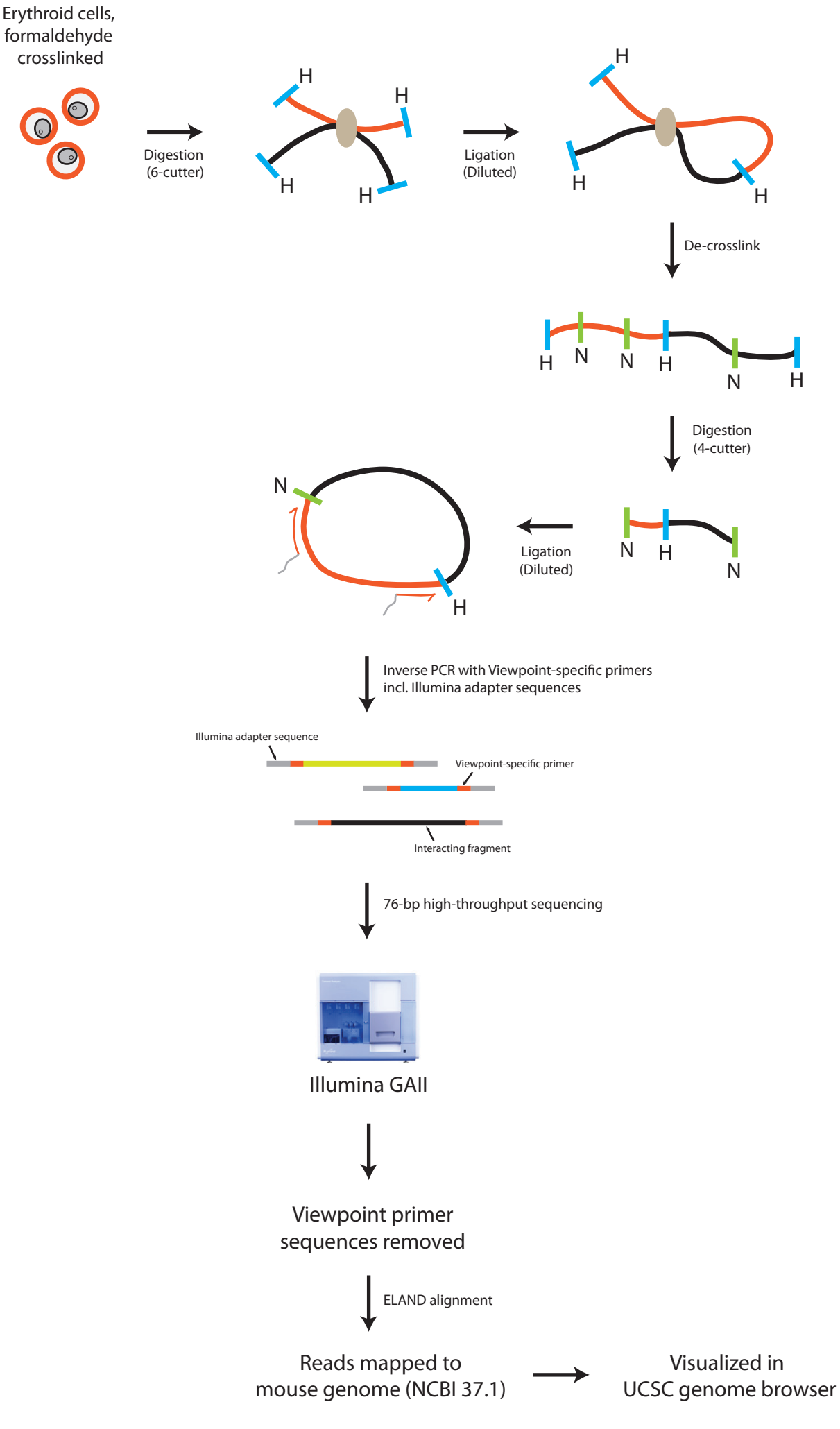
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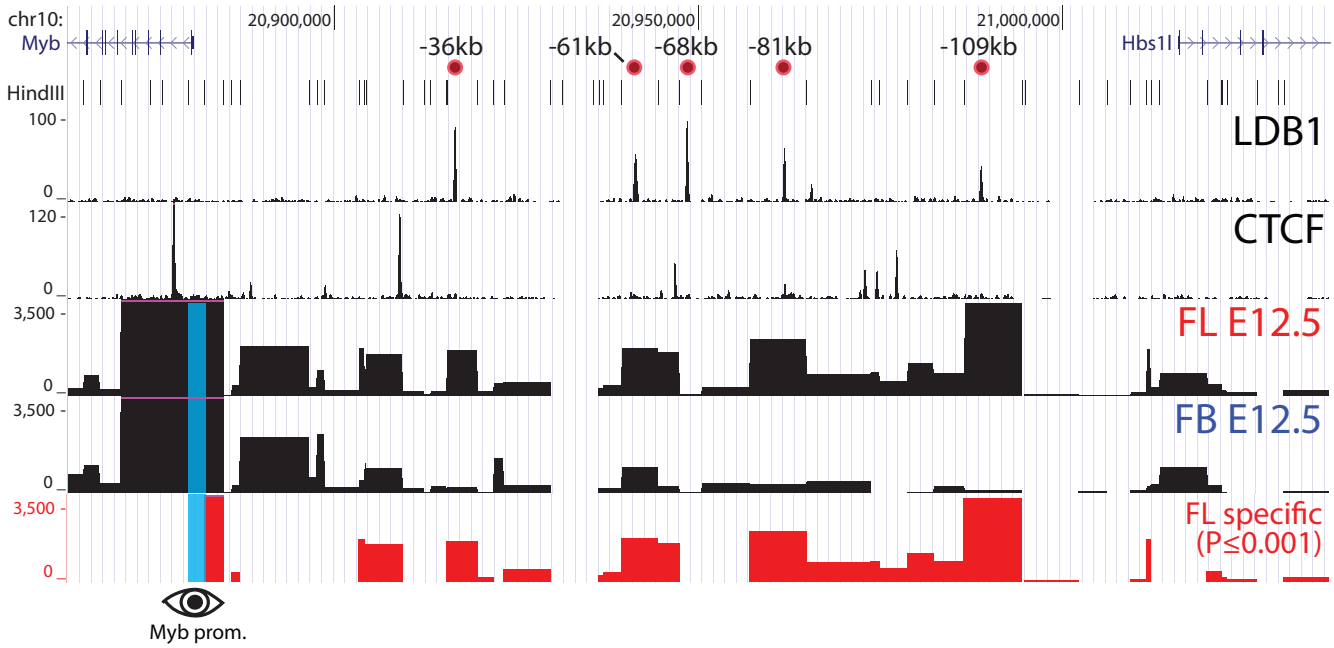
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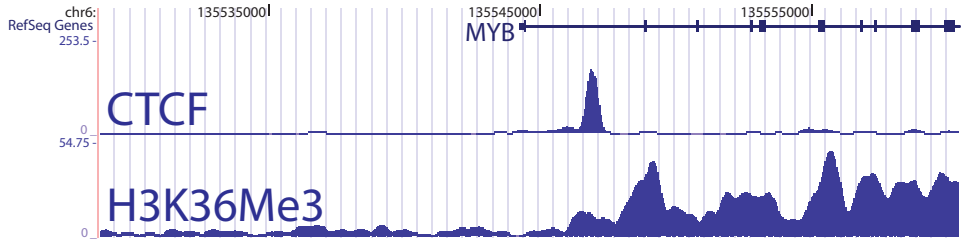


ENCODE - K562

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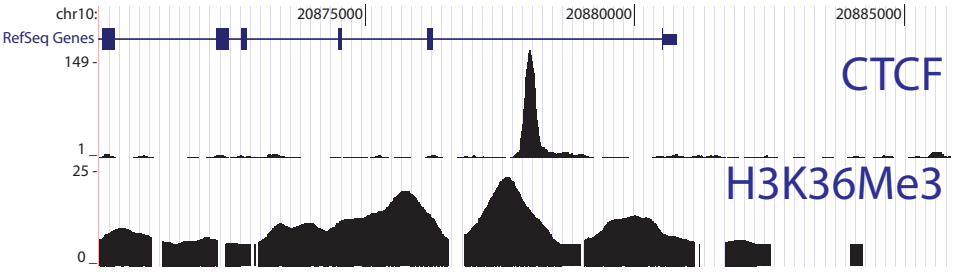


Wong et al. 2011

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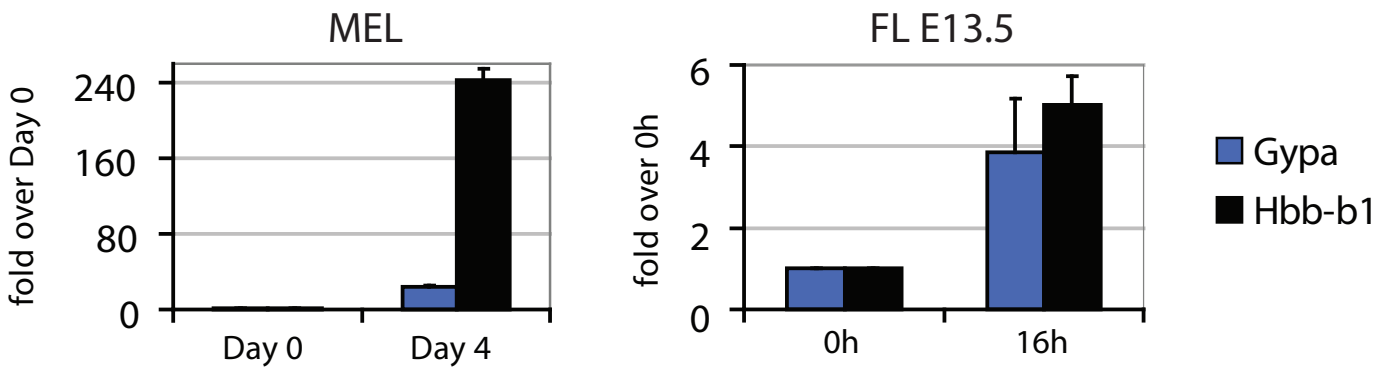
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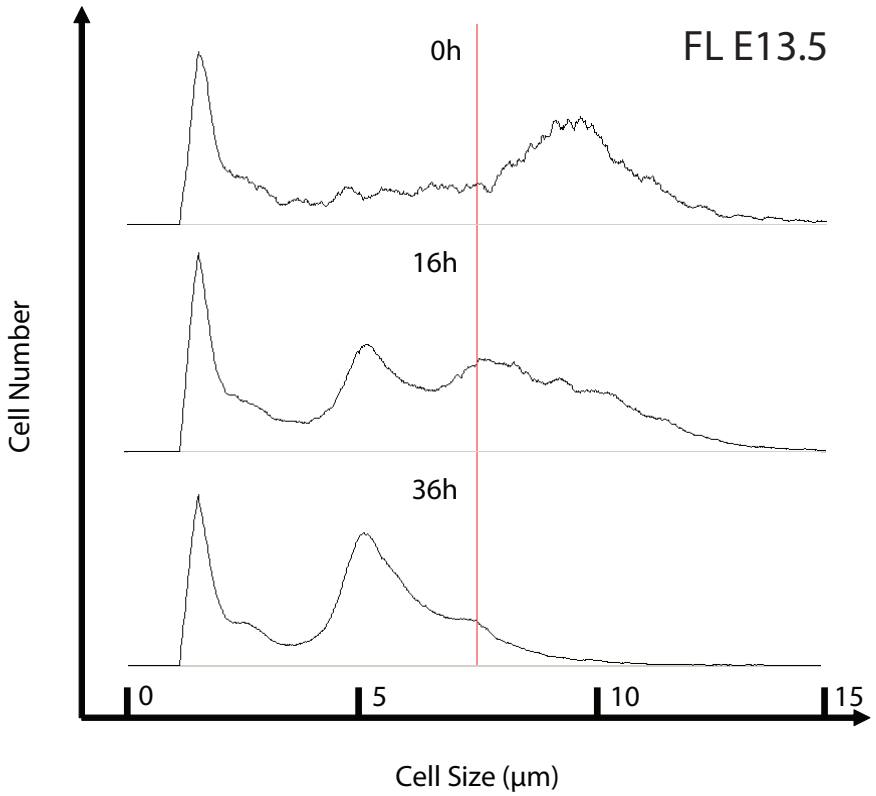
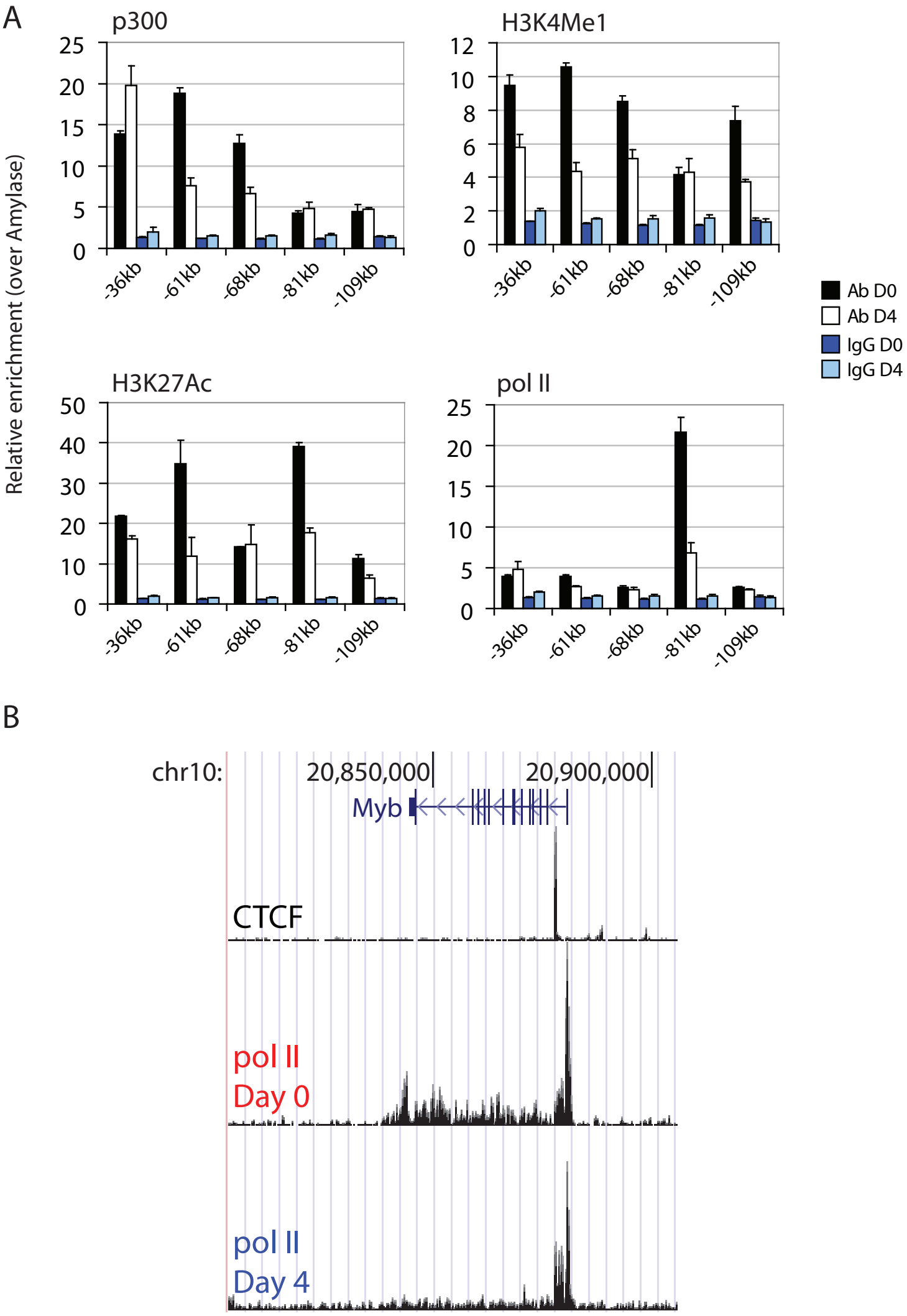
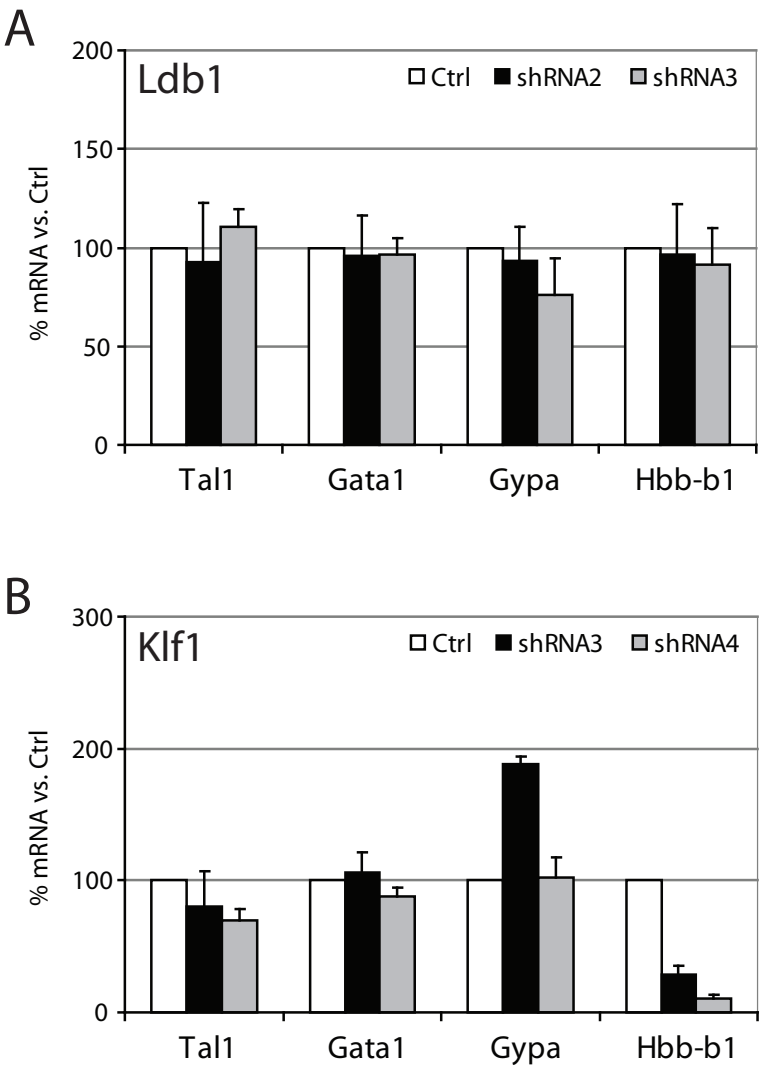
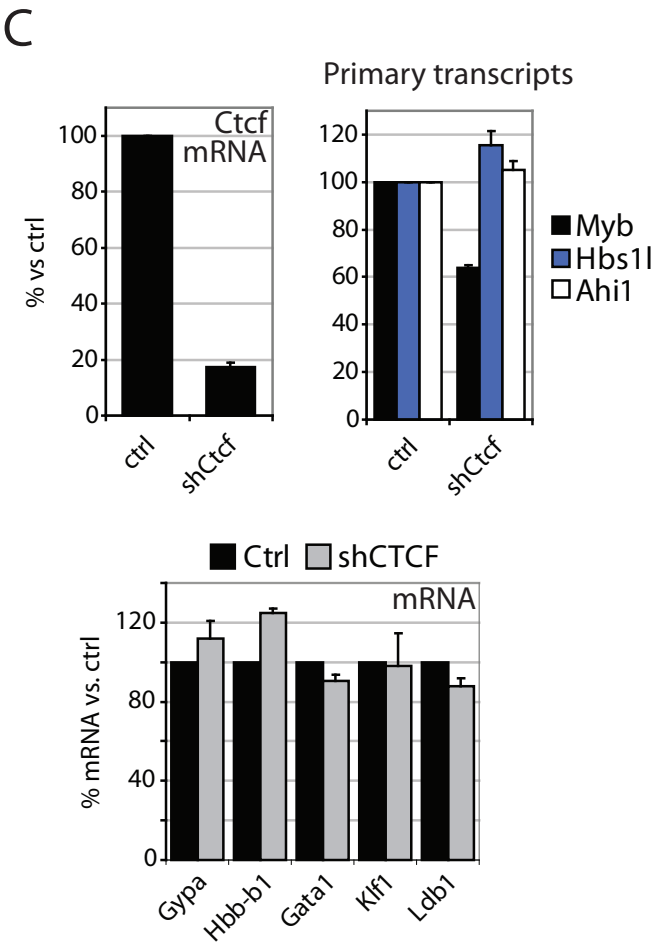
Hbb-b1

B


FL E13.5
0h
16h
36h
Cell Number
0
5
10
15
Cell Size (µm)


A
p300
H3K4Me1
H3K27Ac
pol II
Relative enrichment (over Amylase)
-36kb
-61kb
-68kb
-81kb
-109kb
Ab D0
Ab D4
IgG D0
IgG D4
B
chr10:
20,850,000
20,900,000
Myb
CTCF
pol II
Day 0
pol II
Day 4


A
Ldb1
Ctrl
shRNA2
shRNA3
% mRNA vs. Ctrl
200
150
100
50
0
Tal1
Gata1
Gypa
Hbb-b1
B
Klf1
Ctrl
shRNA3
shRNA4
% mRNA vs. Ctrl
300
200
100
0
Tal1
Gata1
Gypa
Hbb-b1


C
Primary transcripts
Ctcf
mRNA
% vs ctrl
100
80
60
40
20
0
ctrl
shCtcf
120
100
80
60
40
20
0
ctrl
shCtcf
Myb
Hbs1l
Ahi1
Ctrl
shCTCF
mRNA
% mRNA vs. ctrl
120
80
40
0
Gypa
Hbb-b1
Gata1
Klf1
Ldb1