A dynamic CTCF chromatin binding landscape promotes DNA hydroxymethylation and transcriptional induction of adipocyte differentiation

SUPPLEMENTARY DATA

Figure S1. Genomic distribution of conCTS and dynCTS.
Genomic annotation of conCTS and dynCTS was performed using CEAS from the cistrome analysis platform [1]. The percentage of CTS found within gene promoters, downstream regions, exons, introns and intergenic domains are shown in comparison with the their genomic coverage (Genome).

Figure S2. Effect of CTCF silencing on mitotic clonal expansion and cell viability.
3T3-L1 pre-adipocytes (day 0) were transfected using siRNAs directed against CTCF (si-CTCF) or a control set of non-targeting siRNAs (si-Control) and concomitantly induced to differentiate using the regular MDI protocol (A) or rosiglitazone (Rosi) (B). After 3 days, cells were harvested, stained using trypan blue and counted. The number of viable and non-viable cells is reported relative to the total number of cells at day 0. Results are means ± S.D from 2 independent experiments performed in triplicates.

Figure S3. Validation of Adipoq, Lgals12, Pnpla8, Fabp4 and Mgl1 as PPARG target genes in 3T3L1 cells.
3T3-L1 adipocytes were transfected using siRNAs directed against PPARG (si-PPARG) or a control set of non-targeting siRNAs (si-Control). RT-qPCR assays were performed after 3 days and results expressed for each analyzed gene as relative mRNA expression levels compared to those obtained with cells transfected with si-Control, arbitrarily set to 1. Results
are means ± S.D from a representative experiment performed in triplicates. Statistical significance was assessed using Student’s $t$ tests for unpaired data. *** $p < 0.001$.

**Figure S4. CTCF is required for PPARG-mediated gene transcriptional activations involved in adipogenesis.**

**A-B)** The Integrated Genome Browser (IGB) was used to visualize PPARG and CTCF ChIP-seq signals obtained from 3T3-L1 at the indicated stages of the differentiation process. Normalized wig files were used and the scale was kept identical for the different tracks related to CTCF. Shown are regions spanning 25 kb on each side of the TSS of $\text{Fabp4}$ (A) and $\text{Mgl1}$ (B), which are indicated by arrows. DynCTS are indicated on top of the tracks using a letter that refers to clusters identified in Fig.2.

**C-D)** 3T3-L1 cells were transfected using siRNAs directed against CTCF (si-CTCF) or a control set of non-targeting siRNAs (si-Control) and concomitantly induced to differentiate in the absence (-Rosi) or the presence (+Rosi) of the PPARG agonist rosiglitazone. RT-qPCR assays were performed after 3 days and results expressed for each analyzed gene as relative mRNA expression levels compared to those obtained with cells transfected with si-Control and not exposed to rosiglitazone, arbitrarily set to 1. Results are means ± S.D from a representative experiment performed in triplicates. Statistical significance was assessed using Student’s $t$ tests for unpaired data. * $p < 0.05$; ** $p < 0.01$.

**Figure S5. Additional chromatin features that characterize active transcriptional regulatory regions are found at gainCTS co-bound by PPARG.**

Analyses were performed as in Fig.7 and show average DHS-seq (A) and FAIRE-seq (B) signal levels in 3T3-L1 pre-adipocytes (day 0) and adipocytes (day ≥ 6) at gainCTS. All gainCTS were centered (arrowhead) and a region spanning 2.5 kb on each side was analyzed.
Figure S6. DynCTS from cluster A behave as transcriptional regulatory regions repressed during adipocyte differentiation.

A-D) Average H3K4me1 (A), H3K4me2 (B), H3K4me3 (C) and H3K27ac (D) ChIP-seq signal levels at days -2, 0, 2 and 7 of the 3T3-L1 differentiation process at dynCTS from cluster A. All dynCTS were centered (arrowhead) and a region spanning 2.5 kb on each side was analyzed.

E) Similar analyses as in A-D were performed using ChIP-seq for the indicated TFs or cofactors obtained in differentiated 3T3-L1 adipocytes (day ≥ 6).

Figure S7. GainCTS co-bound by PPARG comprise both promoters and enhancers activated during differentiation.

Analyses were performed as in Fig.7 and S5 using gainCTS, which were previously separated into 2 classes with respect to their distance to the nearest RefSeq gene transcriptional start site (TSS), i.e < 2.5 kb or > 2.5 kb from gene TSS, respectively.

Figure S8. Controls showing lack of PPARG recruitment and recognition motif enrichment in gainCTS – PPARG.

A) Average PPARG ChIP-seq signal levels from 3T3-L1 adipocytes at gainCTS + PPARG and gainCTS – PPARG. All gainCTS were centered (arrowhead) and a region spanning 2.5 kb on each side was analyzed.

B) Top 10 transcription factor recognition motifs identified using CENDIST for both gainCTS + PPARG and gainCTS – PPARG.
Figure S9. CTCF silencing does not impact on TET enzyme expression in differentiating 3T3-L1 cells.

3T3-L1 cells were transfected using siRNAs directed against CTCF (si-CTCF) or a control set of non-targeting siRNAs (si-Control) and concomitantly induced to differentiate in the absence (-Rosi) or the presence (+Rosi) of the PPARG agonist rosiglitazone. RT-qPCR assays were performed after 3 days and results expressed for each analyzed gene as relative mRNA expression levels compared to those obtained with cells transfected with si-Control and not exposed to rosiglitazone, arbitrarily set to 1. Results are means ± S.D from a representative experiment performed in triplicates.

Figure S10. PPARG is barely expressed in HEK293T cells and not co-immunoprecipitated by Flag-CTCF.

A) Normalized PPARG mRNA expression in NCI60 human cell-lines shows basal PPARG levels in HEK293T cells (arrow). Data were obtained from BioGPS [2].

B) Western blot for PPARG was performed using cell extracts from 3T3-L1 cells and HEK293T expressing Flag-CTCF (Input). Proteins co-immunoprecipitated with Flag-CTCF were also analyzed. PPARG appears in 3T3-L1 adipocytes as 3 bands including PPARG1 and PPARG2, as previously described [3]. Only the smallest from of PPARG is barely observable in the HEK293T input and was not co-immunoprecipitated by Flag-CTCF.

Figure S11. Dynamic CTCF cistrome during differentiation of human adipose stromal cells identify gained CTS associated with PPARG signalling.

A) CTS from differentiating human adipose stromal cells (hASC) (days -2, 0 and 9) [4] were identified and analyzed as described for 3T3-L1 cells. Note that CTCF ChIP-seq data from day 3 were removed from the analyses because of its significantly lower quality compared to
CTCF ChIP-seq data from days -2, 0 and 9 as judged by using NGS QC Generator [5]. 37,149 conCTS and 16,348 dynCTSs were identified. Identification of 8 different dynamic pattern of CTCF binding during hASC adipogenesis was performed using k-means clustering on dynCTS. CTCF chromatin binding intensities are shown for all CTS from the 8 different clusters (clusters A-H) at days -2, 0 and 9 of the differentiation process. The number of CTS within each cluster is provided as well as the average binding profile (red curve).

B) The presence of CTS from the 8 different clusters identified in A within 25 kb of the TSS of RefSeq genes induced during adipogenesis and bound by PPARG (Induced+PPARG) or other genes. The fraction of recovered genes is shown for each CTS cluster relative to that obtained with “Other genes”, which was arbitrarily set to 1. Statistical significance was assessed using Chi-squared with a Holm's correction tests. *** $p < 0.001$

Figure S12. Transcriptional expression profiles of genes associated with various combinations of gainCTS and PPARG binding sites during adipogenesis.

Average RMA-normalized mRNA expression levels of genes associated with the indicated combinations of gainCTS and PPARG recruitment sites (taking a region spanning 25 kb on each side of their TSS) at days -2, 0, 2 and 7 of the 3T3-L1 differentiation process. Results are means ± S.E.M.

Figure S13. Genomic co-localisation of CTCF, TET1 and 5hmC.

Average TET1 (A-B) ChIP-seq signals and 5hmC levels issued from hMeDIP-seq (C) at CTS specifically detected in mouse ES cells, heart, cerebellum or cortex. Cell/tissue-specific CTS were determined using all available CTCF peaks from ENCODE. Details of the TET1 ChIP-seq and hMeDIP-seq data used are provided in Table S1. All CTS were centered (arrowhead) and a region spanning 2.5 kb on each side was analyzed.
**Figure S14. Evidences for the biological relevance of gainCTS from 3T3-L1 adipocytes.**

**A)** GainCTS + PPARG and gainCTS – PPARG from 3T3-L1 cells were compared to DHS sites identified in the indicated mouse cells and tissues by the ENCODE consortium. The percentage of sites that lies within DHS sites from a given cell/tissue was reported.

**B)** GainCTS + PPARG from 3T3-L1 cells were compared to PPARG binding sites identified using ChIP-seq in primary brown, inguinal and epididymal adipocytes [6]. The percentage of gainCTS + PPARG overlapping with PPARG binding sites from the different primary adipocytes was reported.

**Figure S15. Functional genomic analysis of changes in CTCF binding intensity at conCTS during adipocyte differentiation.**

**A)** Violin plots showing the differential CTCF ChIP-seq signals at conCTS and dynCTS obtained using Manorm for the indicated stages of 3T3-L1 adipocytes differentiation. The data show that changes in CTCF signal intensity are significantly greater at dynCTS than at conCTS. Statistical significance was assessed using Mann-Whitney’s tests for unpaired data. *** $p < 0.001$.

**B)** CTCF chromatin binding intensities are shown for all CTS from the 7 different clusters (clusters A-G) identified using k-means clustering from conCTS using CTCF ChIP-seq signals at days -2, 0, 2 and 7 of the 3T3-L1 differentiation process. The number of CTS within each cluster is provided as well as the average binding profile (red curve).

**C)** % conCTS from clusters A-G that overlap transcriptional regulatory regions active at days -2, 0, 2 and 7 of the 3T3-L1 differentiation process. Active regulatory regions were defined as those significantly enriched for H3K4me1 or H3K4me3 and H3K27ac identified in [4].
D) Average RMA-normalized mRNA expression levels of genes associated with CTS (genes with at least 1 CTS within 25 kb of their TSS) from the 7 different clusters identified in B at days -2, 0, 2 and 7 of the 3T3-L1 differentiation process. Results are means ± S.E.M.

E) The indicated categories of RefSeq genes were analyzed for the presence of CTS from the 7 different clusters identified in B within 25 kb of their TSS. The fraction of recovered genes is shown for each CTS cluster relative to that obtained with “Other genes”, which was arbitrarily set to 1. Statistical significance was assessed using Chi-squared with a Holm's correction tests. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

F) Average 5hmC levels issued from hMeDIP-seq data obtained using 3T3-L1 pre-adipocytes (day 0) or adipocytes (day 8) at conCTS from cluster G (using sites localized at active enhancers as for Fig.8A). All CTCF sites were centered (arrowhead) and a region spanning 2.5 kb on each side was analyzed.

SUPPLEMENTARY REFERENCES

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Fig. S6

A  H3K4me1

B  H3K4me2

C  H3K4me3

D  H3K27ac

E  Transcriptional Regulators
Distance to gene TSS of gainCTS

< 2.5 kb (11 %)  > 2.5 kb (89 %)

A
H3K4me1
Average ChIP-seq signal intensity

B
H3K4me2
Average ChIP-seq signal intensity

C
H3K4me3
Average ChIP-seq signal intensity

D
H3K27ac
Average ChIP-seq signal intensity

E
DHS
Average DHS-seq signal intensity

F
FAIRE
Average FAIRE-seq signal intensity

G
Transcriptional Regulators
Average ChIP-seq signal intensity
B

GainCTS + PPARG

| RANK | Name                      | Family                      | Score  | Binding Range | Threshold | Count1 | Count2 | Z0Score | Z1Score | P-value |
|------|---------------------------|-----------------------------|--------|---------------|-----------|--------|--------|---------|---------|---------|
| 1    | V$\text{SJTCCF\_ES}$     | jaspar_BetaBetaAlpha_zinc_finger | 16.3413 | 200 2.8323    | 0.2651163 | 0.2837209 | 14.2033 | 1.13802 | 0       |
| 2    | V$\text{SJTJaspac\_CTCF}$ | jaspar_BetaBetaAlpha_zinc_finger | 12.6112 | 320 3.0479    | 0.2604651 | 0.2604651 | 11.6202 | 0.990975 | 0       |
| 3    | V$\text{SJTCCF\_RXRA}$   | jaspar_Hormone_nuclear_Receptor | 8.20674 | 600 3.29      | 0.4186047 | 0.3116279 | 7.15685 | 1.04089 | 0       |
| 4    | V$\text{SJCQUTF\_Q6}$    | ERE                        | 7.19997 | 480 3.1835    | 0.2093023 | 0.1906877 | 6.79056 | 0.499413 | 0       |
| 5    | V$\text{SJTJaspac\_NFC}$ | jaspar_NFL_CCAAT_binding    | 5.71372 | 400 2.9389    | 0.2576744 | 0.2976744 | 5.43989 | 0.273529 | 0       |
| 6    | V$\text{SJTCCF\_RXRA}$   | ERE                        | 5.69637 | 680 3.1495    | 0.2651163 | 0.2     | 4.48552 | 1.21064 | 0       |
| 7    | V$\text{SJTJaspac\_HNF4}$ | jaspar_Hormone_nuclear_Receptor | 5.63993 | 560 3.2354    | 0.2186047 | 0.172093 | 5.10255 | 0.537373 | 0       |
| 8    | V$\text{SJTJaspac\_ESR1}$ | jaspar_Hormone_nuclear_Receptor | 5.27775 | 680 3.0434    | 0.2186047 | 0.1674419 | 4.17081 | 1.10694 | 3.33e-16 |
| 9    | V$\text{SJTJaspac\_Q3}$  | ERE                        | 5.03723 | 520 2.3059    | 0.2232558 | 0.1953488 | 4.24316 | 0.794069 | 4.55e-15 |
| 10   | V$\text{SJTJaspac\_Q6\_Q3}$ | ERE                      | 4.96027 | 400 2.1066    | 0.2372039 | 0.2372039 | 3.26928 | 1.6912 | 1.08e-14 |

GainCTS - PPARG

| RANK | Name                      | Family                      | Score  | Binding Range | Threshold | Count1 | Count2 | Z0Score | Z1Score | P-value |
|------|---------------------------|-----------------------------|--------|---------------|-----------|--------|--------|---------|---------|---------|
| 1    | V$\text{SJTJaspac\_CTCF}$ | jaspar_BetaBetaAlpha_zinc_finger | 26.204 | 80 3.0403    | 0.1544554 | 0.239634 | 25.0837 | 1.12029 | 0       |
| 2    | V$\text{SJTCCF\_ES}$     | jaspar_BetaBetaAlpha_zinc_finger | 24.5128 | 80 3.1112    | 0.1326733 | 0.2534653 | 23.6979 | 0.814914 | 0       |
| 3    | V$\text{SJTJaspac\_Tcf21\_}$ | jaspar_CP2                   | 5.09074 | 280 3.1352   | 0.0990099 | 0.1148515 | 3.74634 | 1.3444 | 3.33e-8 |
| 4    | V$\text{SJTAP\_Q3}$      | AP2                        | 5.05403 | 840 2.9826   | 0.1584158 | 0.0970297 | 4.13674 | 0.917286 | 4.15e-8 |
| 5    | V$\text{SJTJaspac\_RXRA}$ | jaspar_Hormone_nuclear_Receptor | 4.92267 | 640 3.3001   | 0.1069307 | 0.1168317 | 3.79695 | 1.12572 | 9.01e-8 |
| 6    | V$\text{SJTJaspac\_RXRA}$ | jaspar_Hormone_nuclear_Receptor | 4.8654 | 200 2.5247   | 0.09108911 | 0.1363373 | 4.13945 | 0.725954 | 1.26e-7 |
| 7    | V$\text{SJTJaspac\_HNF4\_Q3}$ | jaspar_Hormone_nuclear_Receptor | 4.86317 | 680 3.2386   | 0.1465347 | 0.09306931 | 3.8037 | 0.929795 | 1.27e-7 |
| 8    | V$\text{SJTJaspac\_ESR1}$ | jaspar_Hormone_nuclear_Receptor | 4.70325 | 360 2.8057   | 0.1069307 | 0.1188119 | 3.60809 | 1.09517 | 3.15e-7 |
| 9    | V$\text{SJTJaspac\_Q3}$  | AP2                        | 4.68197 | 600 2.8628   | 0.180198 | 0.1267327 | 3.45288 | 1.22909 | 3.54e-7 |
| 10   | V$\text{SJTJaspac\_Q6\_Q3}$ | AP2                      | 4.43661 | 280 2.9337   | 0.08712871 | 0.1049505 | 3.4364 | 1.00241 | 1.32e-6 |
Fig. S9

**Tet1**

Relative mRNA expression

Rosi - +

- si-Control
- si-CTCF

**Tet2**

Relative mRNA expression

Rosi - +

- si-Control
- si-CTCF

**Tet3**

Relative mRNA expression

Rosi - +

- si-Control
- si-CTCF
A

Normalized PPARG mRNA expression

B

3T3-L1 adipocytes

HEK293T + Flag-CTCF

WB:

PPARG

input

IP anti-Flag
| TF binding                                      | # genes | mRNA expression |
|------------------------------------------------|---------|----------------|
| GainCTS + PPARG and gainCTS - PPARG            | 21      |                |
| GainCTS + PPARG                                | 212     |                |
| GainCTS - PPARG and PPARG - CTCF              | 377     |                |
| GainCTS - PPARG                                | 396     |                |
Fig. S13

A

TET1 (Xu et al. 2011)_ES cells

Cell/tissue-specific CTS

ES cells
Heart
Cerebellum
Cortex

Average ChIP-seq signal intensity

B

TET1 (Wu et al. 2011)_ES cells

C

5hmC_ES cells
| Reference | Cell/Tissue | Experiment | Differentiation stages | Series Accession number | database |
|-----------|-------------|------------|------------------------|-------------------------|----------|
| Nielsen R et al., Genes Dev, 2008 | 3T3-L1 | ChIP-seq RXR | Day 6 | GSE13511 | Gene Expression Omnibus |
| Raghav SK et al., Mol Cell, 2012 | 3T3-L1 | ChIP-seq SMRT | Day 6 | E-MTAB-1031 | ArrayExpress [http://www.ebi.ac.uk/arrayexpress/] |
| Siersbaek R et al., EMBO J, 2011 | 3T3-L1 | DHS-seq | Days 0, 6 | GSE27826 | Gene Expression Omnibus |
| Waki H et al., PLoS Genet, 2011 | 3T3-L1 | FAIRE-seq | Days 0, 8 | DRR000382 | DNA data bank of Japan [http://trace.ddbj.nig.ac.jp/DRASearch/] |
| Sérandour AA et al., Nucleic Acid Res, 2012 | 3T3-L1 | hMeDIP-seq | Days 0, 8 | GSE38407 | Gene Expression Omnibus |
| Neri F et al., Genome Biol, 2013 | Brain, MEF | hMeDIP-seq | N/A | GSE57582 | Gene Expression Omnibus |
| Colquitt BM et al., Proc Natl Acad Sci USA, 2013 | HBC, GBC, mOSN (Main olfactory epithelium) | hMeDIP-seq | N/A | GSE38604 | Gene Expression Omnibus |
| Mellén M et al., Cell, 2012 | Granule cells (Cerebellum) | hMeDIP-seq | N/A | GSE42080 | Gene Expression Omnibus |
| Siersbaek MS et al., Mol Cell Biol, 2012 | Primary adipocytes | ChIP-seq PPARγ | Day 7 | GSE41481 | Gene Expression Omnibus |
| Wu H et al., Nature, 2011 | ES | ChIP-seq TET1 | N/A | GSE659799 | Gene Expression Omnibus |
| Xu Y et al., Mol Cell, 2011 | ES | hMeDIP-seq | N/A | GSE28532 | Gene Expression Omnibus |
| Database CTCFBSDB 2.0 | Multiple | Mouse CTCF recruitment sites from ChIP-seq | N/A | http://insulatordb.uthsc.edu/ |
| Mouse ENCODE | Multiple | CTCF recruitment sites from ChIP-seq, DHS sites from DHS-seq | N/A | https://genome.ucsc.edu/ENCODE/ |
| Mikkelisen TS et al., Cell, 2010 | 3T3-L1, hASC | Gene expression profiling by microarray | Days -2, 0, 2, 7 | GSE20606 | Gene Expression Omnibus |

Table S1. Summary of the functional genomic and transcriptomic data used in this manuscript.
### RT-qPCR primer sequence

| Primer Sequence | Targeted Gene |
|-----------------|---------------|
| CATGCTCAACATCTCCCCCCTTCTCC | Rplp0 |
| GGGAAAGTTGAATCCGTCTCCACAG |  |
| CCGTGATGGAAGACCACCTCG | Pparg |
| AGGCTGTGAGTACGTTGGGTC |  |
| ACGACACAAAAAGGGTCAGGAT | Adipoq |
| TCTTGGCAGACTGGGCAAGGAT |  |
| GGGCTTCCCTCCAGACAGA | Lgals12 |
| CATTGAGTGCAACTTCACGC |  |
| AAAATGTGGCAGGCGTATTAG | Pnpla8 |
| AAGGCCGAGGGTTTATCAG |  |
| GTG CTG TCT TTG TGG GAA CCT GG | Fabp4 |
| TGT GCC AAA GCC CAC TCC CAC T |  |
| TGGACACCATCCAGAAAGGAC | Mgll |
| CCTCTCTCGGACACTAGGA |  |
| GGTTGCGAGGACAGTGCG | Rps28 |
| TAACGCAACCTTCAGCCTTC |  |
| CTC CTA TCA GCA CCC TGA GC | Rxra |
| CCT TGA GGA CGC CAT TGA GG |  |
| GAG CTT GTT CCT CGA TGT GG | Tet1 |
| CAA ACC CAC CTG AGG CTG TT |  |
| GTT GTT GTC AGG GTG AGA ATC | Tet2 |
| TCT GTC TIC TGG CAA ACT TAC A |  |
| CCG GAT TGA GAA GGT CAT CTAC | Tet3 |
| AAG ATA ACA ATC ACG GCG TTC TT |  |

### hMeDIP-qPCR primer sequence

| Primer Sequence | Targeted Regions |
|-----------------|-----------------|
| CAGAGAGGCGCAGTTTGGAGG | GainCtcf + Pparg #1 |
| CGTGCGATACCCACTTCCACC |  |
| GTCTGGGGAAATGTTCAAGGGA | GainCtcf + Pparg #2 |
| CATGTCTGTGGTGTGCTTGTGTC |  |
| AAGCCTCTAGTTGGGACGAC | GainCtcf + Pparg #3 |
| AGTGCAACAACGCGACAAAG |  |
| CACGCTTGGCTGCCTGGGAA | GainCtcf + Pparg #4 |
| TCTGGCACCAGCAGCTCAAGC |  |
| TAGCTTACTGGTGCTTGGG | GainCtcf + Pparg #5 |
| GCGAGCATTGGTGCTTGCTTG |  |
| TGAAGGCAGAGTGGCTGAGA | GainCtcf - Pparg #1 |
| CACAGGCTGGCTTACTTGGA |  |
| CTGCTGCTTCTAGTGCTGGTG |  |
| GGCCTGCAATGAGTGGAGACC |  |
| ATTACAGATCAGCGGGGTGT | GainCtcf - Pparg #2 |
| GCAGCTTCCACAGGTCAT |  |
| ACTGCACTTTGGCTCTACC | GainCtcf - Pparg #3 |
| CAGGCCGGGAAAGAAGTCAT |  |
| Primers                                                              | Function                |
|----------------------------------------------------------------------|-------------------------|
| GCCCAGTTGCTAGTAGCTGCTTT                                            | GainCtcf - Pparg #4     |
| AGCGTTCGCGGGAACATTCT                                              | GainCtcf - Pparg #5     |
| CCCGGGAACACTTCTGCTTT                                              | GainCtcf - Pparg #6     |
| GTTCCTTCTCGGACCATTGC                                              | Control #1              |
| CGGTGTCCCAATCTTTGTT                                               | Control #2              |
| CCGTCTGGCCAAGAAATCTACT                                           | Control #3              |
| GCCACCAGTATTTTCAAAGCG                                              | Control #4              |
| GGGAGACAGGAAATAGGAGA                                              | Control #5              |
| ACCTCCTTACACCTAGGAG                                              | Control #6              |

Table S2. Real-time PCR primers used in this study.