

Supplementary Information

Pre-established ATF4 occupancy and chromatin organization instruct selective transcription activation during integrated stress response

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Supplementary Data 3 ATF4 peaks in C2C12 treated with Thap for 6h

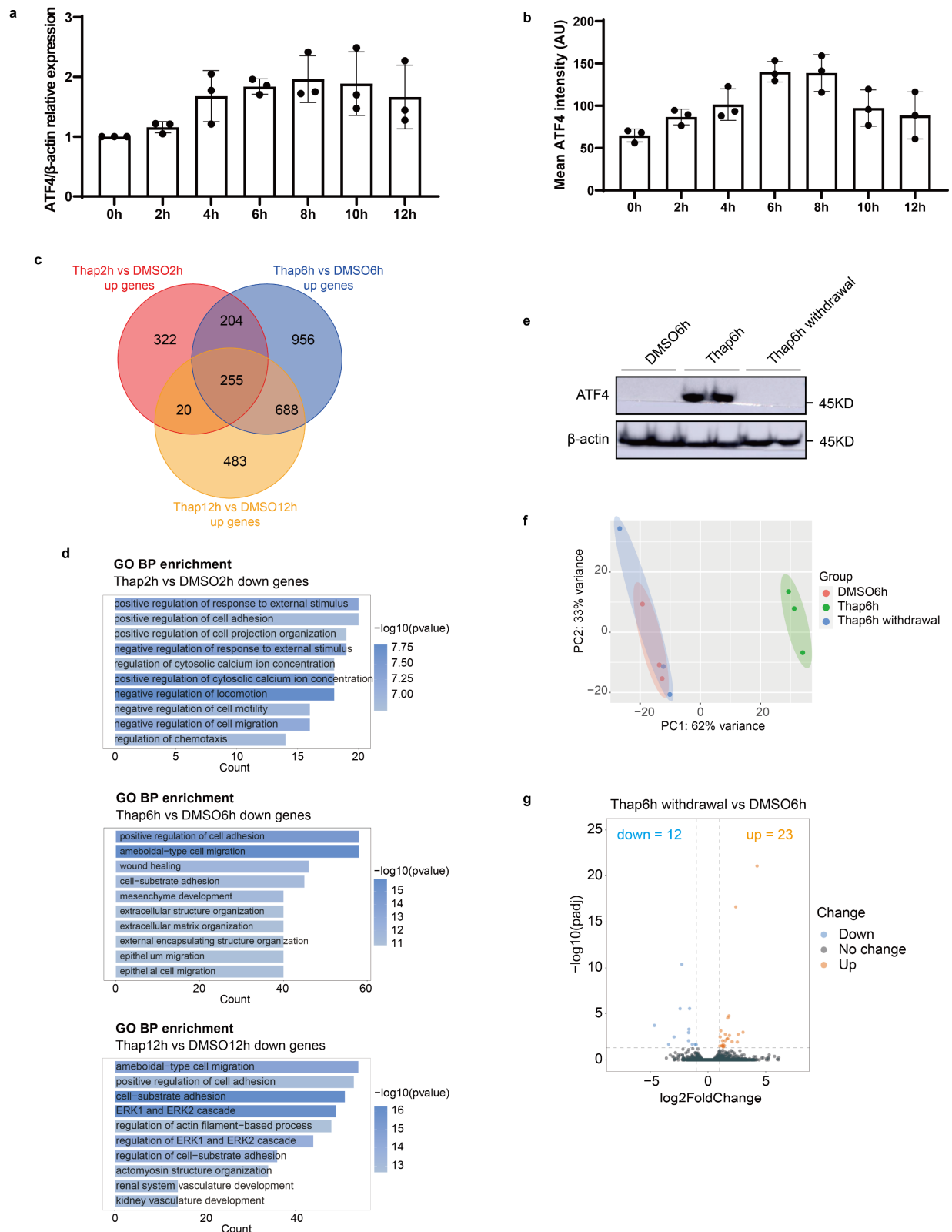
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Supplementary Figure 1 Transcriptional changes induced by ISR are reversible

a-b Quantitative analysis for Western Blot (**a**) and Immunofluorescence (**b**) of ATF4 in C2C12 cells treated with Thap for 0h, 2h, 4h, 6h, 8h, 10h, and 12h, respectively.

Values are mean $\pm$ SD. N = 3 biological replicates. AU: Arbitrary Units.

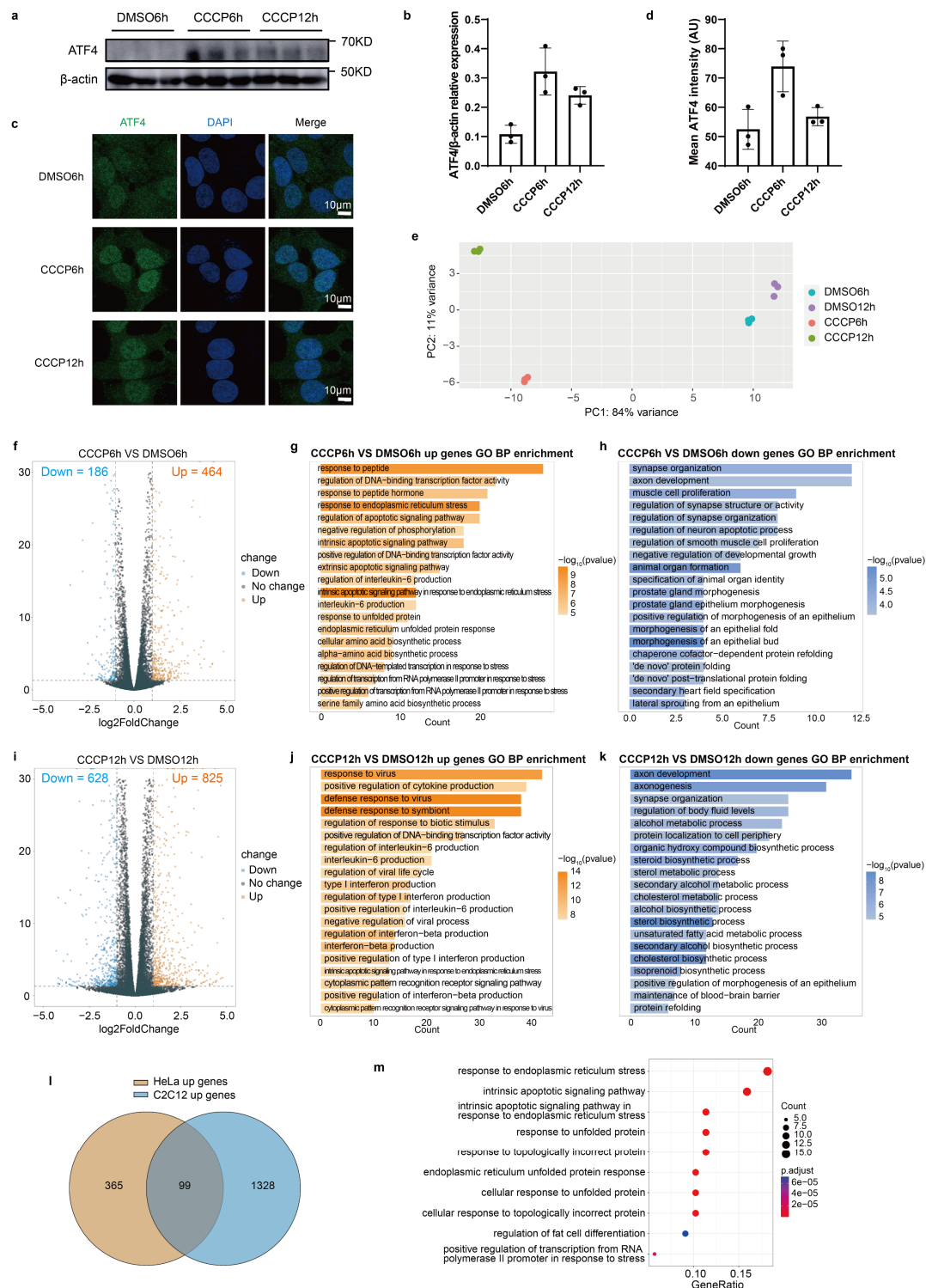
c Venn diagram showing the overlaps of upregulated expression genes in C2C12 treated with Thap versus DMSO for 2 hours, 6 hours, and 12 hours.

d The top ten most enriched Gene Ontology (GO) biological process (BP) terms associated with downregulated genes of C2C12 treated with Thap versus DMSO for 2 hours, 6 hours, and 12 hours. Enrichment analyses were performed using the ClusterProfiler R package with a one-tailed hypergeometric test followed by a Benjamini-Hochberg correction.

e Western blot analysis of ATF4 expression in C2C12 cells treated with DMSO for 6 hours, Thap for 6 hours, and Thap for 6 hours followed by a 48-hour recovery in Thap-free medium (Thap6h withdrawal). β -actin was used as the loading control. Experiments were repeated independently at least 3 times with similar results.

f PCA plot of gene expression data obtained via RNA-seq data for three biological replicates for C2C12 cells treated with DMSO for 6 hours (DMSO6h, red dots), Thap for 6 hours (Thap6h, green dots), and Thap for 6 hours followed by a 48-hour recovery in Thap-free medium (Thap6h withdrawal, blue dots). Note that the datasets for Thap6h withdrawal samples cluster together with DMSO6h, indicating the transcriptional changes induced during ISR are largely reversible.

g Volcano plot showing differentially expressed genes ($|\log_2$ fold change| > 1 and $p_{adj} < 0.05$ determined by two-sided unpaired t-test) between C2C12 cells treated with DMSO for 6 hours and those treated with Thap for 6 hours followed by a 48-hour recovery in Thap-free medium.



Supplementary Figure 2 RNA-Seq reveals progressive transcriptional reprogramming during ISR in HeLa cells

a Western blot of ATF4 in HeLa cells treated with DMSO for 6h and 10 μ M CCCP for 6h, 12h, respectively. β -actin was used as the loading control. Experiments were repeated independently at least 3 times with similar results.

b Quantitative analysis of ATF4 in HeLa cells treated with DMSO for 6h, and 10 μ M CCCP for 6h and 12h, respectively. Values are mean $\pm$ SD. n = 3 biological replicates.

c Immunofluorescence images showing ATF4 signals (green) in HeLa cells treated with DMSO for 6h and 10 μ M CCCP for 6h and 12h, respectively. DAPI staining highlights nuclei (blue). Scale bar = 10 μ m. Experiments were repeated independently at least 3 times with similar results.

d Quantitative analysis of ATF4 signals in HeLa cells treated with DMSO for 6h and 10 μ M CCCP for 6h, 12h, respectively. Values are mean $\pm$ SD. n = 3 biological replicates. AU: Arbitrary Units.

e PCA plot of RNA-seq datasets of HeLa cells treated with DMSO and CCCP for different durations.

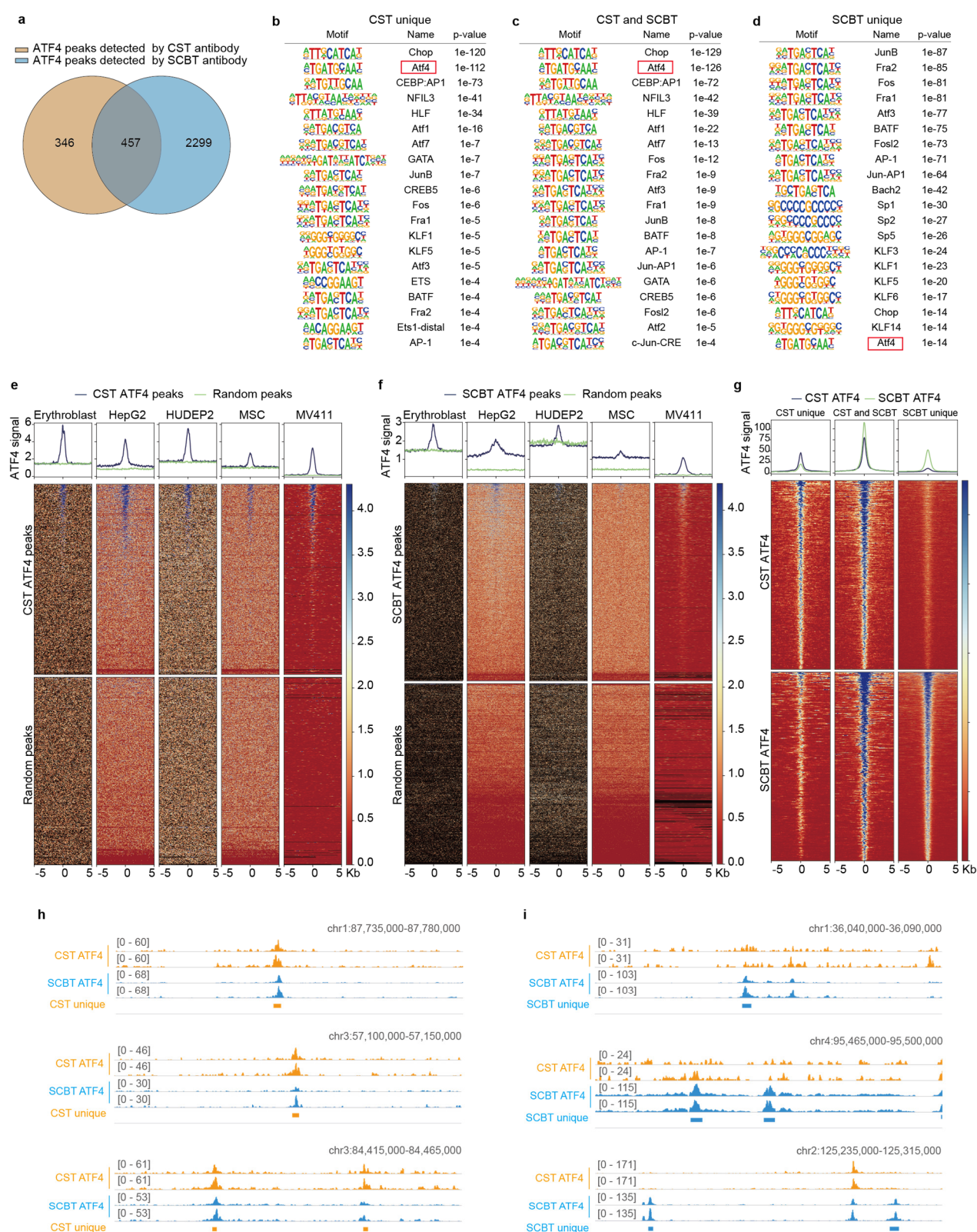
f Volcano plot depicting differentially expressed genes ($\text{padj} < 0.05$ and $|\log_2\text{FC}| > 1$) between the HeLa cells treated with DMSO and CCCP for 6 hours. Statistical analysis was performed using two-sided unpaired t-test.

g-h Dot graphs indicate the top ten most enriched GO BP terms for upregulated (**g**) or downregulated (**h**) genes of HeLa cells treated with CCCP versus DMSO for 6 hours. Enrichment analyses were performed using the ClusterProfiler R package with a one-tailed hypergeometric test followed by Benjamini-Hochberg correction.

i-k Similar analyses as in (**f-h**), but for differentially expressed genes ($\text{padj} < 0.05$ and $|\log_2\text{FC}| > 1$) between the HeLa cells treated with DMSO and CCCP for 12 hours.

l The Venn plot showing the overlap of upregulated genes in HeLa and C2C12 cells after inducing ISR for 6 hours.

m Dot graph indicating the top ten most enriched GO BP terms for commonly upregulated genes in both HeLa and C2C12 cells. The upregulated genes in C2C12 are converted into homologous human genes using the R package homologene. Enrichment analyses were performed using the ClusterProfiler R package with a one-tailed hypergeometric test followed by Benjamini-Hochberg correction.



Supplementary Figure 3 Comparison of ATF4 peaks detected by Cell Signaling Technology (CST) antibody and Santa Cruz Biotechnology (SCBT) antibody

a The Venn plot showing the overlap of ATF4 peaks detected by the CUT&Tag using CST ATF4 antibody and SCBT ATF4 antibody.

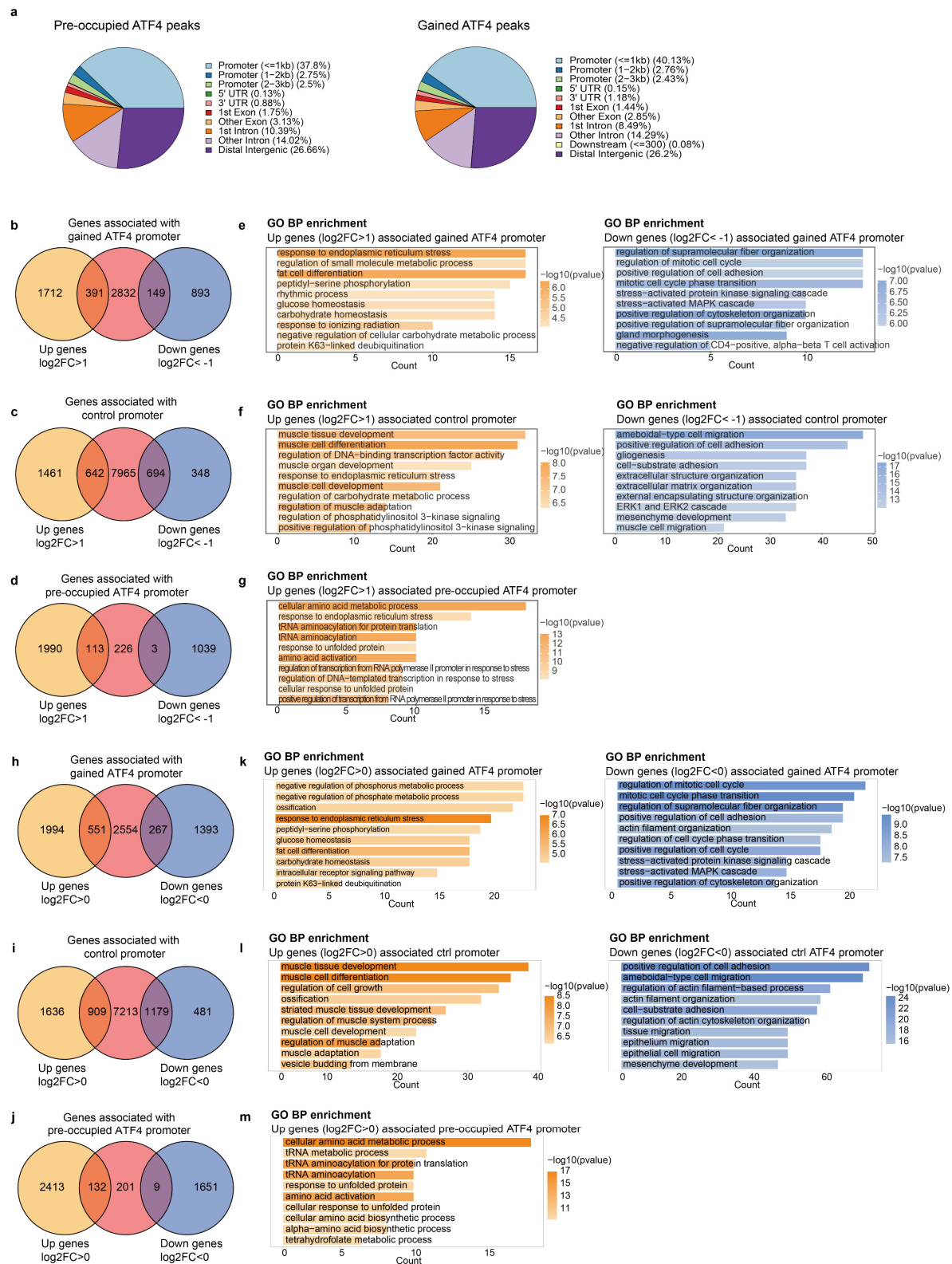
b-d Motif enrichment analysis of the ATF4 peaks detected by only the CST antibody, both CST and SCBT antibody, and only the SCBT antibody. The ATF4 motif was significantly enriched in peaks detected only by CST antibodies and in peaks detected by both CST and SCBT antibodies.

e-f Average intensity profiles and heatmaps of ATF4 signals around the centers of peaks (± 5 kb) detected only by CST antibody (**e**) and SCBT antibody (**f**) in ATF4 ChIP-seq datasets of erythroblast, HepG2, HUDEP2, MSC, MV411.

g Average intensity profiles and heatmaps of ATF4 signals around the centers of peaks (± 5 kb) detected only by CST antibody, both by CST and SCBT antibodies, and only by SCBT antibody in CUT &Tag using the two antibodies. The ATF4 peaks detected only by the CST antibody also showed a noticeable signal in the CUT&Tag experiment using the SCBT antibody, while the ATF4 peaks detected only by the SCBT antibody showed a very weak signal in the CUT&Tag data using the CST antibody.

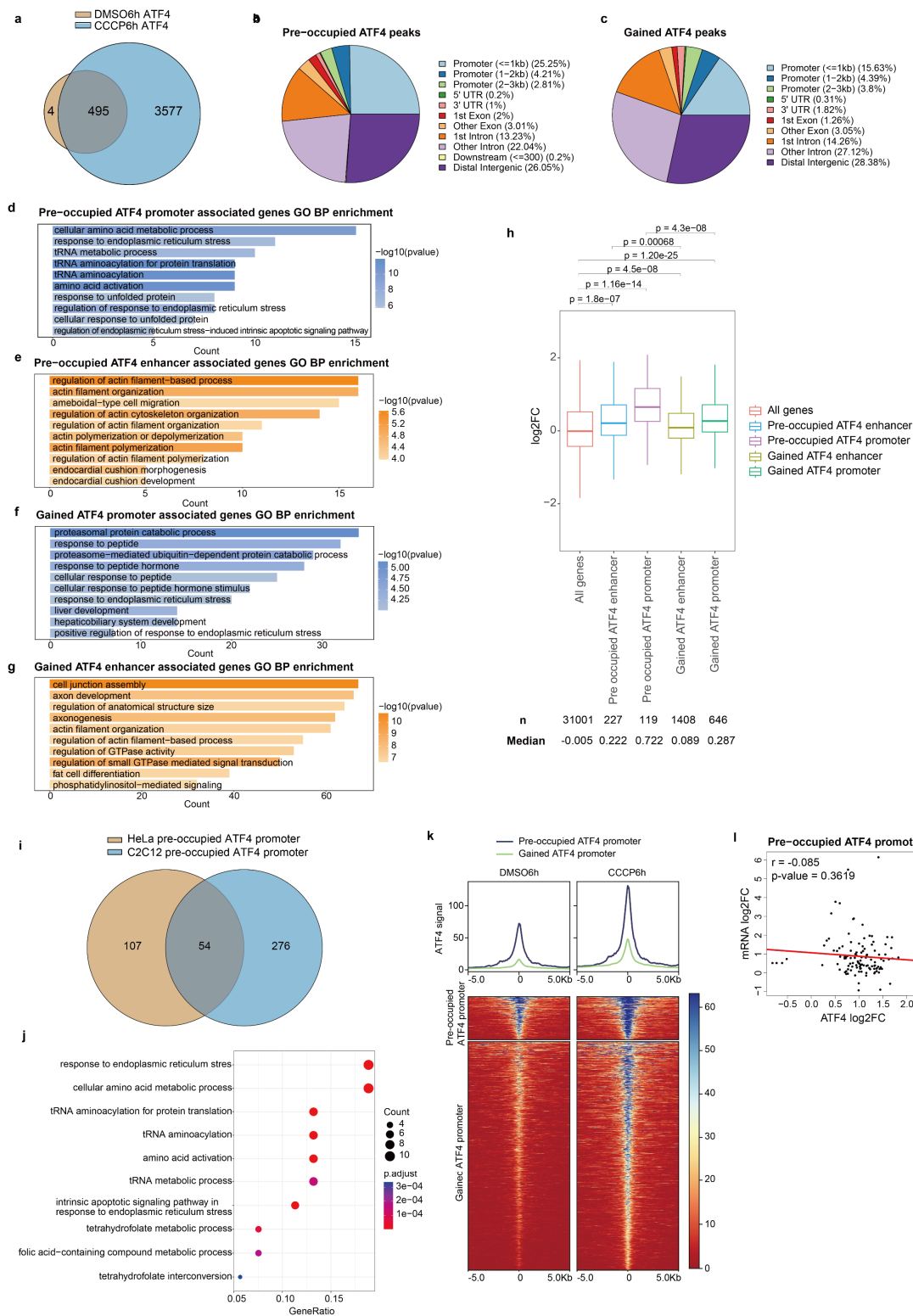
h Representative tracks showing CST ATF4 (orange), SCBT ATF4 (blue) signals at some peaks only detected by the CST antibody.

i Representative tracks showing CST ATF4 (orange), SCBT ATF4 (blue) signals at some peaks only detected by SCBT antibody.



Supplementary Figure 4 Pre-occupied and gained ATF4 peaks are associated with genes of distinct functions

- a** Pie charts showing the distribution of pre-occupied (left) and gained (right) ATF4 peaks across different genomic regions annotated using ChIPseeker.
- b** Venn diagram illustrating the overlap between genes associated with gained ATF4 promoter peaks and genes upregulated ($\log_2FC > 1$, $padj < 0.05$) or downregulated ($\log_2FC < -1$, $padj < 0.05$) during ISR.
- c** Venn diagram illustrating the overlap between genes associated with control ATF4 promoter peaks and genes upregulated ($\log_2FC > 1$, $padj < 0.05$) or downregulated ($\log_2FC < -1$, $padj < 0.05$) during ISR.
- d** Venn diagram illustrating the overlap between genes associated with pre-occupied ATF4 promoter peaks and genes upregulated ($\log_2FC > 1$, $padj < 0.05$) or downregulated ($\log_2FC < -1$, $padj < 0.05$) during ISR.
- e-g** Bar plots showing the top ten enriched Gene Ontology (GO) biological process (BP) terms for genes upregulated (left, $\log_2FC > 1$, $padj < 0.05$) or downregulated (right, $\log_2FC < -1$, $padj < 0.05$) during ISR and associated with gained ATF4 promoter peaks (**e**), control ATF4 promoter peaks (**f**), or pre-occupied ATF4 promoter peaks (**g**). No GO BP terms were enriched for downregulated genes ($\log_2FC < -1$, $padj < 0.05$) associated with pre-occupied ATF4 promoter peaks due to the small number of genes (**g**). Enrichment analyses were performed using the ClusterProfiler R package with a one-tailed hypergeometric test followed by a Benjamini-Hochberg correction.
- h-i** Similar analyses as in (**b-g**), but using $\log_2FC > 0$ or < 0 as the cutoff to identify upregulated or downregulated genes, respectively.



Supplementary Figure 5 ATF4 activates stress-related gene expression during the ISR in HeLa cells

a Venn diagram showing the overlap of ATF4 peaks identified in HeLa cells treated with either DMSO or CCCP for 6 hours.

b-c Pie charts showing the distribution of pre-occupied (**b**) and gained (**c**) ATF4 peaks in HeLa cells across different genomic regions annotated using ChIPseeker.

d-g GO enrichment analysis for genes associated with pre-occupied ATF4 promoters (**d**), pre-occupied ATF4 enhancers (**e**), gained ATF4 promoters (**f**), and gained ATF4 enhancers (**g**). Enrichment analyses were performed using the ClusterProfiler R package with a one-tailed hypergeometric test followed by a Benjamini-Hochberg correction.

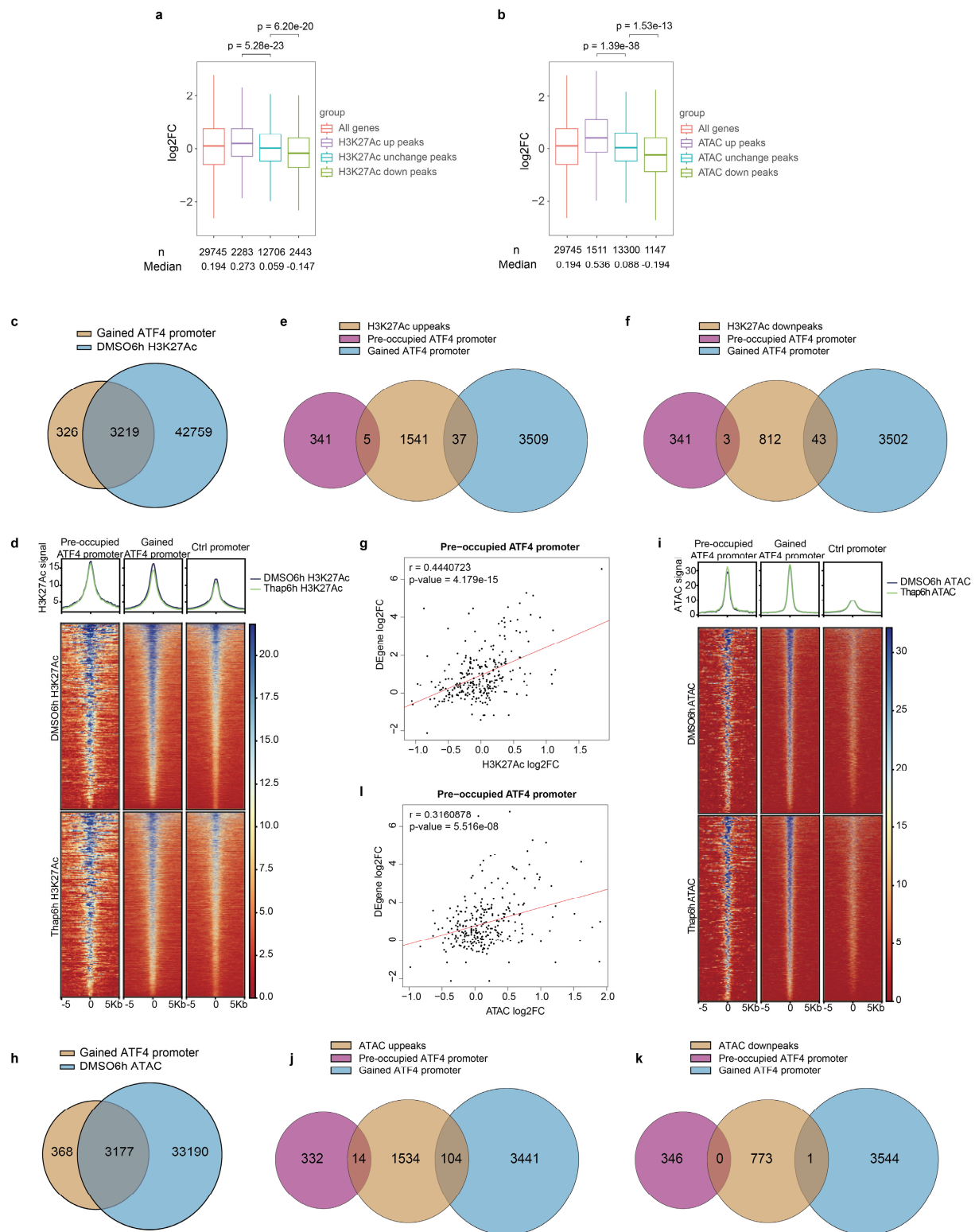
h Boxplots comparing the extent of expression changes for genes associated with different classes of ATF4 peaks in HeLa cells. In the boxplot, the center line indicates the median, box limits represent the upper and lower quartiles, and whiskers extend to $1.5\times$ the interquartile range. The values beyond the whiskers are not shown in the boxplots. P-values were calculated using a two-tailed t-test. The number of genes and the median expression changes for each group are shown at the bottom. Genes associated with pre-occupied ATF4 promoters exhibit the most robust transcription activation.

i The Venn plot showing the overlap of pre-occupied ATF4 promoter peaks in HeLa and C2C12 cells. C2C12 peaks were converted to the human genome sequence using LiftOver for comparison with HeLa cell peaks.

j Dot graph indicating the top ten most enriched GO BP terms for pre-occupied ATF4 peaks, both in HeLa and C2C12 cells. Enrichment analyses were performed using the ClusterProfiler R package with a one-tailed hypergeometric test followed by a Benjamini-Hochberg correction.

k Averaged line graph and heatmaps displaying ATF4 occupancy (RPKM) around the center (± 5 kb) of pre-occupied and gained ATF4 promoter peaks in HeLa cells treated with DMSO or CCCP for 6 hours.

l Scatter plots with linear regression (red line) showing the correlation between changes in ATF4 signal and gene transcription levels. Statistical analysis was performed using two-sided Spearman correlations. Spearman correlation coefficients (r) and associated P-values are indicated.



Supplementary Figure 6 ATF4 peaks do not exhibit significant changes in H3K27Ac and chromatin accessibility changes during ISR

a-b Boxplots showing the fold changes of transcript levels for genes associated with up, down, and unchanged H3K27Ac (**a**) and ATAC-seq (**b**) peaks. In the boxplots, the center line indicates the median, box limits represent the upper and lower quartiles, and whiskers extend to $1.5\times$ the interquartile range. The values beyond the whiskers are not shown in the boxplots. P-values were calculated using a two-tailed t-test. The number of genes and the median expression changes for each group are shown at the bottom.

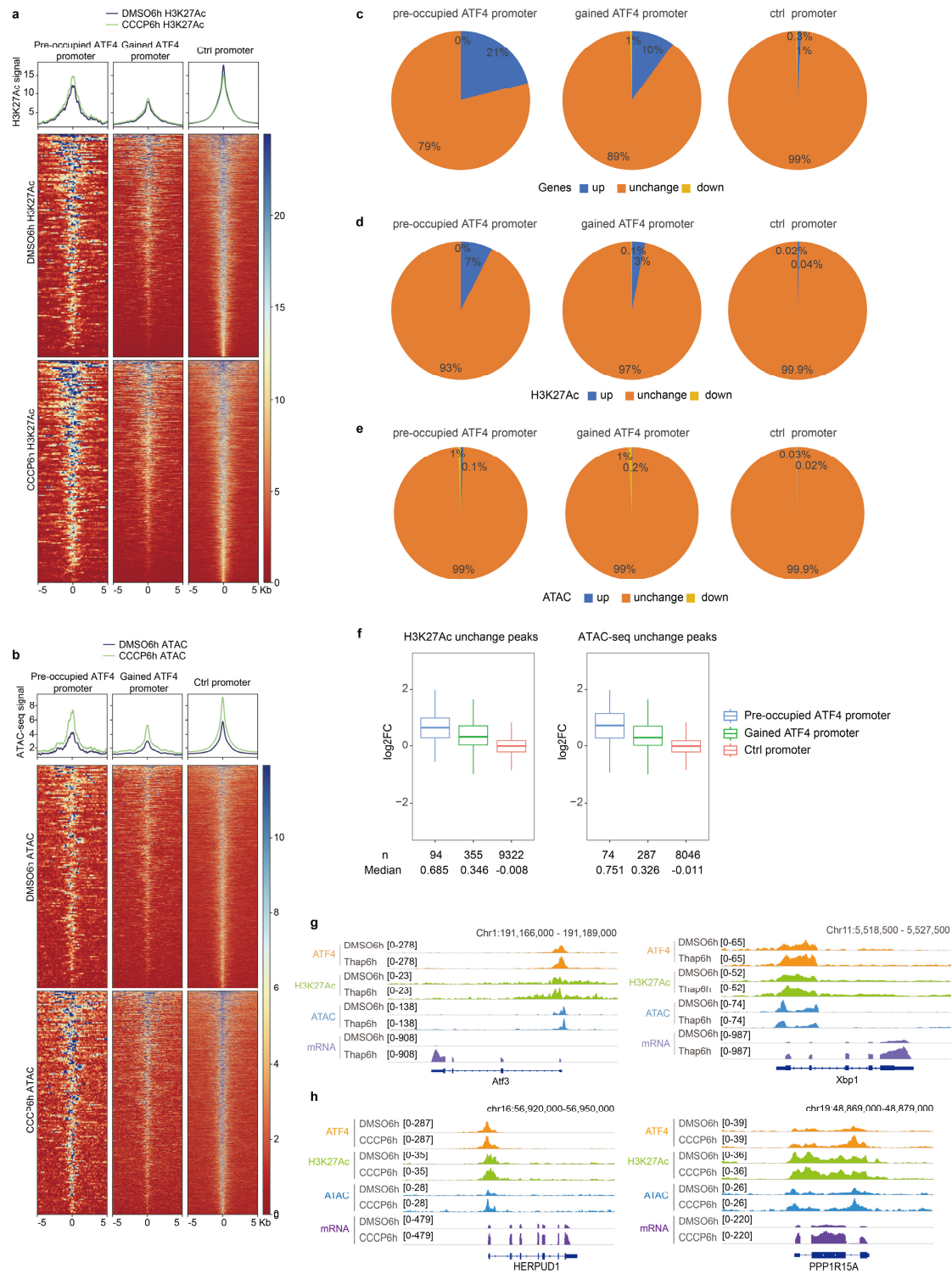
c Venn diagram illustrating the overlap between gained ATF4 promoter peaks and H3K27Ac peaks in C2C12 cells treated with DMSO for 6 hours.

d Average intensity profiles and heatmaps of H3K27Ac signals around the centers of pre-occupied ATF4 promoters, gained ATF4 promoters, and control promoter peaks (± 5 kb) in C2C12 cells treated with DMSO or Thap for 6 hours.

e-f Venn diagrams depicting the overlap between H3K27Ac peaks with increased (**e**) or decreased (**f**) signal and ATF4 peaks. Few pre-occupied or gained ATF4 promoter peaks exhibit significant changes in H3K27Ac levels.

g Scatter plots with linear regression (red line) showing the correlation between changes in H3K27Ac signal and gene transcription levels. Statistical analysis was performed using two-sided Spearman correlations. Spearman correlation coefficients (r) and associated P-values are indicated.

h-l Similar analyses as in (**c-g**), but for ATAC-seq data.



Supplementary Figure 7 H3K27Ac or chromatin accessibility changes at ATF4-bound sites are not correlated with transcriptional reprogramming during the ISR in HeLa cells

a Average intensity profiles and heatmaps of H3K27Ac signals around the centers of pre-occupied ATF4 promoters, gained ATF4 promoters, and control promoter peaks (± 5 kb) in HeLa cells treated with DMSO or CCCP for 6 hours.

b Average intensity profiles and heatmaps of ATAC-seq signals around the centers of pre-occupied ATF4 promoters, gained ATF4 promoters, and control promoter peaks (± 5 kb) in HeLa cells treated with DMSO or CCCP for 6 hours.

c Pie charts depicting the proportions of up, down, and unchanged genes at the pre-occupied ATF4 promoter, gained ATF4 promoter, and control promoter peaks.

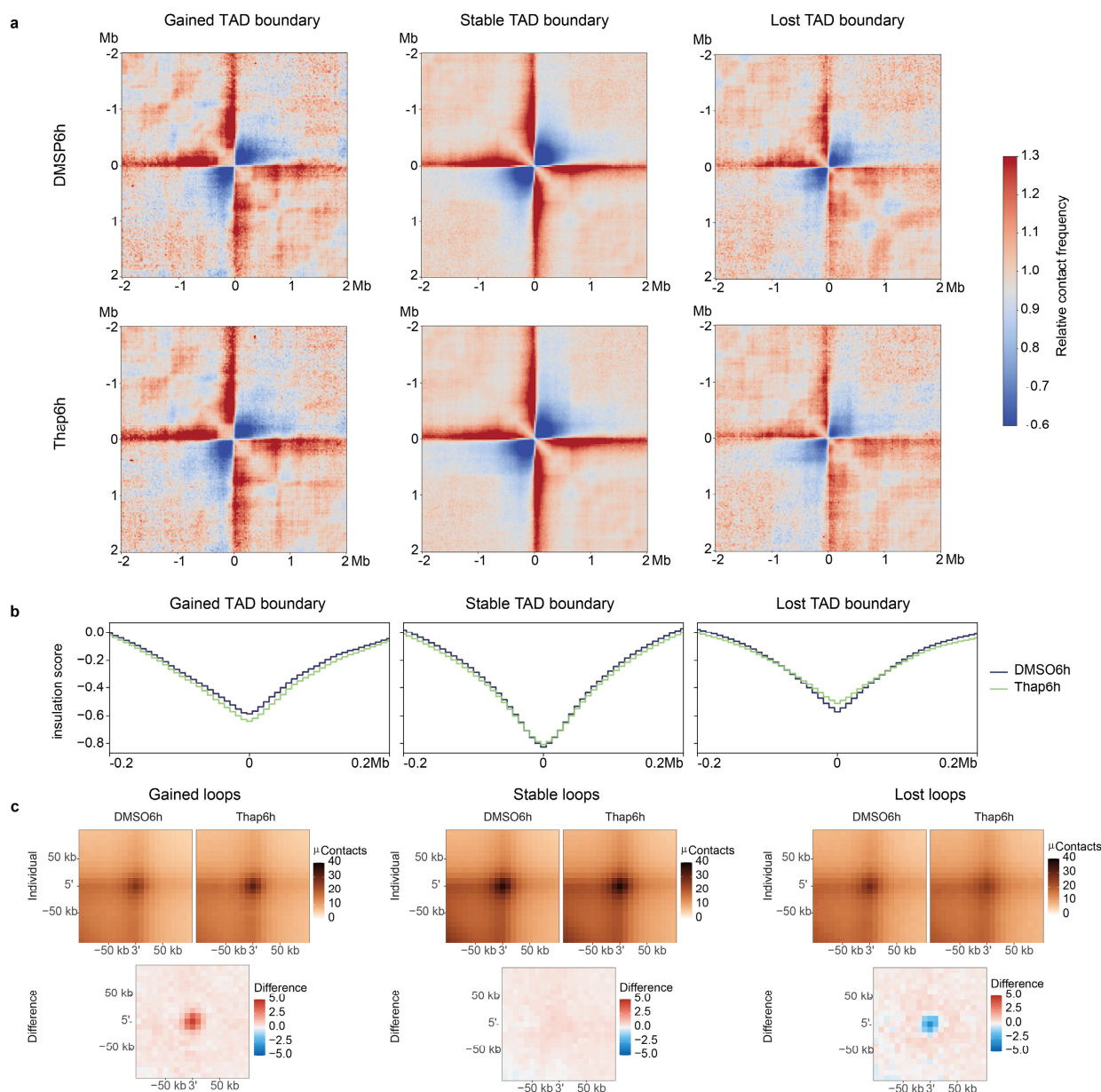
d Pie charts depicting the proportions of up, down, and unchanged H3K27Ac peaks at the pre-occupied ATF4 promoter, gained ATF4 promoter, and control promoter peaks.

e Pie charts depicting the proportions of up, down, and unchanged ATAC-seq peaks at the pre-occupied ATF4 promoter, gained ATF4 promoter, and control promoter peaks.

f Box graph comparing the log₂ fold changes of genes associated with pre-occupied ATF4 promoters, gained ATF4 promoters, and control promoters in unchanged H3K27Ac (left) and ATAC-seq (right) peaks in HeLa cells. In the boxplots, the center line indicates the median, box limits represent the upper and lower quartiles, and whiskers extend to 1.5 $\times$ the interquartile range. The values beyond the whiskers are not shown in the boxplots. P-values were calculated using a two-tailed t-test. The number of genes and the median expression changes for each group are shown at the bottom.

g Representative tracks showing ATF4 CUT&Tag (orange), H3K27Ac CUT&Tag (green), ATAC-seq (blue), and RNA-seq (purple) signals at the *Atf3* and *Xbp1* gene loci in C2C12 cells treated with DMSO or Thap for 6 hours.

h Representative tracks showing ATF4 CUT&Tag (orange), H3K27Ac CUT&Tag (green), ATAC-seq (blue), and RNA-seq (purple) signals at the *HERPUD1* and *PPP1R15A* gene loci in HeLa cells treated with DMSO or CCCP for 6 hours.



Supplementary Figure 8 Hi-C reveals changes in higher-order chromatin organization during ISR

a Pileup heatmaps of genomic regions flanking gained, stable, or lost TAD boundaries (±2 Mb) in C2C12 cells treated with Thap or DMSO for 6 hours. Bin size: 10 kb.

b Averaged insulation profiles for 400 kb genomic regions centered around gained, stable, or lost TAD boundaries (±200 kb) in DMSO and Thap-treated cells. The insulation profiles show a downward shift at gained TAD boundaries and an upward shift at lost TAD boundaries, indicating the strengthening or weakening of these boundaries, respectively.

c Pileup heatmaps displaying the aggregated intensity of gained, stable, or lost chromatin loops in C2C12 cells treated with DMSO or Thap for 6 hours. Difference heatmaps (Thap – DMSO) are shown below. The 200-kb genomic regions centered on loop anchors (± 100 kb) were used for aggregation. Bin size: 10 kb.

a-b Average intensity profiles and heatmaps of H3K27Ac (**a**) and ATAC-seq (**b**) signals around the centers of gained TAD boundary, stable TAD boundary, and lost TAD boundary (± 5 kb) in C2C12 cells treated with DMSO or Thap for 6 hours.

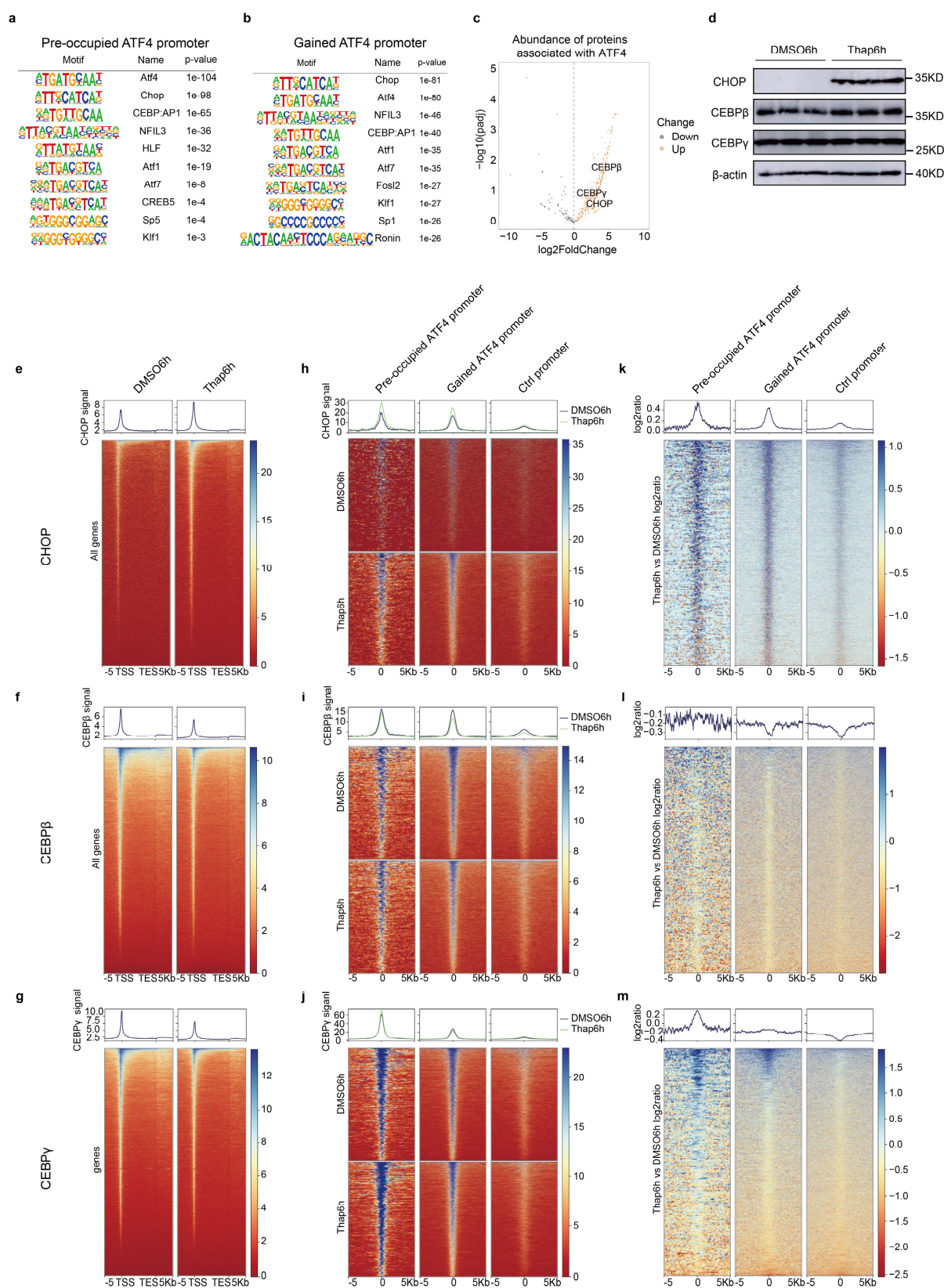
c Boxplot comparing the log₂ fold changes of genes located in gained TAD boundary, lost TAD boundary, and stable TAD boundary in C2C12 cells. In the boxplot, the center line indicates the median, box limits represent the upper and lower quartiles, and whiskers extend to 1.5 $\times$ the interquartile range. The values beyond the whiskers are not shown in the boxplots. P-values were calculated using a two-tailed t-test. The number of genes for each group is displayed.

d-e Average intensity profiles and heatmaps of H3K27Ac (**d**) and ATAC-seq (**e**) signals around the centers of gained loop anchors, stable loop anchors, and lost loop anchors (± 5 kb) in C2C12 cells treated with DMSO or Thap for 6 hours.

f Boxplot comparing the log₂ fold changes of genes located in gained loop anchors, lost loop anchors, and stable loop anchors in C2C12 cells. In the boxplot, the center line indicates the median, box limits represent the upper and lower quartiles, and whiskers extend to 1.5 $\times$ the interquartile range. The values beyond the whiskers are not shown in the boxplots. P-values were calculated using a two-tailed t-test. The number of genes for each group is displayed.

g Averaged insulation profiles for 400 kb genomic regions centered on TAD boundaries (± 200 kb) that coincide with pre-occupied ATF4 promoter peaks, gained ATF4 promoter peaks, and control promoter peaks in DMSO and Thap-treated cells.

h Pileup heatmaps showing the intensity of chromatin loops anchored at pre-occupied ATF4 promoters, gained ATF4 promoters, and control promoters in C2C12 cells treated with DMSO or Thap for 6 hours. Difference heatmaps (Thap – DMSO) are shown below. The 200-kb genomic regions centered on the loop anchors (± 100 kb) were used for aggregation. Bin size: 10 kb.



Supplementary Figure 10 Genome-wide redistribution of ATF4 heterodimerization partners during ISR

a, b Motif enrichment analysis of the pre-occupied ATF4 promoters (**a**) and the gained ATF4 promoter (**b**). The binding motifs for the CHOP and CEBP transcription factors are among the most significantly enriched motifs.

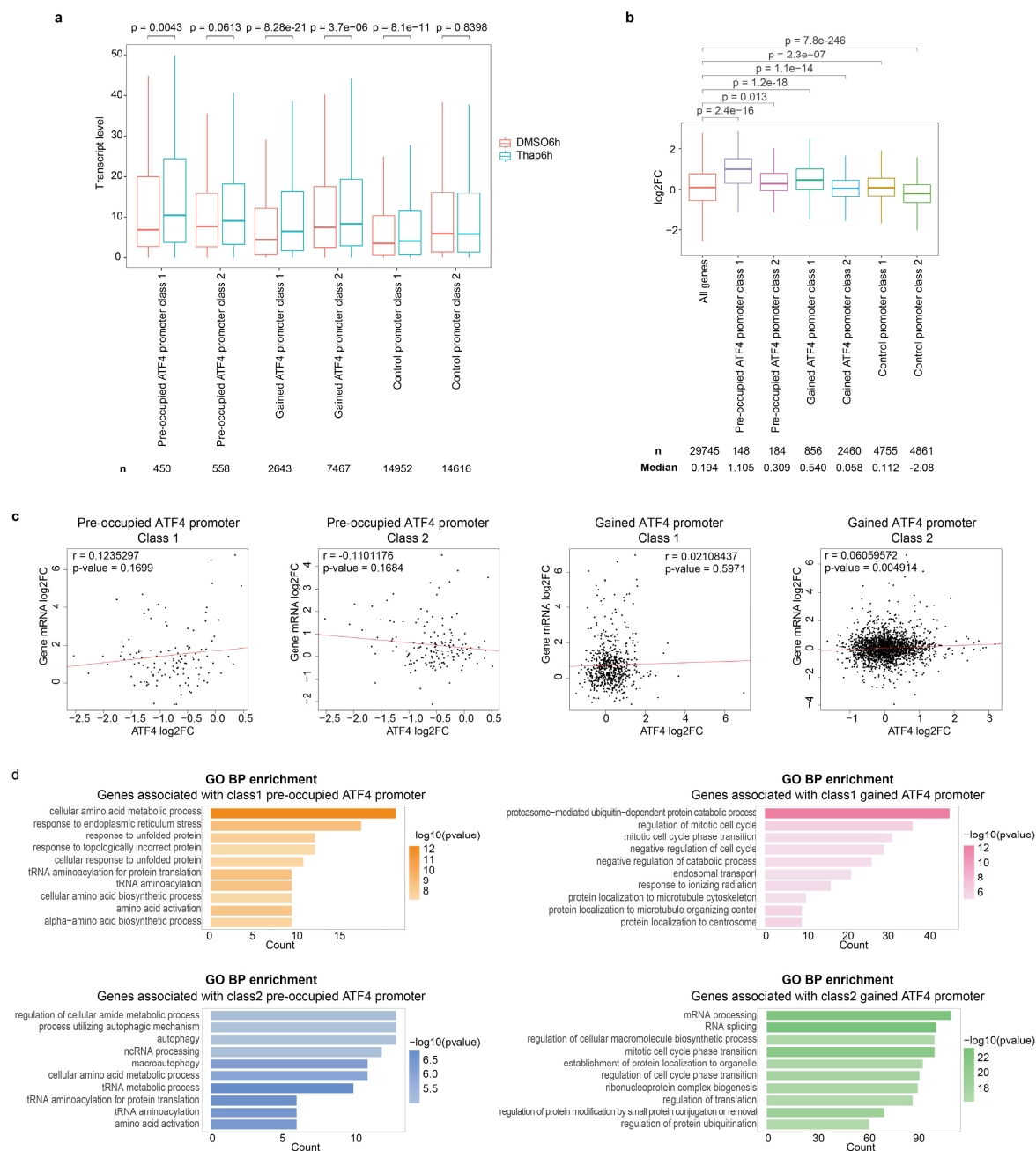
c Volcano plots showing proteins with altered interaction with ATF4 during ISR identified by Immunoprecipitation Mass Spectrometry (IP-MS). Proteins with increased interaction with ATF4 are highlighted in orange, while those with decreased interaction are shown in gray. Statistical analysis was performed using two-sided unpaired t-test.

d Western blot analysis of CHOP, CEBP β , and CEBP γ in C2C12 cells treated with DMSO or Thap for 6 hours. β -actin was used as the loading control. Thap treatment results in an elevation of CHOP protein levels, while CEBP β and CEBP γ levels remain unchanged.

e-g Average intensity profiles and heatmaps showing CHOP (**e**), CEBP β (**f**), and CEBP γ (**g**) occupancy across all genes and their flanking regions in C2C12 cells treated with DMSO or Thap for 6 hours.

h-j Average intensity profiles and heatmaps depicting the occupancy of CHOP (**h**), CEBP β (**i**), and CEBP γ (**j**) at genomic regions centered around pre-occupied ATF4 promoters, gained ATF4 promoters, or control non-ATF4 promoters (± 5 kb) in C2C12 treated with DMSO and Thap for 6 hours.

k-m Line plots and heatmaps depicting the log₂ ratio of CHOP (**k**), CEBP β (**l**), and CEBP γ (**m**) occupancy between Thap and DMSO treatments, centered around pre-occupied ATF4 promoters, gained ATF4 promoters, or control non-ATF4 promoters (± 5 kb) in C2C12 treated with DMSO and Thap for 6 hours.



Supplementary Figure 11 Differential transcriptional activation at different classes of ATF4 binding sites

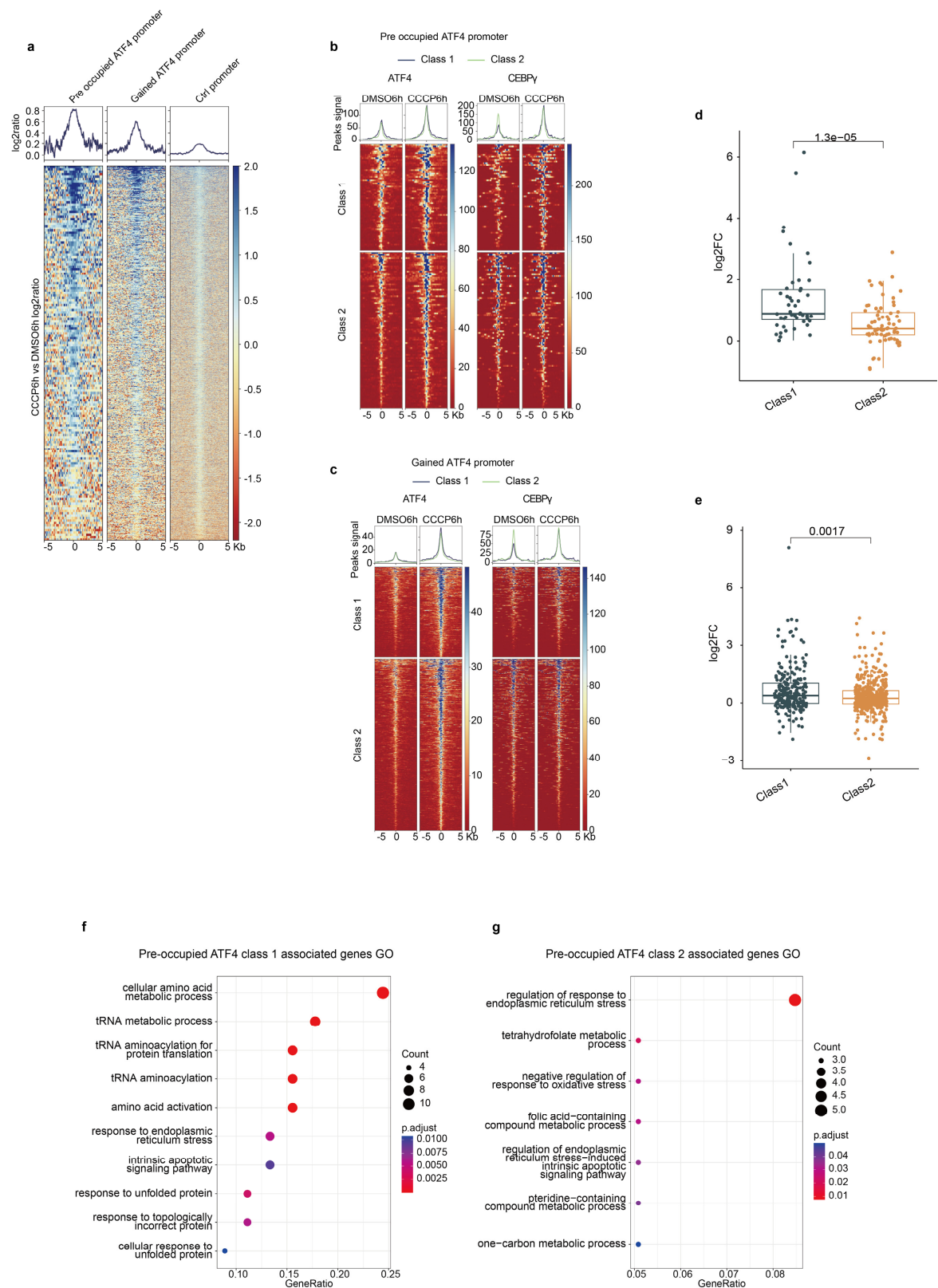
a Boxplot showing transcript levels for genes associated with class 1 and class 2 pre-occupied ATF4 promoters, newly gained ATF4 promoters, and control promoters in C2C12 treated with DMSO and Thap for 6 hours. In the boxplot, the center line indicates the median, box limits represent the upper and lower quartiles, and whiskers extend to 1.5× the interquartile range. The values beyond the whiskers are not shown

in the boxplots. P-values were calculated using a two-tailed Wilcoxon rank sum test. The number of genes for each group is displayed.

b Boxplots showing the fold changes of transcript levels for genes associated with different classes of promoters. In the boxplot, the center line indicates the median, box limits represent the upper and lower quartiles, and whiskers extend to $1.5\times$ the interquartile range. The values beyond the whiskers are not shown in the boxplots. P-values were calculated using a two-tailed t-test. The number of genes and the median expression changes for each group are shown at the bottom.

c Scatter plots with linear regression (red line) depicting the correlation between the log₂ ratio of ATF4 occupancy and the log₂FC of gene expression for class 1 and class 2 sites at pre-occupied ATF4 promoters and gained ATF4 promoters. Statistical analysis was performed using two-sided Spearman correlations. Spearman correlation coefficients (r) and associated P-values are displayed.

d Bar graphs indicate the top ten most enriched GO BP terms for genes associated with different classes of promoters. Enrichment analyses were performed using the ClusterProfiler R package with a one-tailed hypergeometric test followed by a Benjamini-Hochberg correction.



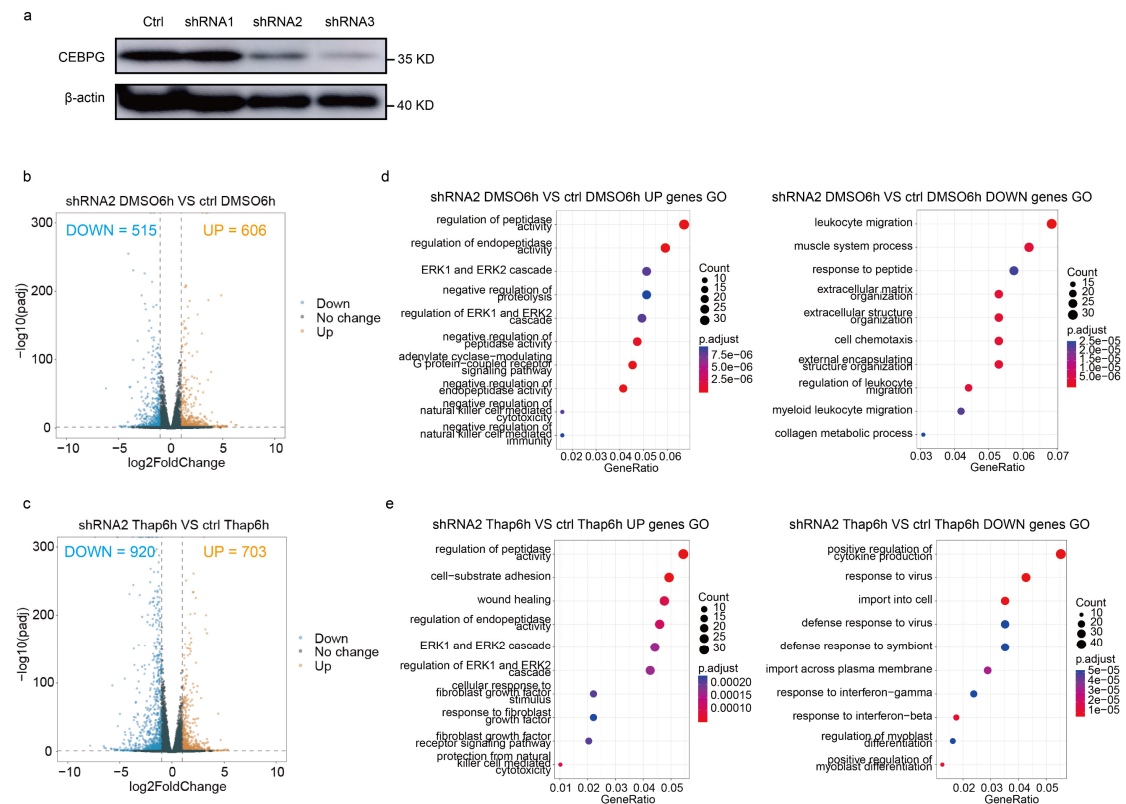
Supplementary Figure 12 Redistribution of CEBPγ correlates with ATF4-mediated transcriptional activation during ISR in HeLa cells

a Average intensity profiles and heatmaps depicting the occupancy of CEBP γ at genomic regions centered around pre-occupied ATF4 promoters, gained ATF4 promoters, or control non-ATF4 promoters (± 5 kb) in HeLa cells treated with DMSO and CCCP for 6 hours.

b-c Average intensity profiles and heatmaps showing normalized ATF4 and CEBP γ signals in DMSO- or Thap-treated C2C12 cells at class 1 and class 2 pre-occupied (**b**) and gained (**c**) ATF4 promoter sites. Two groups are classified based on the CEBP γ log₂ ratio by performing k-means clustering: class 1 sites with increased CEBP γ occupancy and class 2 sites where CEBP γ occupancy remains unchanged.

d-e Boxplots illustrating log₂ fold changes in gene expression associated with class 1 and class 2 sites for pre-occupied ATF4 promoters (**d**) and gained ATF4 promoters (**e**). Genes linked to Class 1 sites exhibit stronger transcriptional activation. P-values were calculated using a two-tailed t-test.

f-g Dot graphs indicate the top ten most enriched GO BP terms for genes associated with two classes of pre-occupied ATF4 promoters. Enrichment analyses were performed using the ClusterProfiler R package with a one-tailed hypergeometric test followed by a Benjamini-Hochberg correction.



Supplementary Figure 13 CEBP γ knockdown affects gene expression upon DMSO and Thap treatment

a Western blotting analysis of CEBP γ expression in CEBP γ -knockdown C2C12 cells (shRNA1, shRNA2, shRNA3) and negative control cells (ctrl).

b-c Volcano plots depicting differentially expressed genes (padj<0.05 and |log2FC|>1) between the CEBP γ -knockdown C2C12 cells (shRNA2) and control cells treated with DMSO (**b**) or Thap (**c**). Statistical analysis was performed using two-sided unpaired t-test.

d-e Dot graphs indicate the top ten most enriched GO BP terms for upregulated genes of CEBP γ -knockdown C2C12 (shRNA2) versus control cells treated with DMSO (**d**) or Thap (**e**). Enrichment analyses were performed using the ClusterProfiler R package with a one-tailed hypergeometric test followed by a Benjamini-Hochberg correction.