

Supplementary Materials for
**ACSS2 coupled with KAT7 regulates histone β -hydroxybutyrylation to
enhance transcription**

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The PDF file includes:

Figs. S1 to S14
Original blots S1 to S9
Legends for tables S1 to S6

Other Supplementary Material for this manuscript includes the following:

Tables S1 to S6

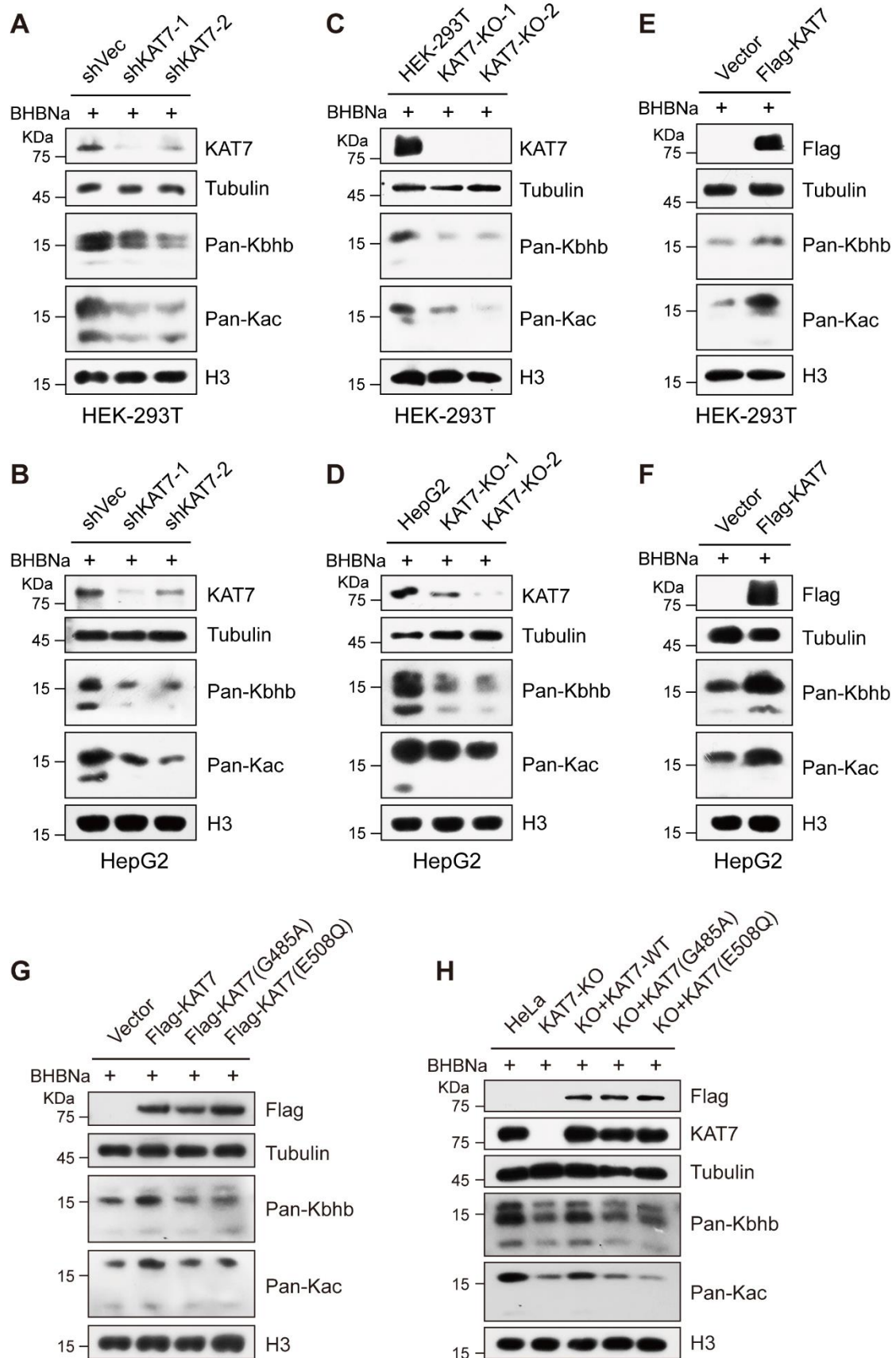


Fig. S1. KAT7 catalyzed β -hydroxybutyrylation on histones depended on its key residues in multiple cell lines.

(A-B) Immunoblot showing the fluctuation of Kbh_b level in KAT7-KD or KAT7-WT 293T (A) or HepG2 (B) cell line in the presence of 10 mM BHB. (C-D) WB analysis illustrating the Kbh_b level in KAT7-KO or KAT7-WT 293T (C) or HepG2 (D) cell line. (E-F) Immunoblot analysis showing the ectopic expression of KAT7 raises the Kbh_b level in 293T (E) or HepG2 (F) cell lines. (G) Detection for the level of Kbh_b or Kac on histones in cells expressing KAT7-WT or its loss function mutants (G485A and E508Q). (H) Rescue assays for the level of Kbh_b or Kac on histones in KAT7-KO HeLa cells overexpressed KAT7-WT, KAT7-G485A, or KAT7-E508Q.

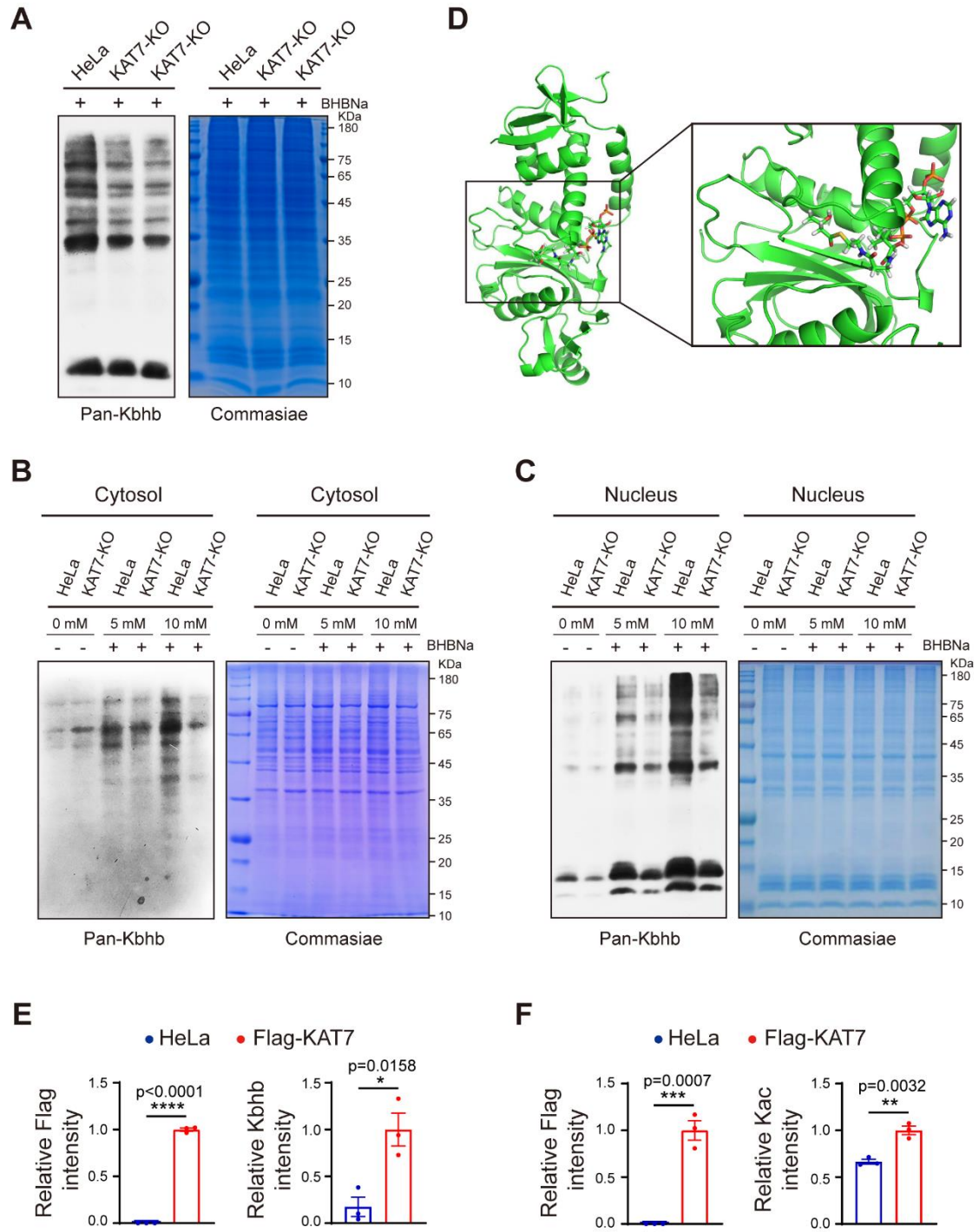


Fig. S2. KAT7 regulates global β -hydroxybutyrylation.

(A) Western blot showing the level of global Kbh_b by comparing KAT7-WT with KAT7-KO HeLa cells in the presence of 10 mM BHB_{Na}. (B-C) Immunoblot illustrating that BHB_{Na} dose-dependently increased the Kbh_b levels on cytoplasmatic or nuclear proteins in WT HeLa cells, while the incremental Kbh_b level was attenuated in KAT7-KO cells. (D) Molecular Docking assays for the simulation of β -hydroxybutyryl-CoA binding to KAT7. (E-F) The relative Kbh_b (E) or Kac (F) intensity of IF in HeLa cells ectopically expressed Flag-KAT7 is shown (Related to Fig. 1E). The blue point represents HeLa cells without ectopic expression of Flag-KAT7 while red point represents HeLa cells with ectopic expression of Flag-KAT7. The bar chart showing the mean $\pm$ SEM of the three independent experiments. $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****).

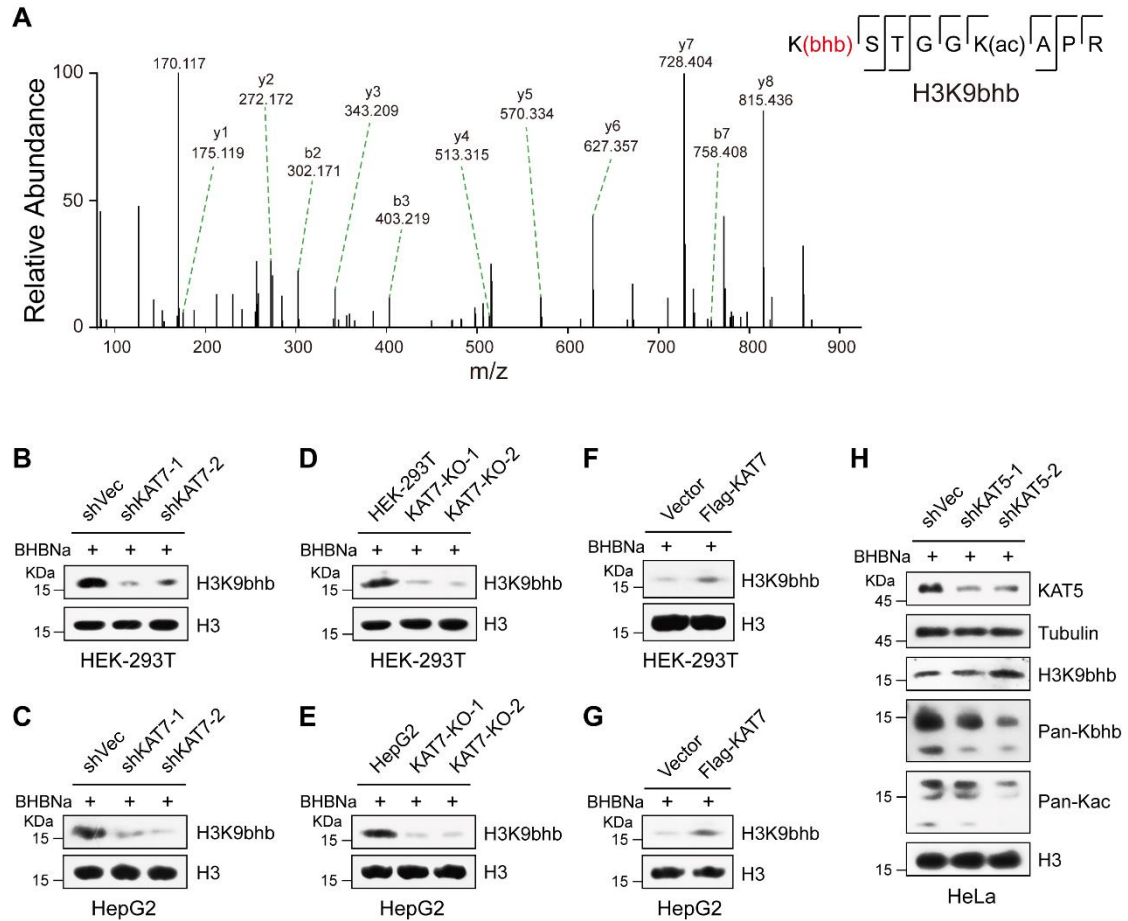


Fig. S4. KAT7 preferably regulates the H3K9bhb level.

(A) The representative MS/MS spectrum of H3K9bhb. The x axis represents m/z and the y axis represents ion relative abundance. (B-C) Immunoblots showing H3K9bhb level in KAT7-KD or KAT7-WT 293T (B) and HepG2 (C) cell lines. (D-E) Immunoblots illustrating H3K9bhb level in KAT7-KO or KAT7-WT 293T (D) and HepG2 (E) cell lines. (F-G) WB analysis indicating H3K9bhb level in KAT7 overexpressed 293T (F) and HepG2 (G) cell lines. (H) Detection of Kbhb and H3K9bhb in KAT5-deficiency HeLa cells.

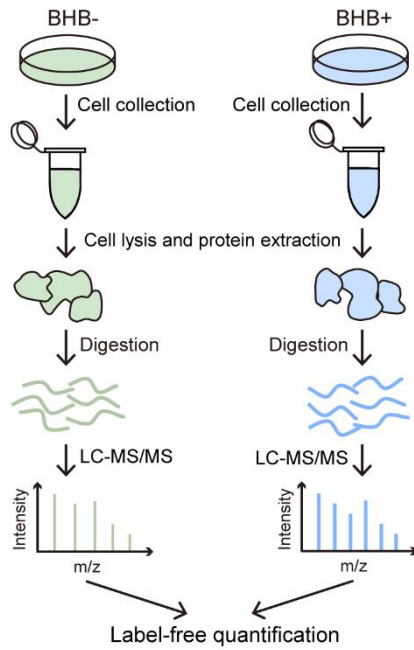
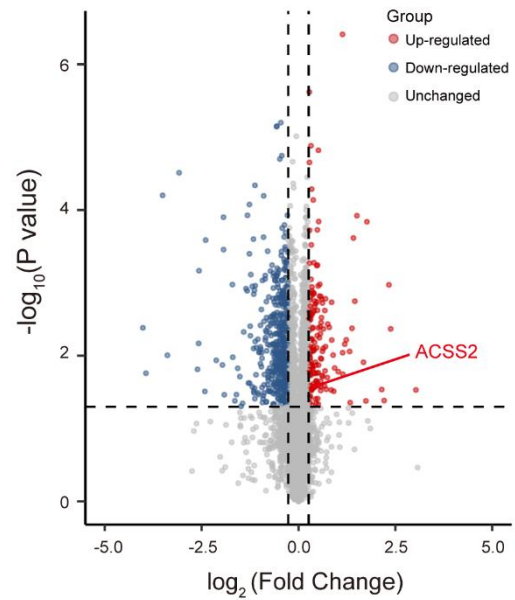
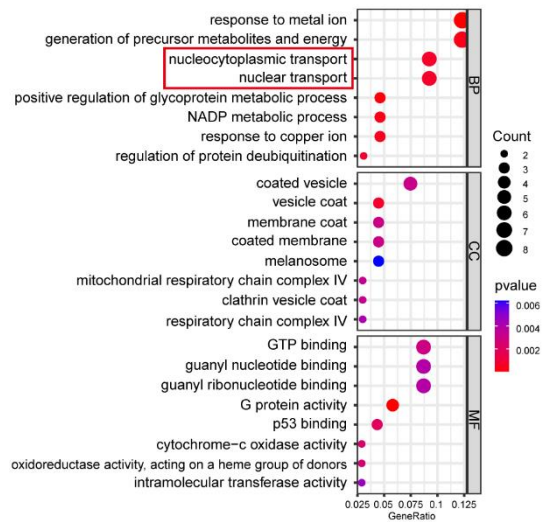
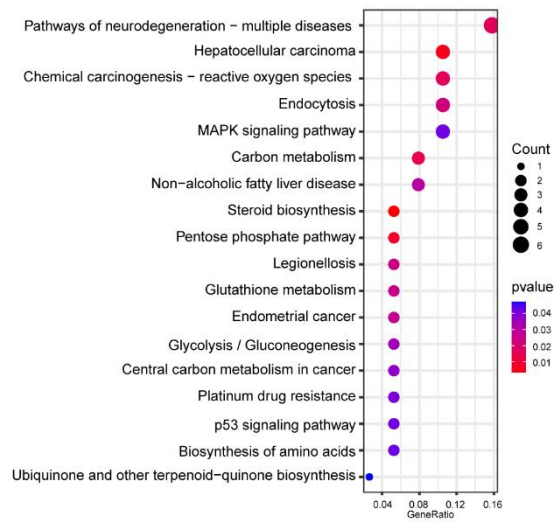
A**B****C****D**

Fig. S5. Data independent acquisition (DIA) quantitative proteome analysis for differential proteins induced by BHB.

(A) The flowchart of label-free quantification proteome by DIA. (B) Volcano plot illustrating the differential proteins with BHB stimulation (1.2-fold change cutoff, $p < 0.05$, $n = 3$ in each group, from independent biological replicates). (C-D) GO (left) or KEGG (right) analysis for upregulated proteins after cells treated with or without BHBNa. The p -value cutoff = 0.05 and q -value cutoff = 0.1 were selected as the cutoff criteria. Benjamini and Hochberg correction was used to adjust p -values.

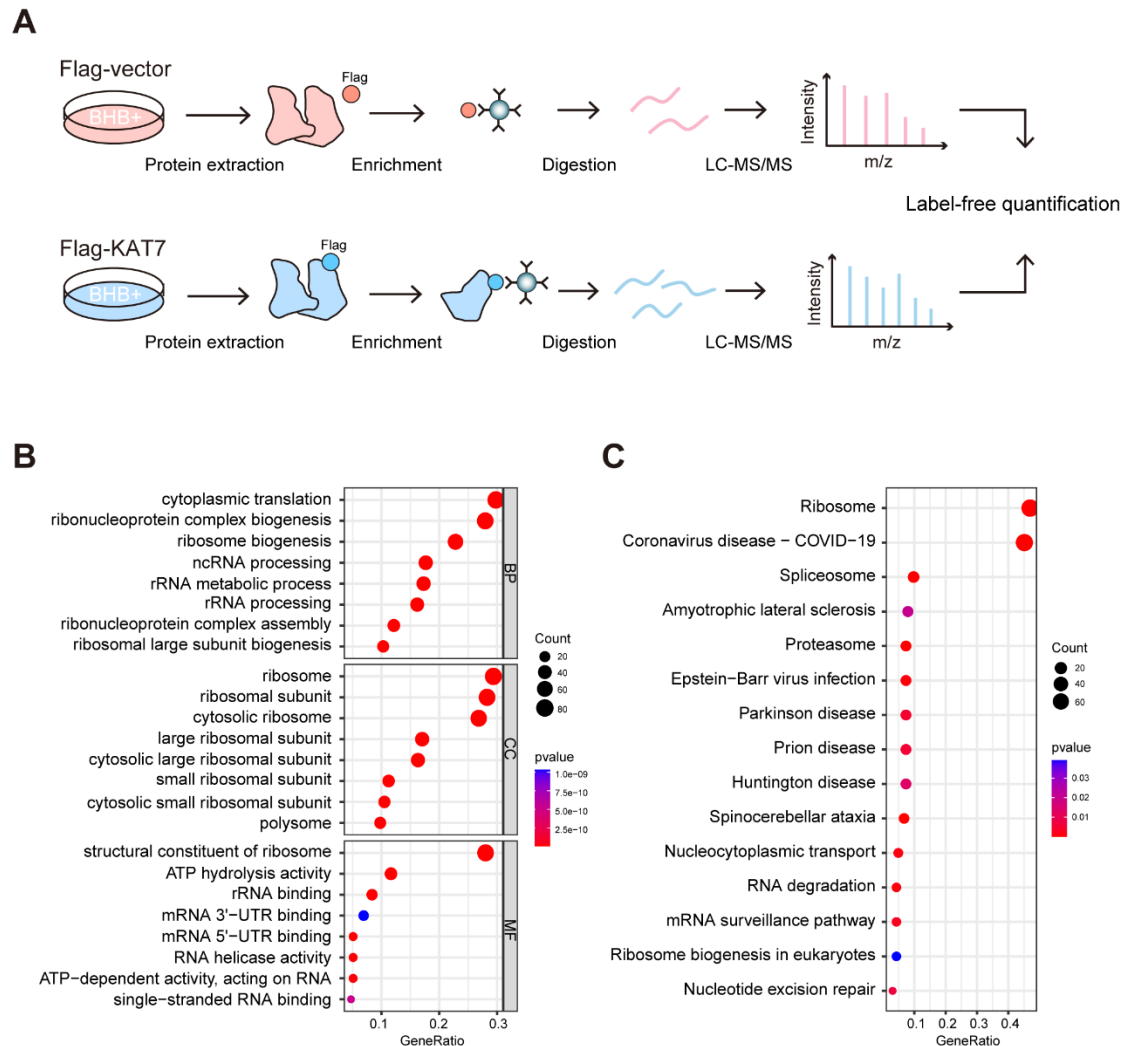


Fig. S6. Identification of ACSS2 as an interacting protein of KAT7.

(A) The flowchart of identification of KAT7 interacting proteins through a label-free quantitative proteomics approach (1.5-fold change cutoff, $p < 0.05$, $n = 3$ in each group, from independent biological replicates). These two groups of cells were treated with BHBNa for 24 h. (B-C) GO (left) and KEGG (right) analysis of proteins identified by KAT7 IP/MS. The p-value cutoff = 0.05 and q-value cutoff = 0.1 were selected as the cutoff criteria. Benjamini and Hochberg correction was used to adjust p-values.

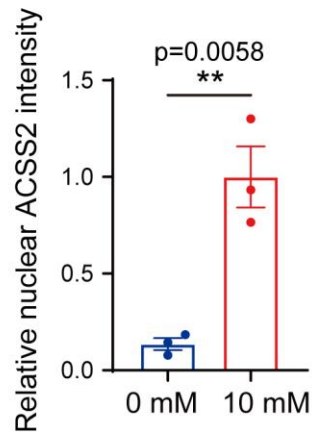
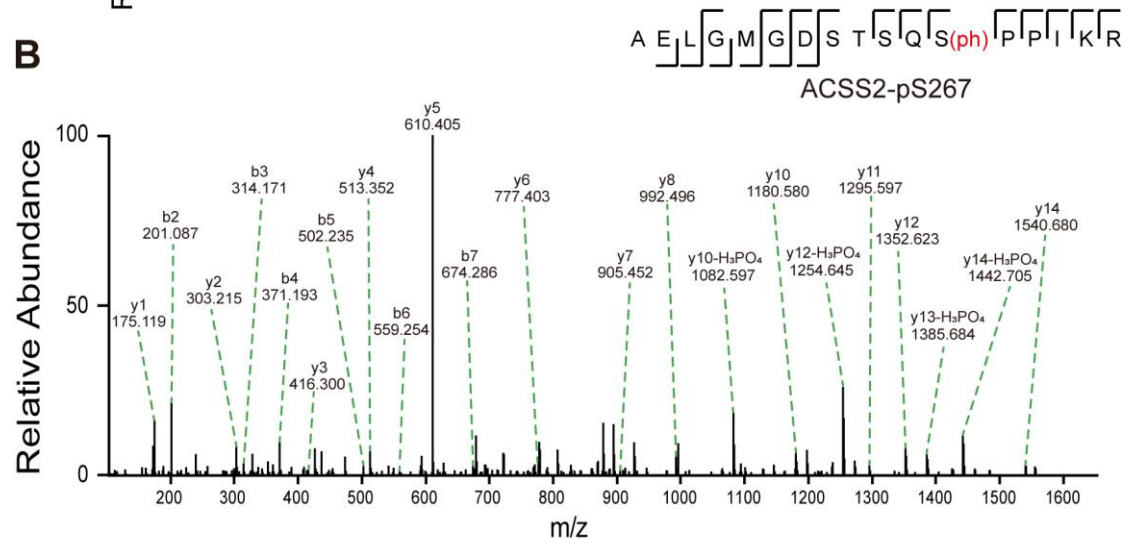
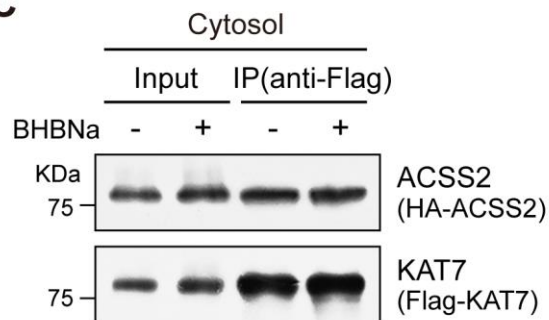
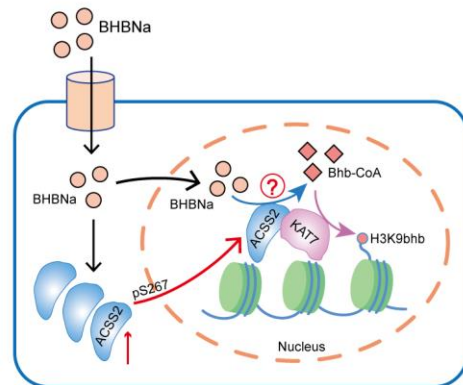
A**B****C****D**

Fig. S7. BHB treatment facilitates the nuclear translocation of ACSS2.

(A) The nuclear distribution intensity of HA-ACSS2 with or without BHBNa treatment is shown (Related to Fig. 3E). Data are the mean $\pm$ SEM of the three independent experiments. $p < 0.01$ (**). **(B)** The representative MS/MS spectrum of S267ph on ACSS2. The x axis represents m/z and the y axis represents ion relative abundance. **(C)** Immunoblot showing ACSS2 level enriched by KAT7 in cytoplasm in the presence or absence of BHB. **(D)** Schematic diagram showing that BHB accumulation promotes the expression of ACSS2 as well as its nuclear translocation, thereafter interacting with KAT7 in the nucleus.

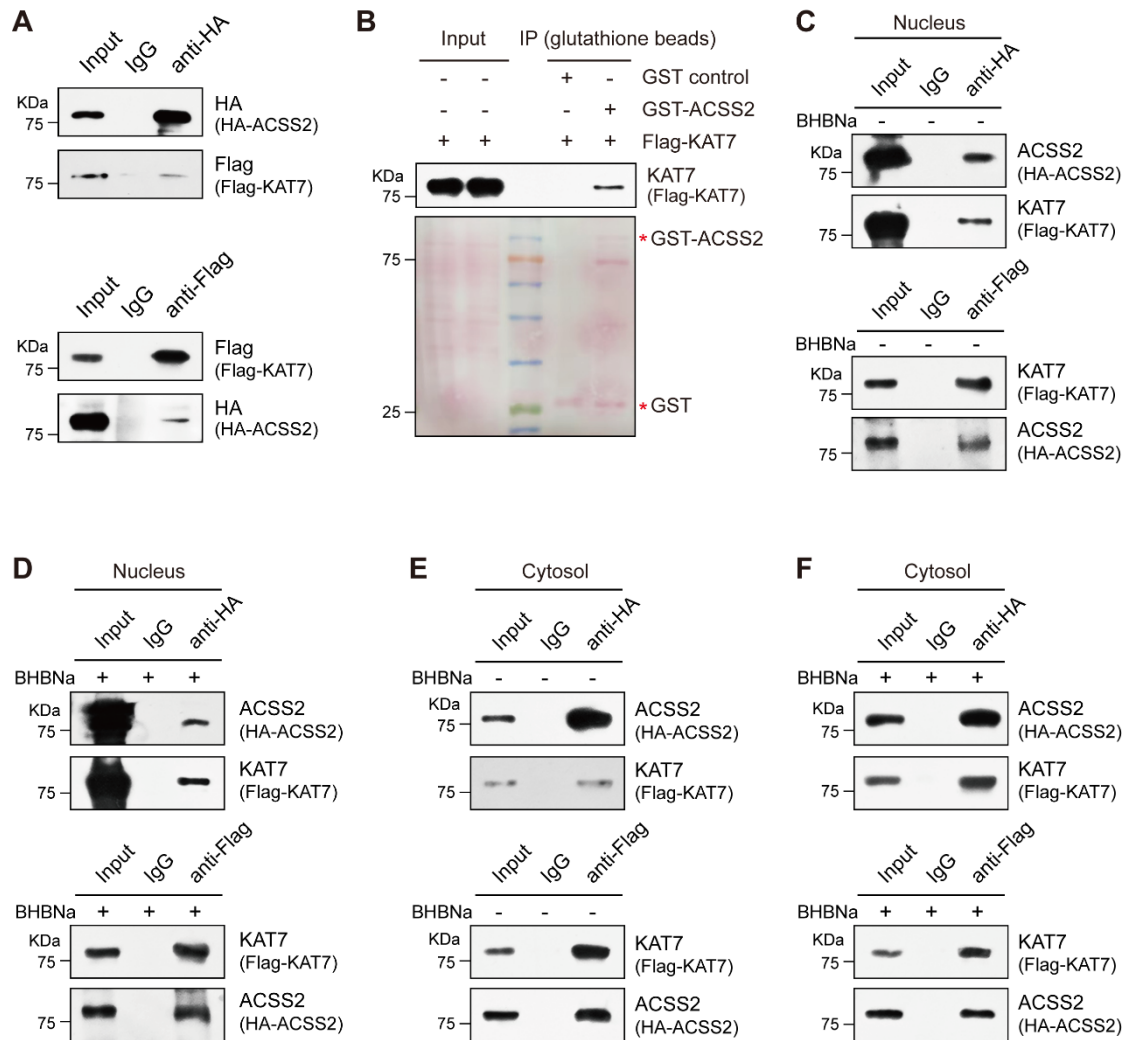


Fig. S8. KAT7 interacted with ACSS2 in the presence or absence of BHB.

(A) Co-immunoprecipitations (IP) and reciprocal IP assays for verifying the interaction between KAT7 and ACSS2 in cells with ectopic expression of KAT7-Flag and ACSS2-HA. (B) Pull down in vitro assay for validating the interaction between KAT7 and ACSS2. (C-D) Co-IP and reciprocal IP assay showing the interaction between KAT7 and ACSS2 in nucleus with or without BHB treatment. (C) representing absence of BHB stimulation while (D) representing presence of BHB treatment. (E-F) Co-IP and reciprocal IP assay showing the interaction between KAT7 and ACSS2 in cytoplasm with or without BHB treatment. (E) representing absence of BHB accumulation while (F) representing presence of BHB treatment.

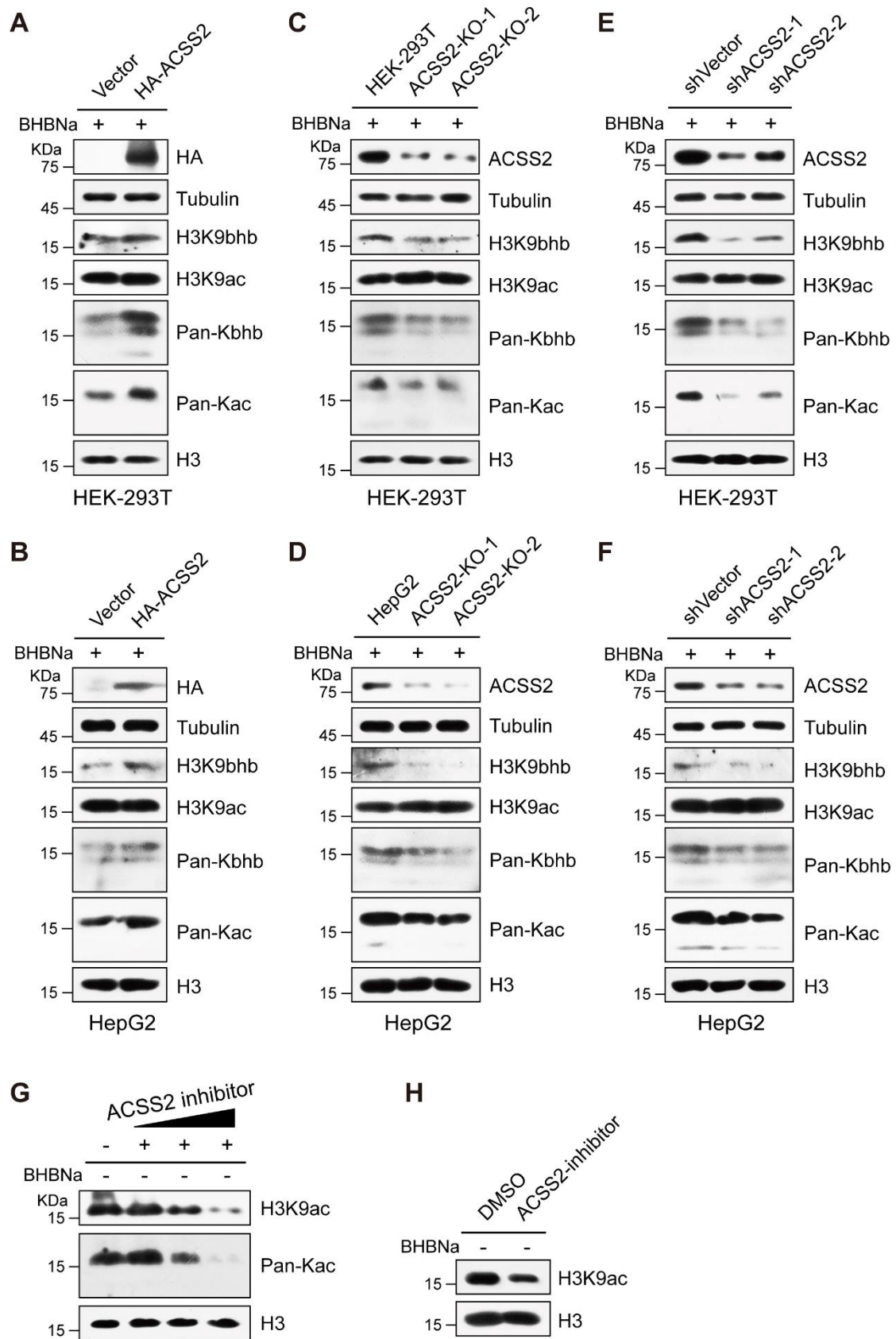


Fig. S9. ACSS2 exerts BHB-CoA synthase to regulate the Kbhb on histones.

(A-B) Immunoblot analysis showing the ectopic expression of ACSS2 elevates the Kbhb level in 293T **(A)** or HepG2 **(B)** cell lines in the presence of 10 mM BHB. **(C-D)** WB analysis illustrating the Kbhb level in ACSS2-KO or ACSS2-WT 293T **(C)** or HepG2 **(D)** cell line. **(E-F)** Immunoblot showing the fluctuation of Kbhb level in ACSS2-KD or ACSS2-WT 293T **(E)** or HepG2 **(F)** cell line. **(G-H)** Immunoblot illustrating the reduced Kac or H3K9ac level leveraging the ACSS2 inhibitors alone in the absence of BHB stimulation.

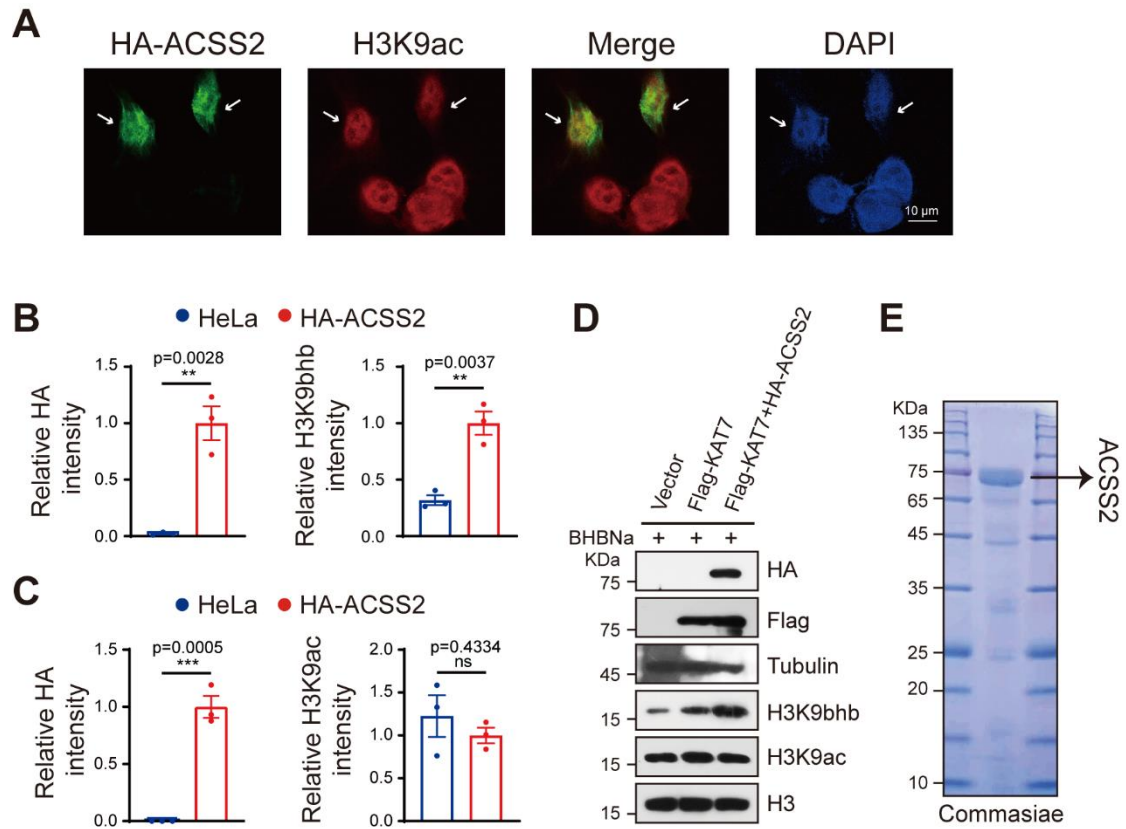


Fig. S10. ACSS2 preferably regulates H3K9bhb but not H3K9ac in the presence of BHB.

(A) IF staining showing the colocalization of H3K9ac and ACSS2 in HeLa cells, whereas slightly increasing trend of H3K9ac level induced by ectopic expression of ACSS2-WT upon BHBNa treatment. (B-C) The relative H3K9bhb (B, Related to Fig. 4F) or H3K9ac (C, Related to fig. S10A) intensity of IF in HeLa cells ectopically expressed HA-ACSS2 is shown. The blue point represents HeLa cells without ectopic expression of HA-ACSS2 while red point represents HeLa cells with ectopic expression of HA-ACSS2. The bar chart showing the mean $\pm$ SEM of the three independent experiments. $p < 0.01$ (**), $p < 0.001$ (***), ns = no substantial. (D) Detection of the level of H3K9bhb or H3K9ac when KAT7 and ACSS2 were co-expressed in HeLa cells upon BHBNa stimulation. (E) Coomassie brilliant blue (CBB) staining of the purified ACSS2 are shown.

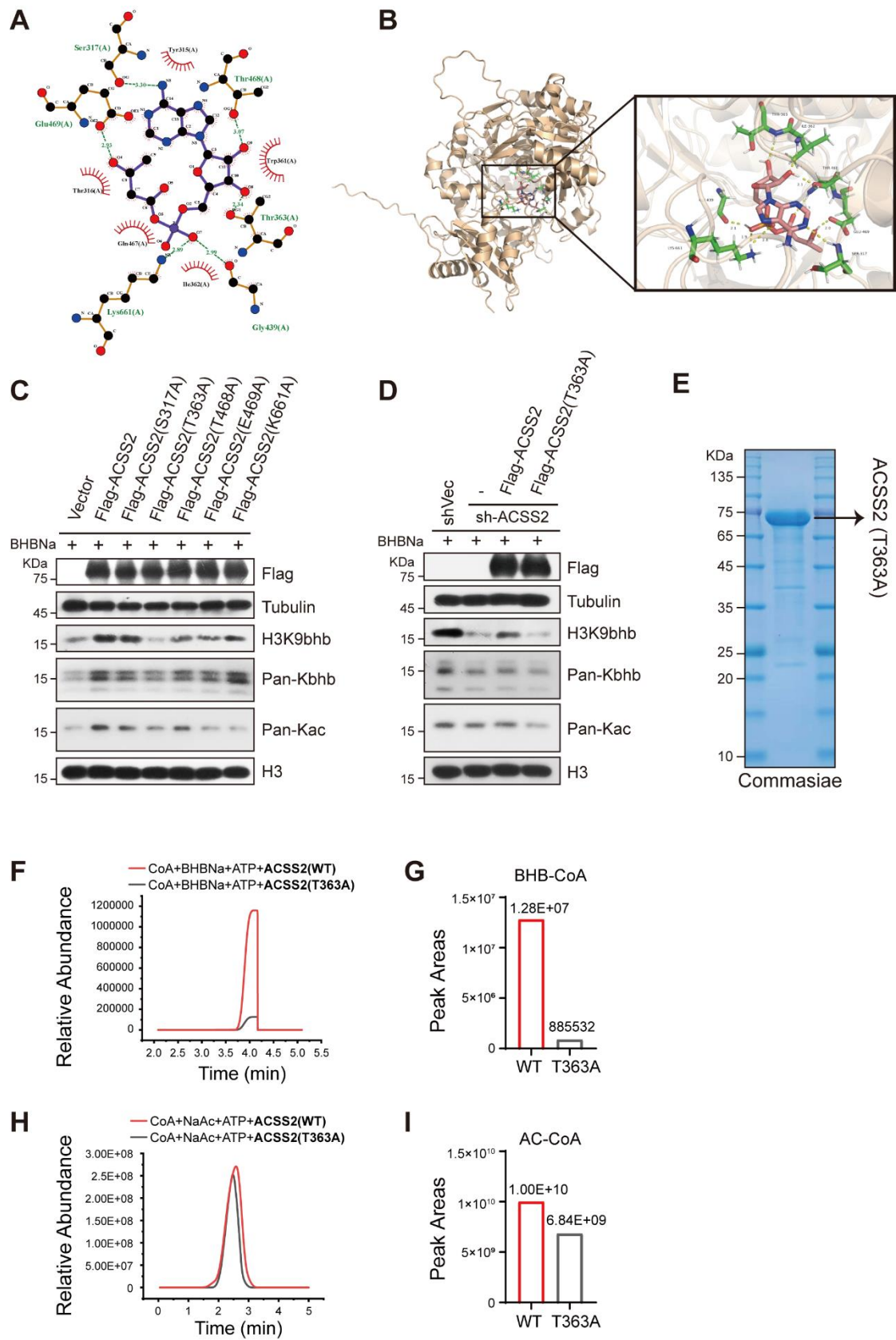


Fig. S11. ACSS2 catalyzes the generation of BHB-CoA relying on its key residues.

(A) Hydrogen bonds and hydrophobic interactions between ACSS2 and Bhb-AMP are shown by LigPlus. (B) Molecular Docking assays for simulation of Bhb-AMP binding to ACSS2. The docking analysis was performed using Surflex-Dock module, a fully automatic docking tool available on Sybyl-X 2.0 program (Tripos Inc.). The 3D structure of ACSS2 was retrieved from AlphaFold2. All the hydrogen atoms were added to define the correct configuration and tautomeric states before the generation of the docking pocket automatically. Then compound Bhb-AMP built by Sybyl program was docked into the pocket for docking-scoring analysis. (C) Immunoblots showing the level of H3K9bhb, Kbhb or Kac in cells treated with BHBNa and transfected by ACSS2-WT or its mutants, including S317A, T363A, T468A, E469A, K661A. (D) The rescue assay for examining the level of H3K9bhb, Kbhb or Kac when ACSS2-WT and mutant ACSS2-T363A were overexpressed in ACSS2-KO HeLa cells in the presence of BHBNa. (E) Coomassie brilliant blue (CBB) staining of the purified loss function mutant ACSS2 (T363A) are shown. (F and H) Identification of BHB-CoA (F) or AC-CoA (H) via HPLC-MS/MS analysis. The BHBNa or NaAC was incubated with CoA and ATP in presence or absence of recombinant ACSS2-WT or ACSS2-T363A, and the resulting sample was thereafter determined by HPLC-MS/MS. The x axis represents elution time and the y axis represents ion relative abundance. (G, I) The bar chart showing the concentration of BHB-CoA (G) or AC-CoA (I) synthesized by ACSS2-WT or ACSS2-T363A. The x axis represents the type of samples (ACSS2-WT or ACSS2-T363A) and y axis represents the chromatographic peak areas of BHB-CoA or AC-CoA. The calculation of peak areas are performed by Xcalibur Qual Browser.

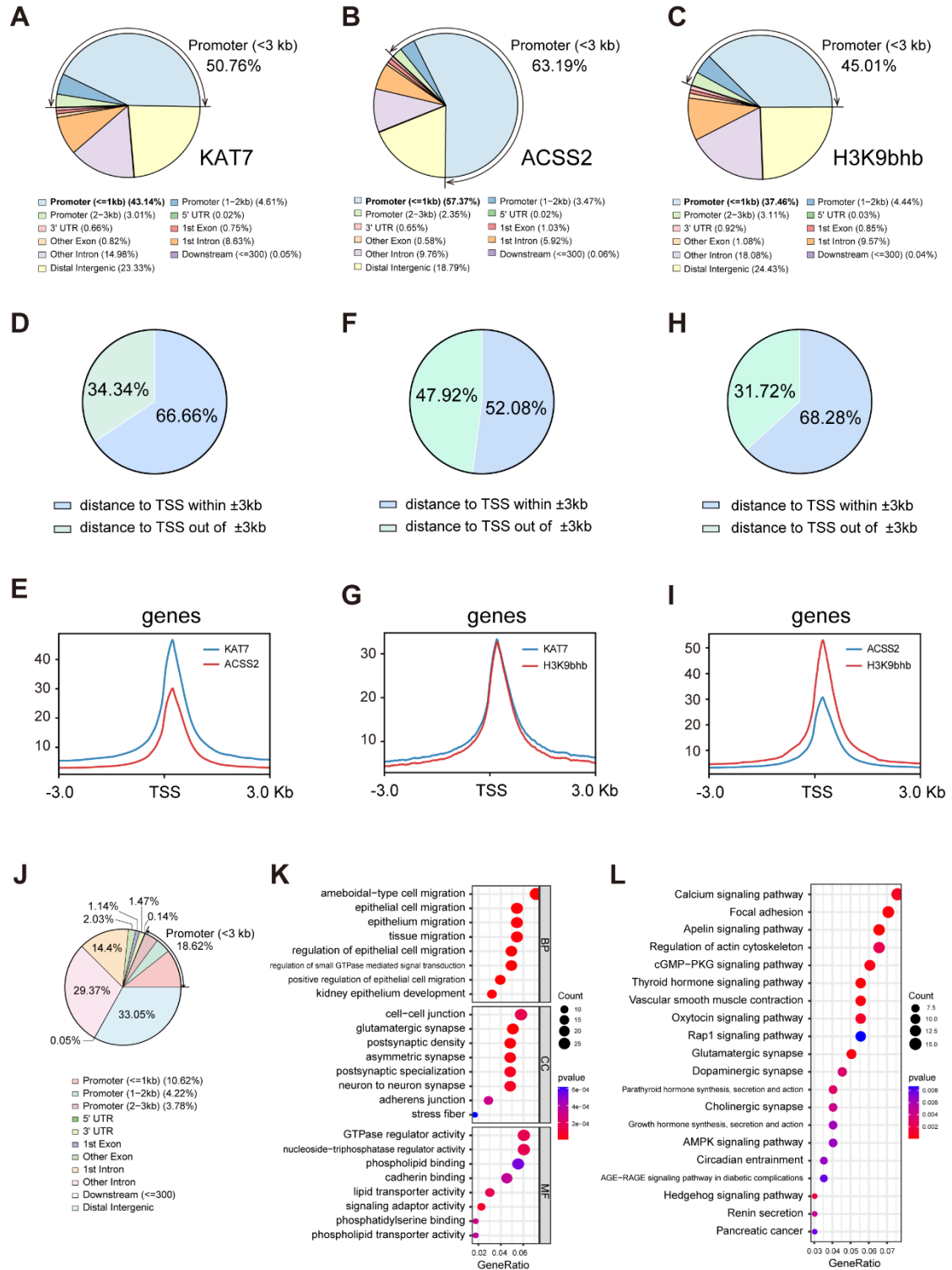


Fig. S12. Genomic localization of KAT7, ACSS2 and Histone H3K9bhb Marks.

(A, B, C) The pie chart showing the profile of KAT7- (A), ACSS2- (B) and H3K9bhb- (C) enriched peaks. (D, E, H) The pie chart describing the proportion of co-peaks localization within ± 3 kb of the TSS of KAT7-ACSS2 (D), KAT7-H3K9bhb (F), and ACSS2-H3K9bhb (H). (E, G, I) Density of CUT&Tag reads within ± 3 kb of the TSS for co-peaks of KAT7-ACSS2 (E), KAT7-H3K9bhb (G), and ACSS2-H3K9bhb (I). (J) Pie chart showing distribution of differential peaks enriched by H3K9bhb antibody (KAT7-WT versus KAT7-KO) across genome, 18.62% of which harbored within ± 3 kb of the TSS. (K-L) GO (left) or KEGG (right) analysis for differential H3K9bhb-marked peaks within ± 3 kb of the TSS between KAT7-WT and KAT7-KO HeLa cells. The p-value cutoff = 0.05 and q-value cutoff = 0.1 were selected as the cutoff criteria. Benjamini and Hochberg correction was used to adjust p-values.

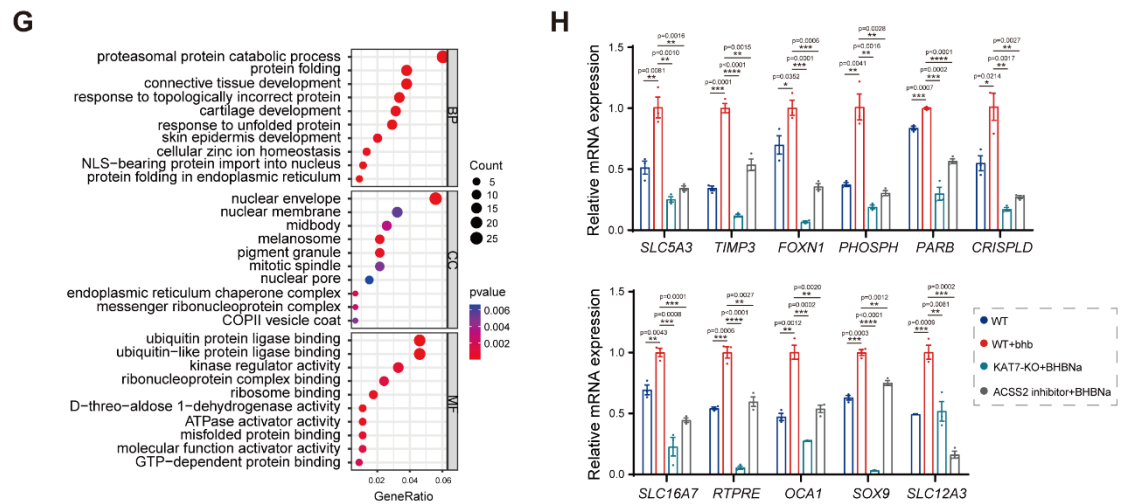
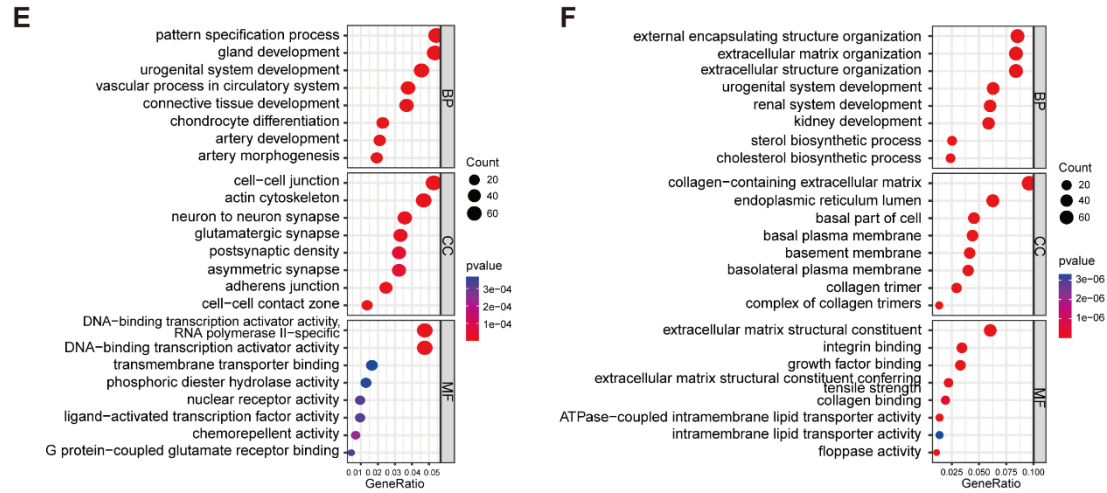
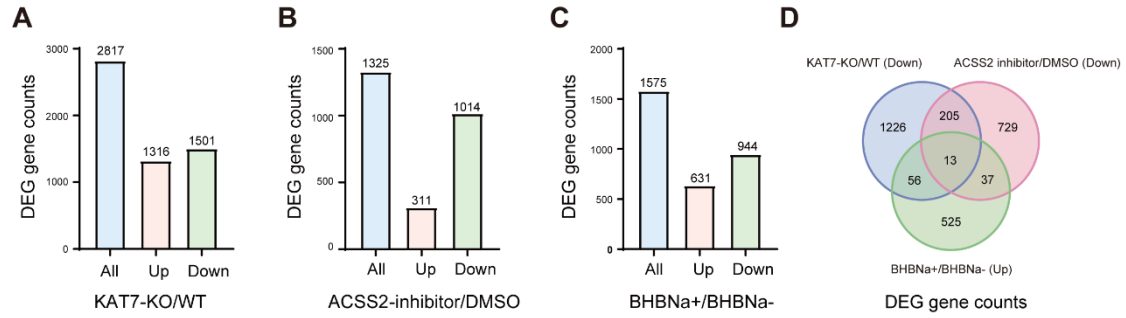


Fig. S13. Systematic profiling of BHB-induced transcriptome regulated by KAT7 and ACSS2.

(A-C) Bar graph showing the number of DEGs (differential expression genes) through RNA-seq analysis: (A) the DEGs in KAT7-deficient cells. (B) the DEGs in cells treated with specific ACSS2 inhibitors, and (C) the DEGs in cells with or without BHB stimulation. (D) Venn diagram illustrating the overlapped genes from RNA-seq analysis among the three groups mentioned above. The down-regulated genes were selected in KAT7-KO versus KAT7-WT group and ACSS2 inhibitors versus DMSO group. The up-regulated genes were selected in BHBNa⁺ versus BHBNa⁻ group. (E-G) GO enrichment analysis of DEGs as in (A-C). The criteria for data analysis is consistent with mentioned above. (H) The RT-qPCR analysis of overlapped candidate genes. The bar chart showing the mean $\pm$ SEM of the three independent experiments. $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****).

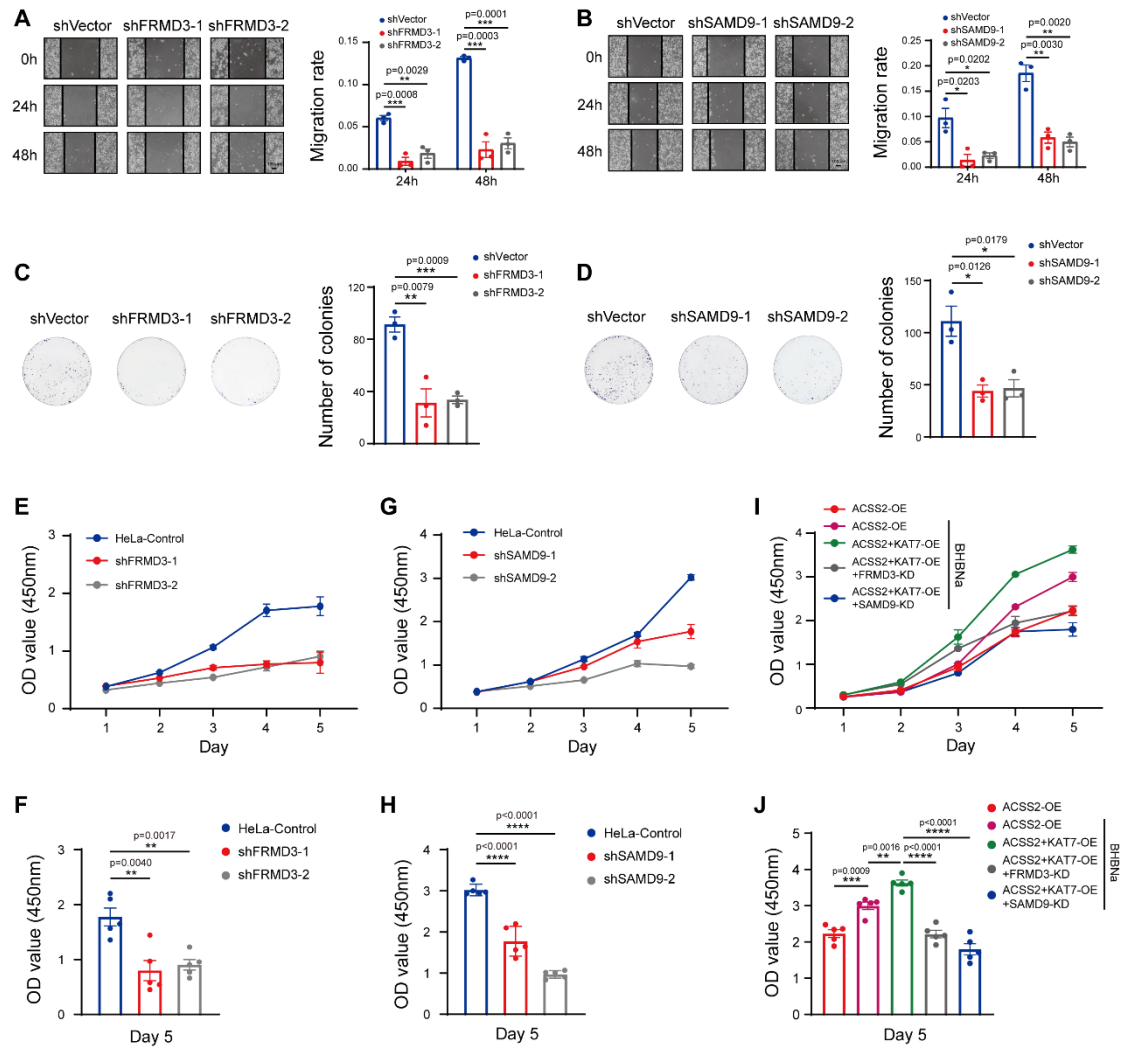
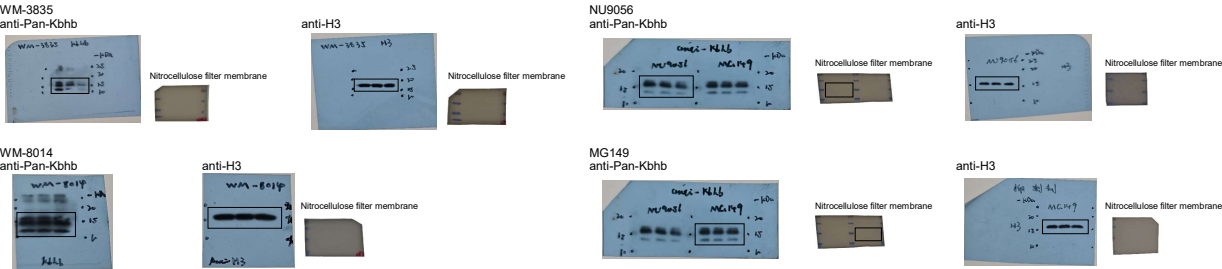


Fig. S14. Detection of migration, clonogenicity, and proliferation of SAMD9 or FRMD3 deficiency cells.

(A-B) The migration assay showing the suppression of the migration rate of HeLa cells after FRMD3 (left) and SAMD9 (right) Knocked down. The bar chart showing the mean $\pm$ SEM of the three independent experiments. $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***). **(C-D)** The clone formation assay illustrating the inhibition of HeLa cells proliferation after FRMD3 (left) and SAMD9 (right) Knocked down. The bar chart showing the mean $\pm$ SEM. $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***). **(E, G)** Line chart showing the growth trend of *FRMD3* KD (E), *SAMD9* KD (G) and Control HeLa cells during 5 days. **(F, H)** The Bar graph showing the growth difference after *FRMD3* (F) or *SAMD9* (H) knocked down in HeLa cells on the last day. The bar chart showing the mean $\pm$ SEM of the three independent experiments. $p < 0.01$ (**), $p < 0.0001$ (****). **(I)** Line chart illustrating the BHB-driven “ACSS2-KAT7” module having an effect on growth rate required H3K9bhb within 5 days. **(J)** The Bar graph showing the growth difference of these cell lines on the last day. The bar chart showing the mean $\pm$ SEM of the three independent experiments. $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****).

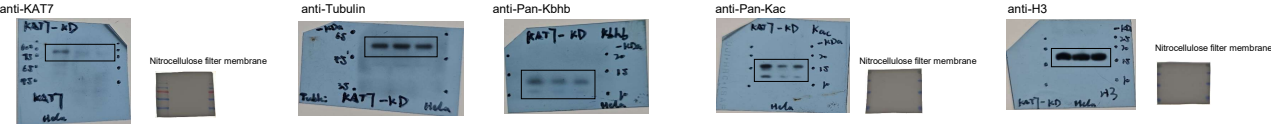
Original blots 1 - Related to Fig. 1 and 2

Fig.1A



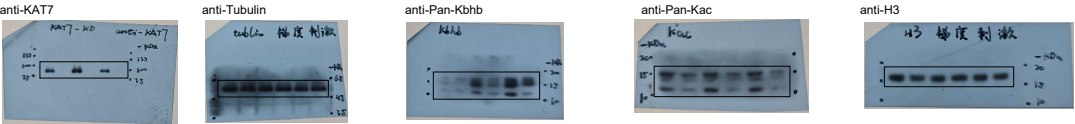
H3 serves as sample processing control. All the blots were using the same samples run in parallel on separate gels.

Fig.1B



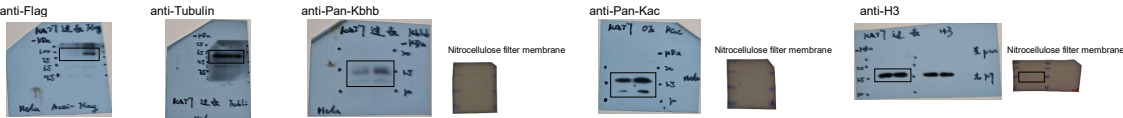
H3 and Tubulin serve as sample processing controls. All the blots were using the same samples run in parallel on separate gels.

Fig.1C



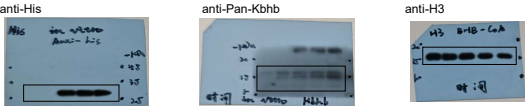
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Fig.1D



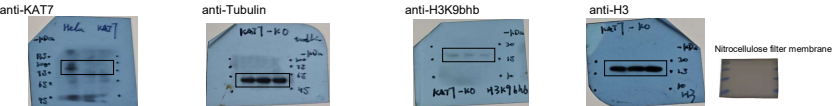
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Fig.1F



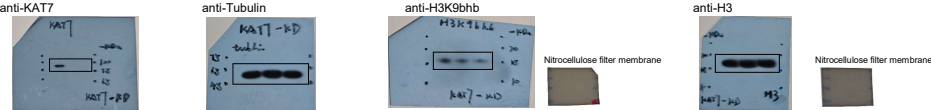
H3 serves as sample processing control. All the blots were using the same samples run in parallel on separate gels.

Fig.2E



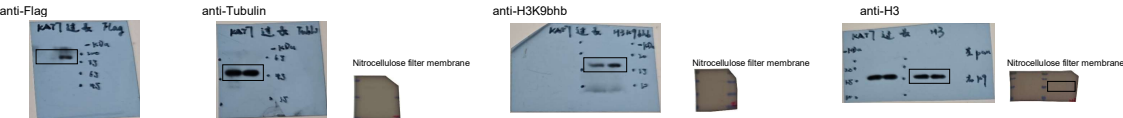
H3 and Tubulin serve as sample processing controls. All the blots were using the same samples run in parallel on separate gels.

Fig.2F



H3 and Tubulin serve as sample processing controls. All the blots were using the same samples run in parallel on separate gels.

Fig.2G



H3 and Tubulin serve as sample processing controls. All the blots were using the same samples run in parallel on separate gels.

Original blots 2 - Related to Fig. 3

Fig.3C

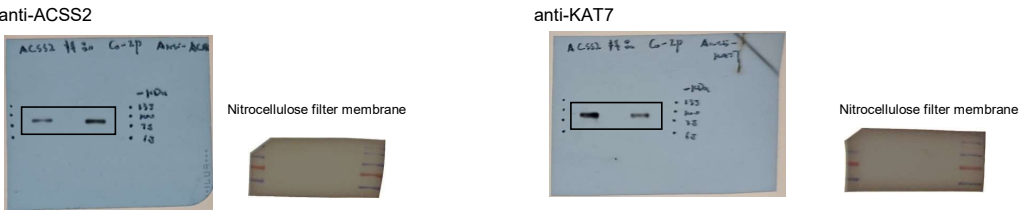


Fig.3D

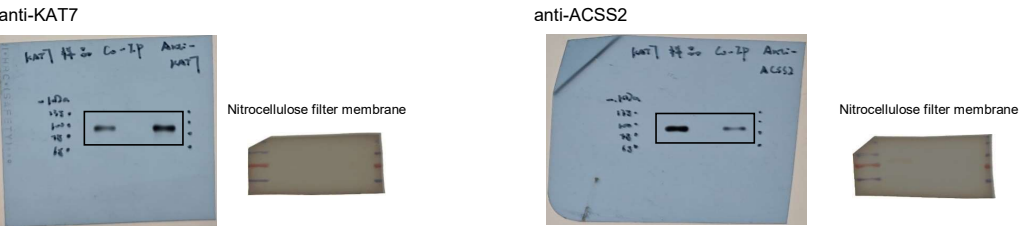
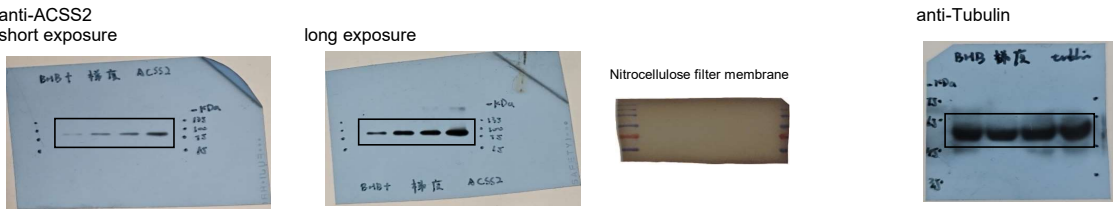
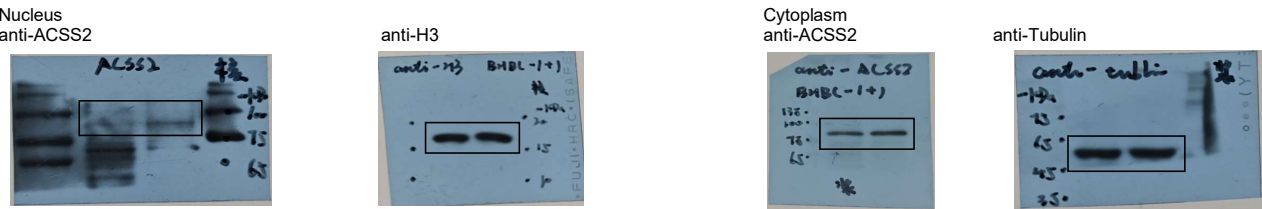


Fig.3F



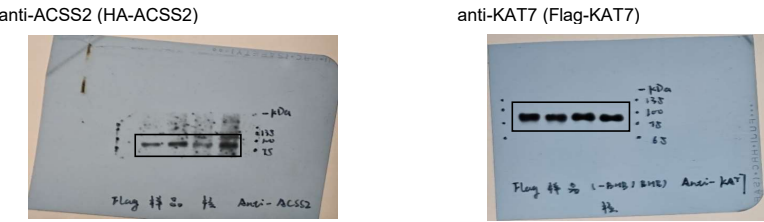
Tubulin serves as sample processing control. All the blots were using the same samples run in parallel on separate gels.

Fig.3G



H3 and Tubulin serve as sample processing controls. All the blots were using the same samples run in parallel on separate gels.

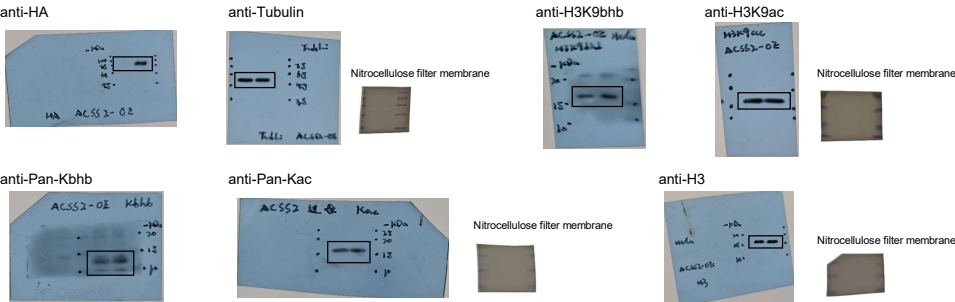
Fig.3H



KAT7 serves as sample processing control. All the blots were using the same samples run in parallel on separate gels.

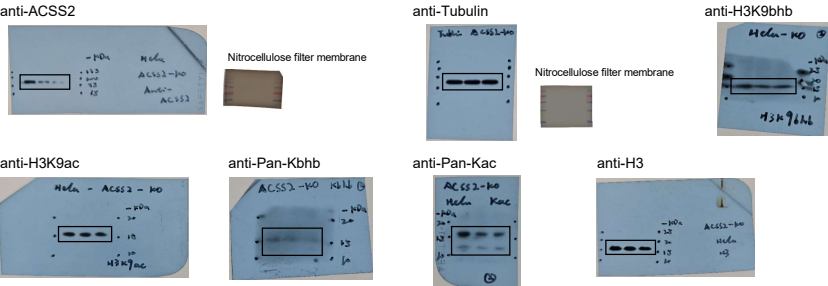
Original blots 3 - Related to Fig. 4

Fig.4A



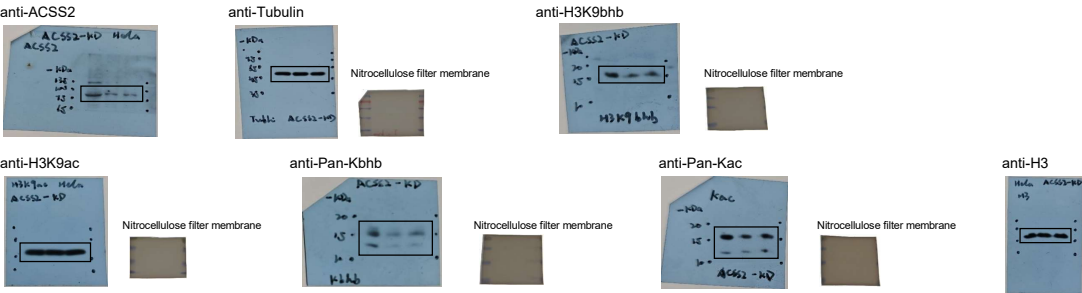
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Fig.4B



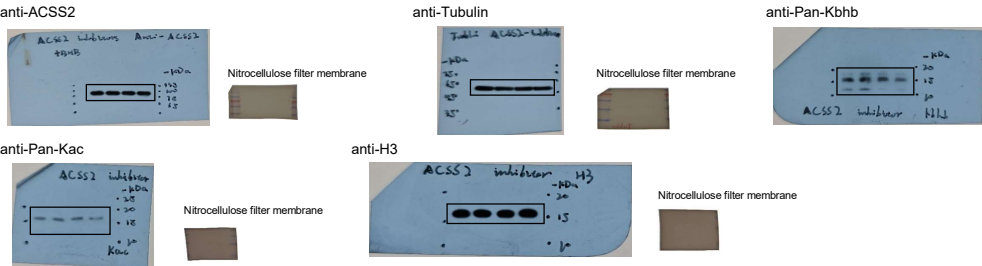
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Fig.4C



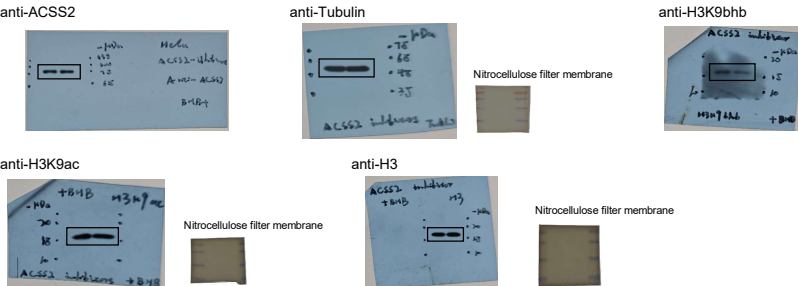
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Fig.4D



H3 and Tubulin serve as sample processing controls. All the blots were using the same samples run in parallel on separate gels.

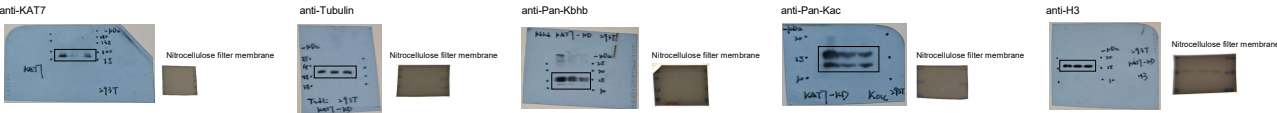
Fig.4E



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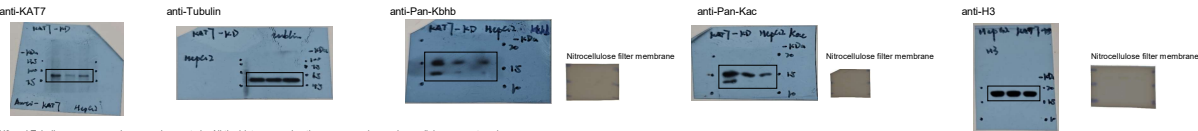
Original blots 4 - Related to fig. S1

SFig.1A



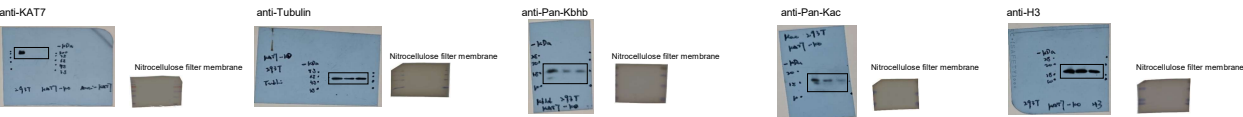
H3 and Tubulin serve as sample processing controls. All the blots were using the same samples run in parallel on separate gels.

SFig.1B



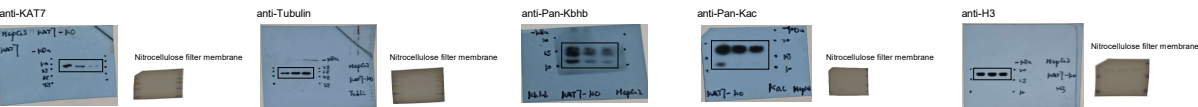
H3 and Tubulin serve as sample processing controls. All the blots were using the same samples run in parallel on separate gels.

SFig.1C



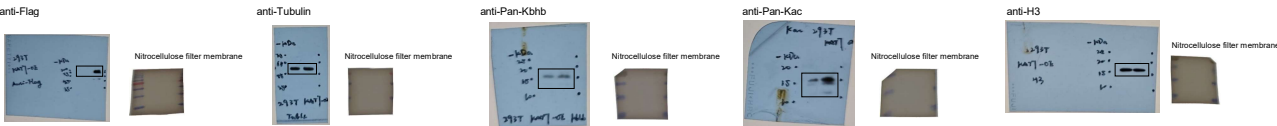
H3 and Tubulin serve as sample processing controls. All the blots were using the same samples run in parallel on separate gels.

SFig.1D



H3 and Tubulin serve as sample processing controls. All the blots were using the same samples run in parallel on separate gels.

SFig.1E



H3 and Tubulin serve as sample processing controls. All the blots were using the same samples run in parallel on separate gels.

SFig.1F



H3 and Tubulin serve as sample processing controls. All the blots were using the same samples run in parallel on separate gels.

SFig.1G



H3 and Tubulin serve as sample processing controls. All the blots were using the same samples run in parallel on separate gels.

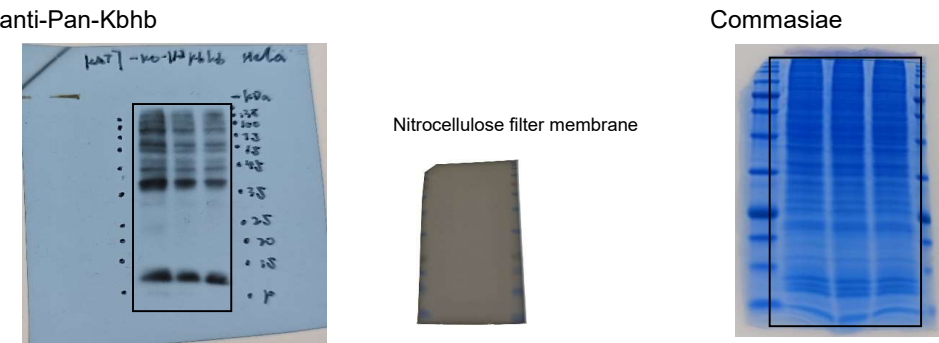
SFig.1H



H3 and Tubulin serve as sample processing controls. All the blots were using the same samples run in parallel on separate gels.

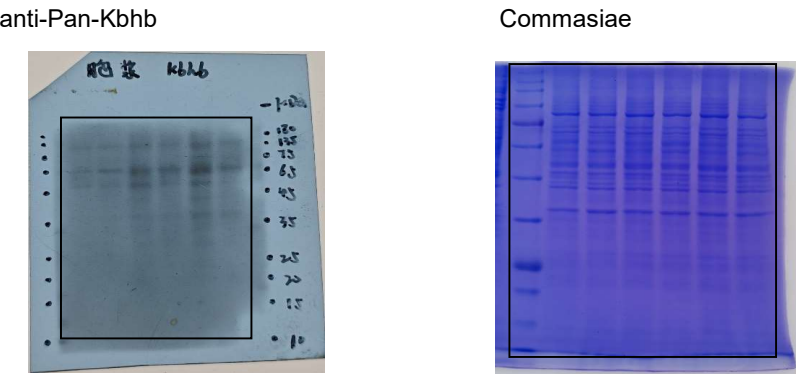
Original blots 5 - Related to fig. S2

SFig.2A



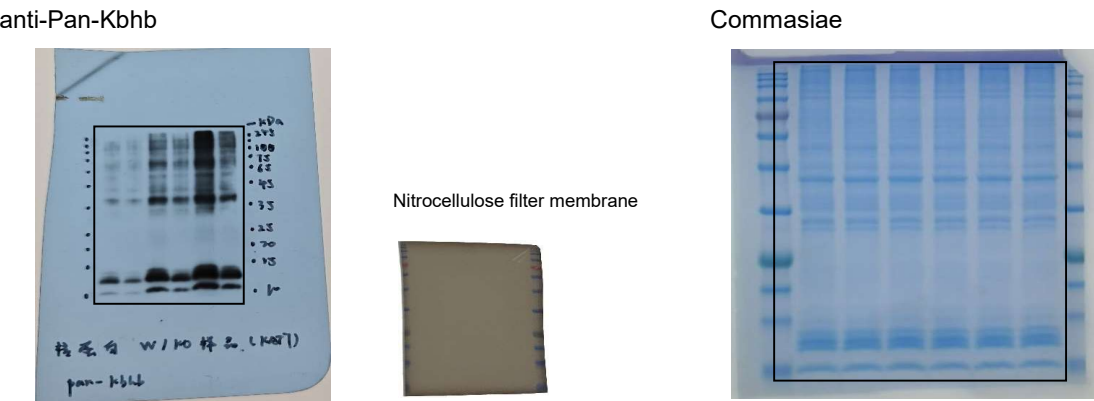
Commasiae serve as sample processing control. All the data were using the same samples run in parallel on separate gels.

SFig.2B



Commasiae serve as sample processing control. All the data were using the same samples run in parallel on separate gels.

SFig.2C



Commasiae serve as sample processing control. All the data were using the same samples run in parallel on separate gels.

Original blots 6 - Related to fig. S4 and S7

SFig.4B

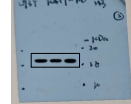
anti-H3K9bhb



Nitrocellulose filter membrane



anti-H3



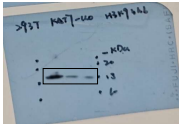
Nitrocellulose filter membrane



H3 serves as sample processing control. All the blots were using the same samples run in parallel on separate gels.

SFig.4D

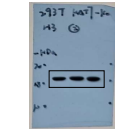
anti-H3K9bhb



Nitrocellulose filter membrane



anti-H3



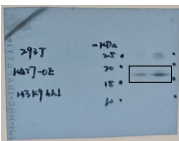
Nitrocellulose filter membrane



H3 serves as sample processing control. All the blots were using the same samples run in parallel on separate gels.

SFig.4F

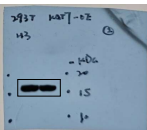
anti-H3K9bhb



Nitrocellulose filter membrane



anti-H3



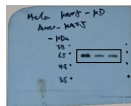
Nitrocellulose filter membrane



H3 serves as sample processing control. All the blots were using the same samples run in parallel on separate gels.

SFig.4H

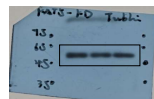
anti-KAT5



Nitrocellulose filter membrane



anti-Tubulin



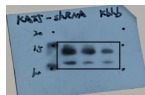
anti-H3K9bhb



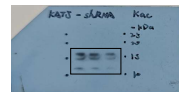
Nitrocellulose filter membrane



anti-Pan-Kbhb



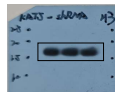
anti-Pan-Kac



Nitrocellulose filter membrane



anti-H3



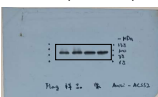
Nitrocellulose filter membrane



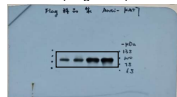
H3 and Tubulin serve as sample processing controls. All the blots were using the same samples run in parallel on separate gels.

SFig.7C

anti-ACSS2 (HA-ACSS2)



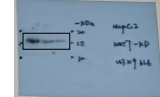
anti-KAT7 (Flag-KAT7)



KAT7 serves as sample processing control. All the blots were using the same samples run in parallel on separate gels.

SFig.4C

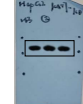
anti-H3K9bhb



Nitrocellulose filter membrane



anti-H3



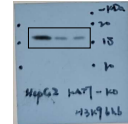
Nitrocellulose filter membrane



H3 serves as sample processing control. All the blots were using the same samples run in parallel on separate gels.

SFig.4E

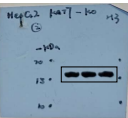
anti-H3K9bhb



Nitrocellulose filter membrane



anti-H3



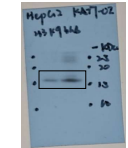
Nitrocellulose filter membrane



H3 serves as sample processing control. All the blots were using the same samples run in parallel on separate gels.

SFig.4G

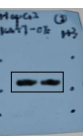
anti-H3K9bhb



Nitrocellulose filter membrane



anti-H3



Nitrocellulose filter membrane



H3 serves as sample processing control. All the blots were using the same samples run in parallel on separate gels.

Original blots 7 - Related to fig. S8

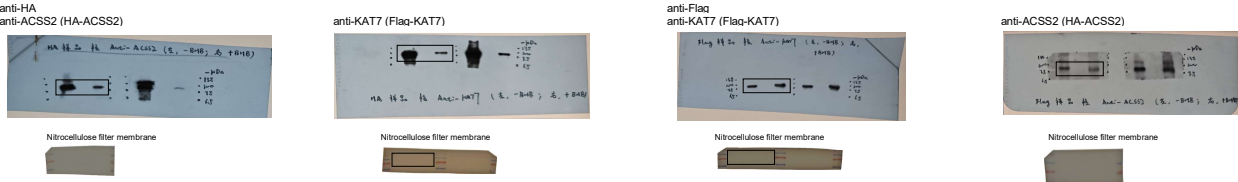
SFig.8A



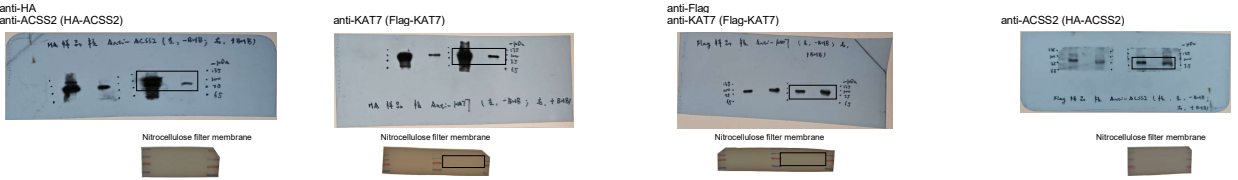
SFig.8B



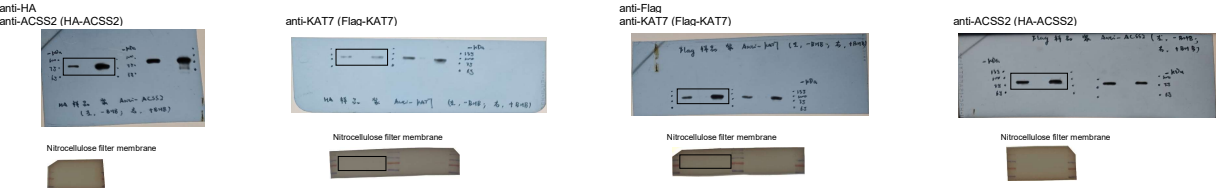
SFig.8C



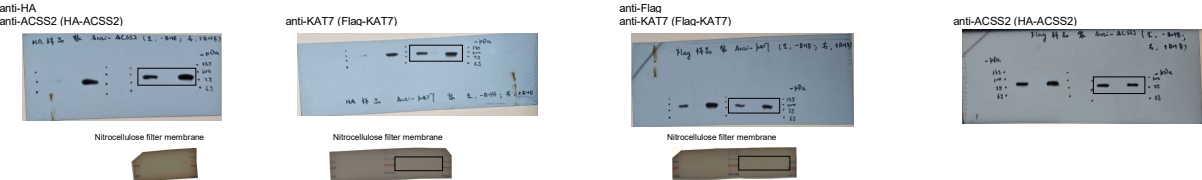
SFig.8D



SFig.8E

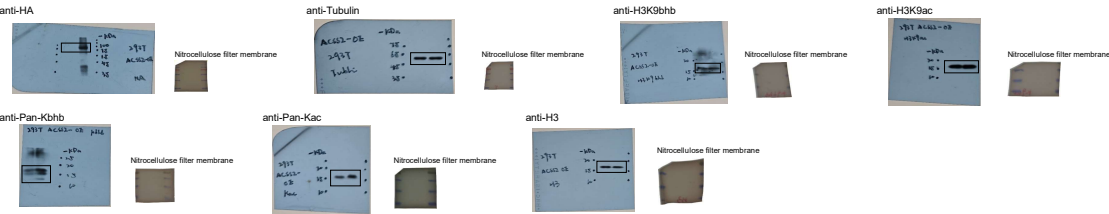


SFig.8F



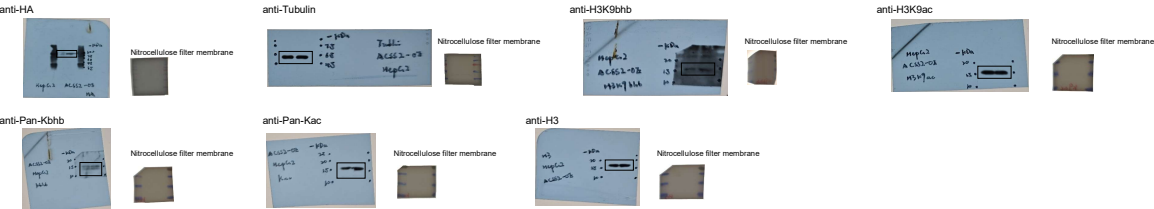
Original blots 8 - Related to fig. S9

SFig.9A



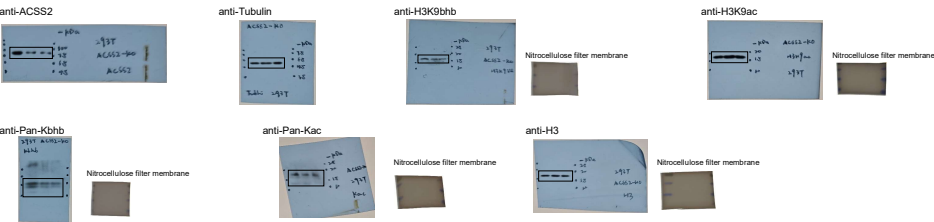
H3 and Tubulin serve as sample processing controls. All the blots were using the same samples run in parallel on separate gels.

SFig.9B



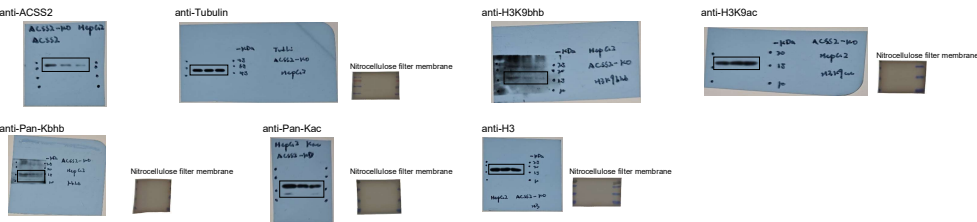
H3 and Tubulin serve as sample processing controls. All the blots were using the same samples run in parallel on separate gels.

SFig.9C



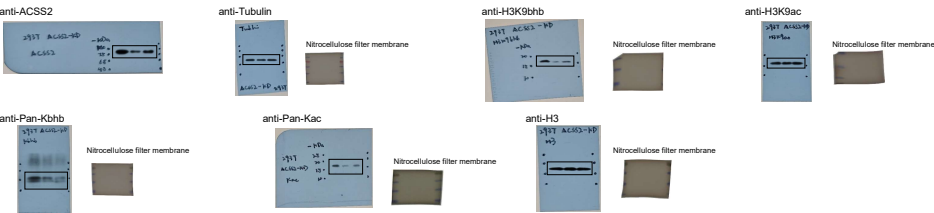
H3 and Tubulin serve as sample processing controls. All the blots were using the same samples run in parallel on separate gels.

SFig.9D



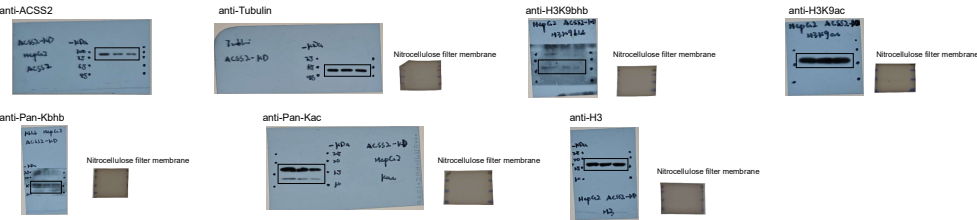
H3 and Tubulin serve as sample processing controls. All the blots were using the same samples run in parallel on separate gels.

SFig.9E



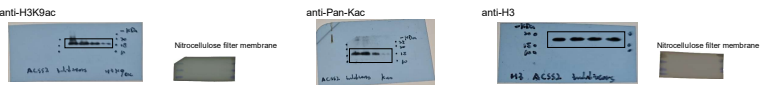
H3 and Tubulin serve as sample processing controls. All the blots were using the same samples run in parallel on separate gels.

SFig.9F



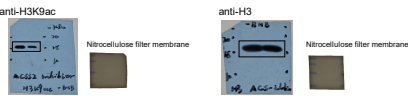
H3 and Tubulin serve as sample processing controls. All the blots were using the same samples run in parallel on separate gels.

SFig.9G



H3 serves as sample processing control. All the blots were using the same samples run in parallel on separate gels.

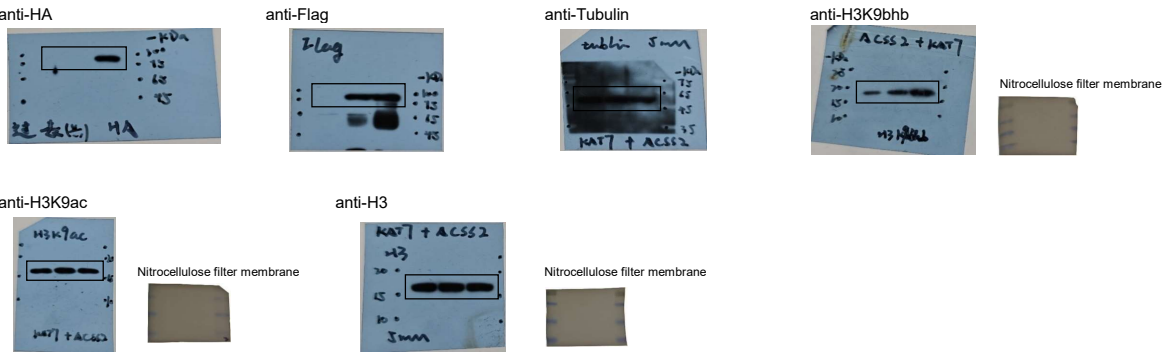
SFig.9H



H3 serves as sample processing control. All the blots were using the same samples run in parallel on separate gels.

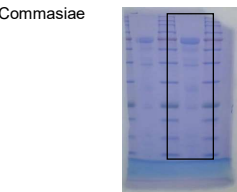
Original blots 9 - Related to fig. S10 and S11

SFig.10D

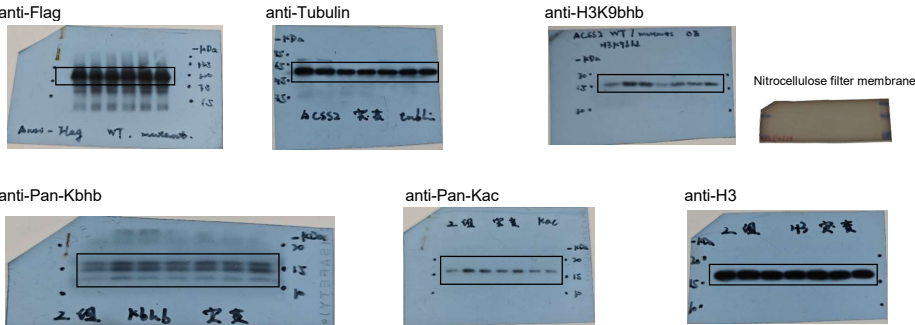


H3 and Tubulin serve as sample processing controls. All the blots were using the same samples run in parallel on separate gels.

SFig.10E

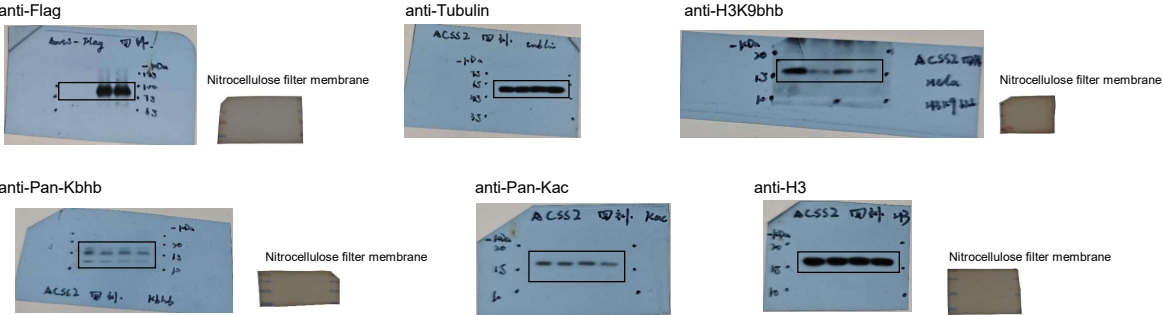


SFig.11C



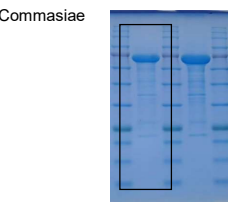
H3 and Tubulin serve as sample processing controls. All the blots were using the same samples run in parallel on separate gels.

SFig.11D



H3 and Tubulin serve as sample processing controls. All the blots were using the same samples run in parallel on separate gels.

SFig.11E



Other Supplementary Material for this manuscript includes the following:

Tables S1 to S6

Table S1 (Microsoft Excel format). Quantification of β -hydroxybutyrylome with KAT7-KO HeLa and KAT7-WT HeLa.

Table S2 (Microsoft Excel format). Quantification results for β -hydroxybutyrylated histones with KAT7-KO HeLa and KAT7-WT HeLa.

Table S3 (Microsoft Excel format). (a) List of overlapped genes from BHB-induced and KAT7-IP proteome. (b) Data dependent acquisition quantitative proteome to identify interacting proteins of KAT7. (c) Data independent acquisition quantitative proteome results for HeLa with or without treatment of BHB.

Table S4 (Microsoft Excel format). shRNA and sgRNA sequences for genes depletion.

Table S5 (Microsoft Excel format). PCR primers for quantifying genes.

Table S6 (Microsoft Excel format). Resources summary statistics.