Molecular Cell, Volume *71*

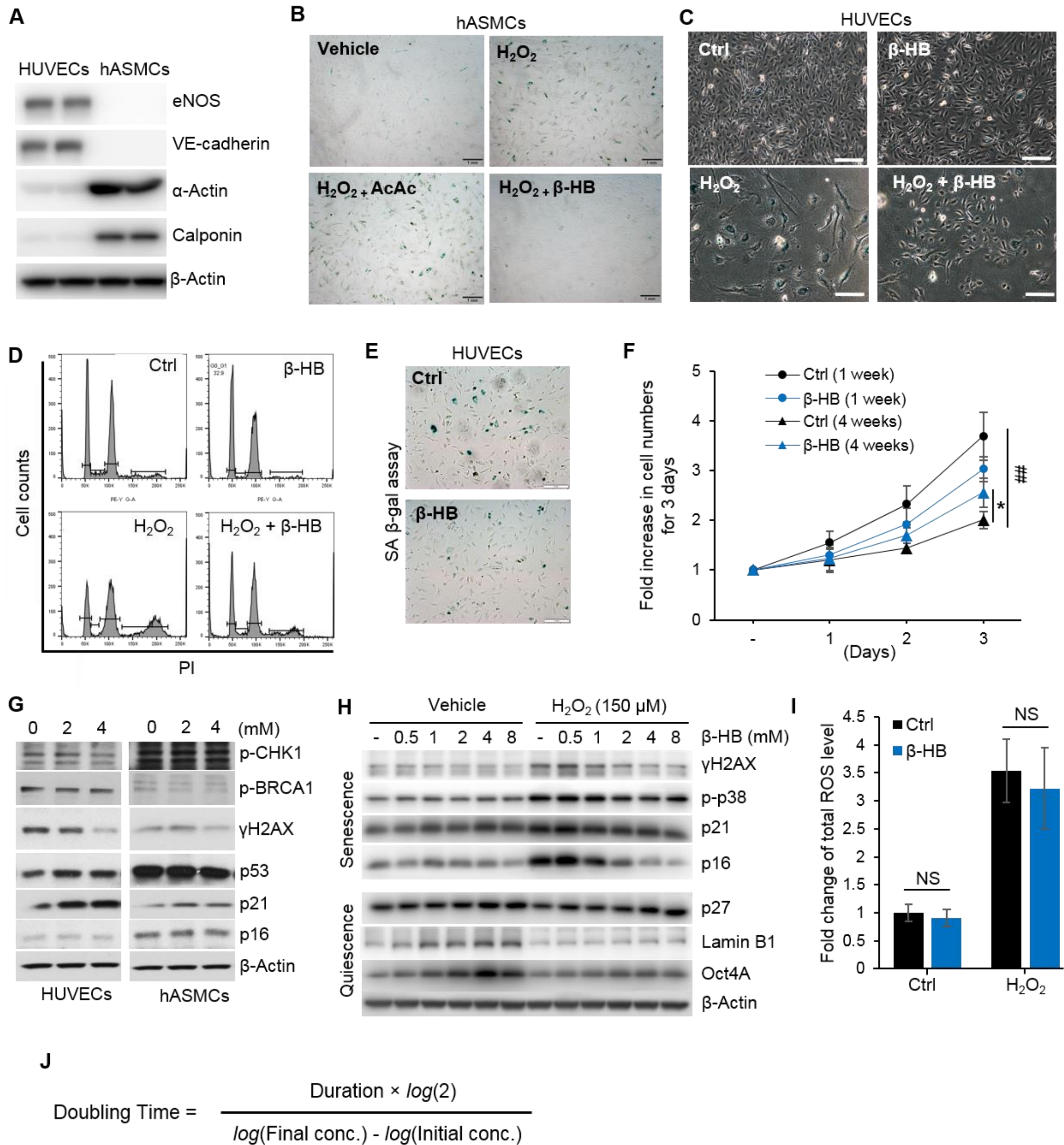
Supplemental Information

β-Hydroxybutyrate Prevents Vascular Senescence through hnRNP A1-Mediated Upregulation of Oct4

Young-min Han, Tatiana Bedarida, Ye Ding, Brian K. Somba, Qiulun Lu, Qilong Wang, Ping Song, and Ming-Hui Zou

Supplemental Figures

Figure S1. Related to Figure 1



$$\text{Doubling Time} = \frac{\text{Duration} \times log(2)}{log(\text{Final conc.}) - log(\text{Initial conc.})}$$

Supplemental Figure 1 (related to Figure 1). Preventive effect of β-hydroxybutyrate (β-HB) on the cellular senescence.

(A) Western blots for endothelial cell markers (eNOS and VE-cadherin) and smooth muscle cell markers (α-Actin and Calponin) in the HUVECs and hASMCs. n = 2.

(B) Representative image of senescence-associated β-galactosidase (SA β-gal) activity assay of hASMCs after hydrogen peroxide (H_2O_2, 150 μM, 3 days) stimulation. Cells were co-treated with acetoacetate (AcAc, 4 mM) or β-hydroxybutyrate (β-HB, 4 mM) for 3 days. Scale bar, 100 µm. n = 3.

(C) Morphological change of HUVECs after H_2O_2 (150 μM, 3 days) stimulation. Cells were co-treated with or without β-HB (4 mM, 3 days). Scale bar, 100 µm. n = 3.

(D) Cell cycle distribution of HUVECs after β-HB (4 mM) treatment for 3 days with or without H_2O_2 (150 μM). Cells were analyzsed by flow cytometry after propidium iodide (PI) staining, based on 10,000 cells. n = 3

(E) Representative images of SA β-gal activity assay of HUVECs with or without β-HB treatment (4 mM) for 4 weeks. n = 3.

(F) HUVECs were maintained with or without β-HB (4 mM) treatment for 1 week or 4 weeks. After treatment, proliferation of HUVECs was analyzed by counting the cell numbers for 3 days. $^{*}p < 0.05$, Ctrl (4 weeks) *vs.* β-HB (4 weeks), $^{\#\#}p < 0.01$ Ctrl (1 week) *vs.* Ctrl (4 weeks). n = 3.

(G) Representative Western blots for DNA repair signaling (p-CHK1 and p-BRCA1), DNA damage (γH2AX) and cell cycle regulatory proteins (p53, p21 and p16) in HUVECs or hASMCs after β-HB treatment (2-4 mM). n = 3.

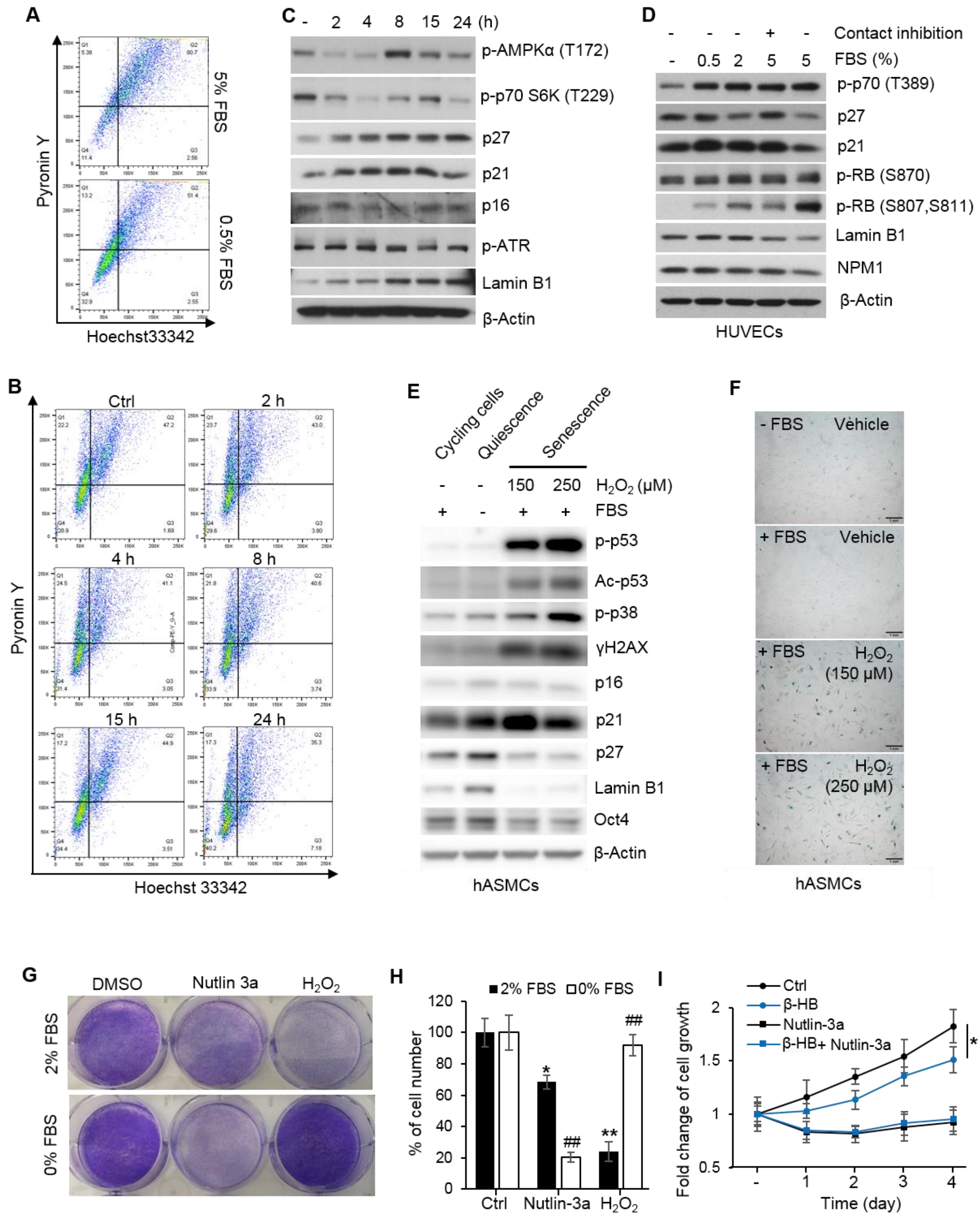
(H) Representative Western blots for senescence (γH2AX, p-p38, p21, and p16) and quiescence (p27, Lamin B1, and Oct4A) markers in hASMCs treated with or without β-HB for 24 h in a dose dependent manner. n = 3.

(I) DCFDA/H2DCFDA cellular reactive oxygen species (ROS) assay to measure total ROS level, stimulated by hydrogen peroxide (H_2O_2, 150 μM) with or without β-HB treatment (4 mM). NS, non-significant.

(J) Equation to calculate doubling time of HUVECs.

Data are presented as mean ± standard error of mean (SEM). NS, non-significant; Ctrl, control.

Figure S2 (related to Figure 2).



Supplemental Figure 2 (related to Figure 2). Induction of cellular quiescence in HUVECs by β-HB treatment.

(A) Cell cycle distribution of quiescent HUVECs (G0 phage) cultured with nutrient complete (5% FBS, EGF, bovine brain extract) or FBS-deprived media (0.5% FBS, EGF, bovine brain extract), assessed by flow cytometry analysis of the intercalation of Pyronin Y and Hoechst 33342. Each samples were analyzed based on 10,000 cells.

(B) Cell cycle distribution of HUVECs with time-dependent treatment of β-HB (2 h-24 h), assessed by flow cytometry analysis of the intercalation of Pyronin Y and Hoechst 33342. Samples were analyzed based on 10,000 cells.

(C) Representative Western blots for quiescence- or senescence-associated signaling proteins (p-AMPKa, p-p70 S6K, p27, p21, p16, p-ATR and Lamin B1) in HUVECs treated with β-HB (4 mM) in a time-dependent manner. n = 3.

(D) Representative Western blots for quiescence markers (p-p70, p27, p21, p-RB, Lamin B1 and NPM1), which were induced by contact inhibition or FBS deprivation in HUVECs. n = 3.

(E) Representative Western blots for quiescence (p27, Lamin B1 and Oct4) and senescence markers (p-p53, Ac-p53, p-p38, γH2AX, p16 and p21) in hASMCs. FBS deprivation-induced quiescent cells and H_2O_2 (150-250 µM, 24 h)-stimulated senescent cells were compared to cycling cells. n = 3.

(F) Representative images of SA β-gal activity assay in hASMCs, cultured with FBS deprivation (24 h) or H_2O_2 (150-250 µM, 24 h) treatment. n = 3.

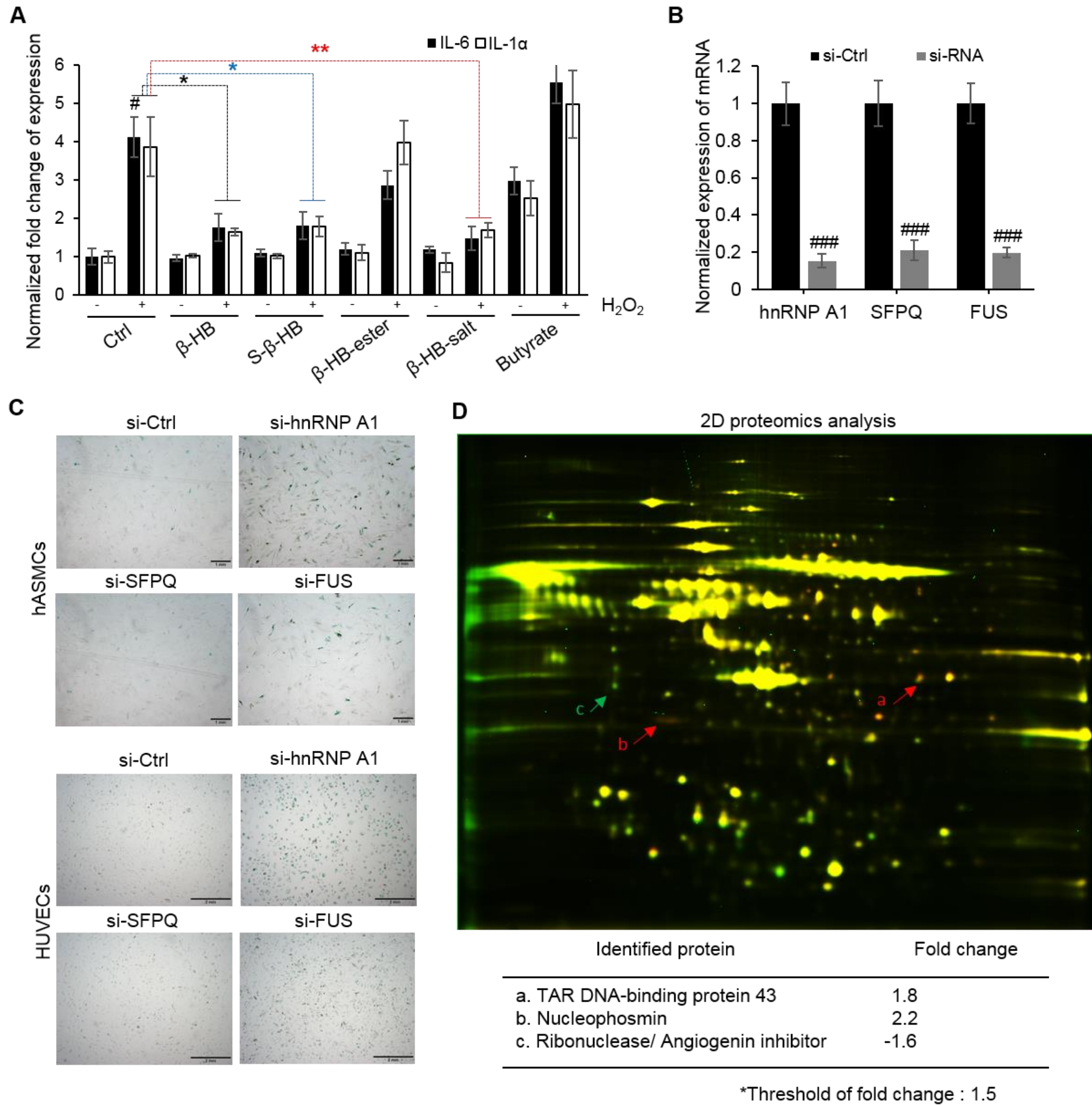
(G) Representative images of crystal violet staining to exhibit the number of HUVECs after Nutlin-3a or H_2O_2 treatment in different conditioned media (2% FBS or 0% FBS). n = 3.

(H) Quantitation of Figure S2G. $^{*}p < 0.05$, $^{**}p < 0.01$ control *vs.* Nutlin-3a or H_2O_2, $^{\#\#}p < 0.01$ 2% FBS *vs.* 0% FBS, n = 3.

(I) Growth rate of HUVECs was analyzed by counting cell numbers every days after treating β-HB and Nutlin-3a. n = 3, $^{*}p < 0.05$ control (4 days) vs. β-HB (4 days).

Data are presented as mean ± standard error of mean (SEM).

Figure S3 (related to Figure 3).



Supplemental Figure 3 (related to Figure 3). Proteomics approach identifying the target protein of β-HB.

(A) Quantitative real-time PCR (qRT-PCR) analysis of IL-6 and IL-1α. β-HB or β-HB analogues (S-β-HB, β-HB-ester, β-HB-salt and Butyrate) was added to HUVECs culture with or without H_2O_2 (150 μM, 24 h) stimulation. n = 3, $^{\#}p < 0.05$ vehicle *vs.* H_2O_2, $^{*}p < 0.05$, $^{**}p < 0.01$ control (Ctrl) + H_2O_2 *vs.* treated group + H_2O_2.

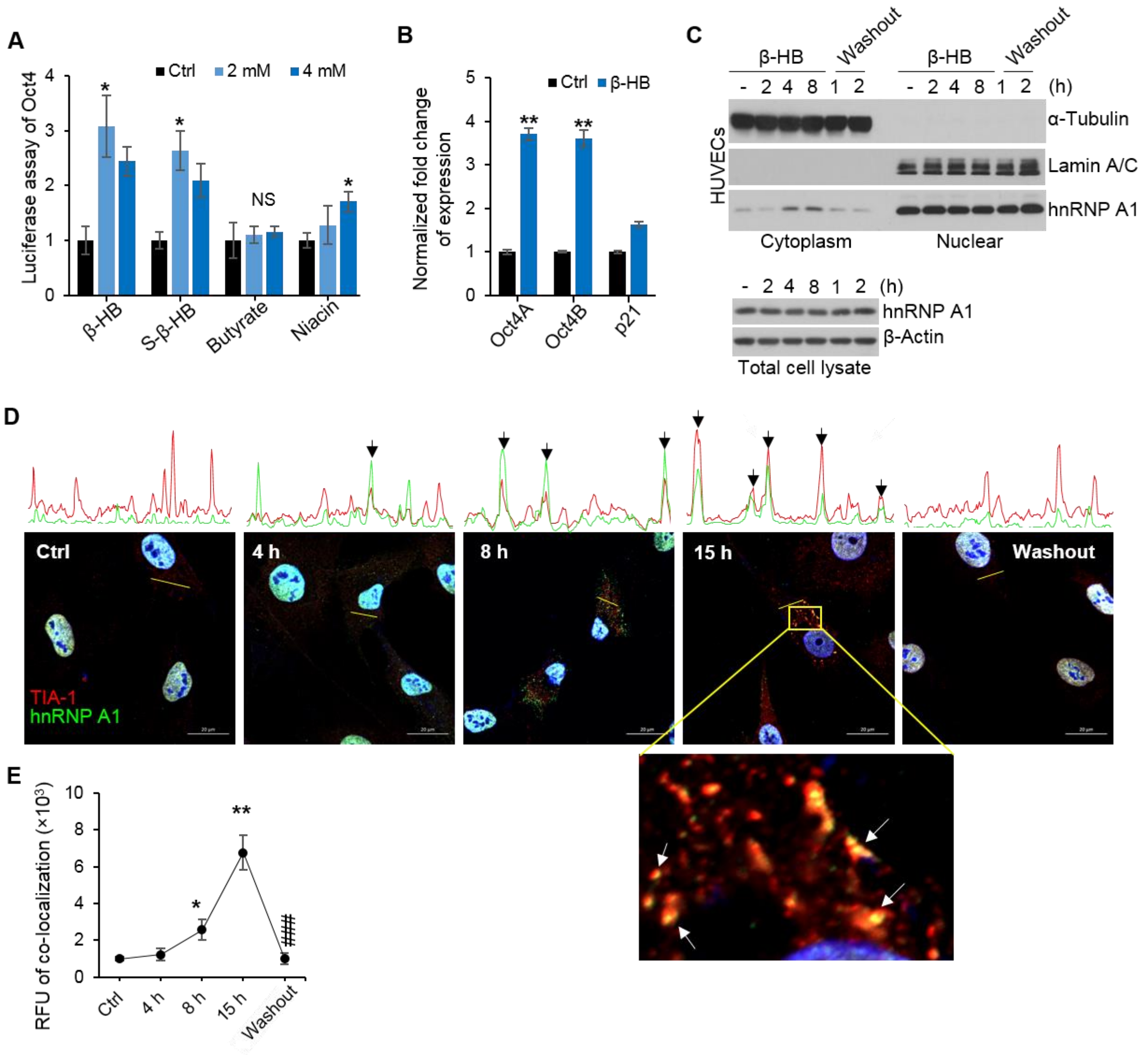
(B) qRT-PCR analysis of hnRNP A1, SFPQ and FUS after silencing each genes to validate silencing efficiency respectively. n = 3, $^{\#\#\#}p < 0.01$ si-Ctrl *vs.* si-RNA.

(C) Representative images of SA β-gal activity assay in hASMCs or HUVECs after silencing hnRNP A1, SFPQ, or FUS. n = 3.

(D) Comparative 2D-DIGE (Difference gel electrophoresis) analysis of HUVECs treated with β-HB (4 mM) for 2 days. Control lysate was labeled with Cy3 (green) dye and β-HB treated lysate with Cy5 (red) dye. Spots were picked and analyzed using MALDI-TOF mass analysis.

Data are presented as mean ± standard error of mean (SEM).

Figure S4 (related to Figure 4).



Supplemental Figure 4 (related to Figure 4). hnRNP A1-mediated stabilization of Oct4 mRNA in response to β-HB.

(A) Luciferase assay for Oct4 transcriptional activity in HUVECs treated with β-HB, S-β-HB, Butyrate, or Niacin, which have structural similarity with β-HB. n = 3. $^{*}p < 0.05$ control *vs.* treated group. NS, non-significant.

(B) qRT-PCR analysis of Oct4A, Oct4B, and p21 in HUVECs treated with β-HB (4 mM, 15 h). n = 3, $^{**}p < 0.01$ control (Ctrl) *vs.* β-HB.

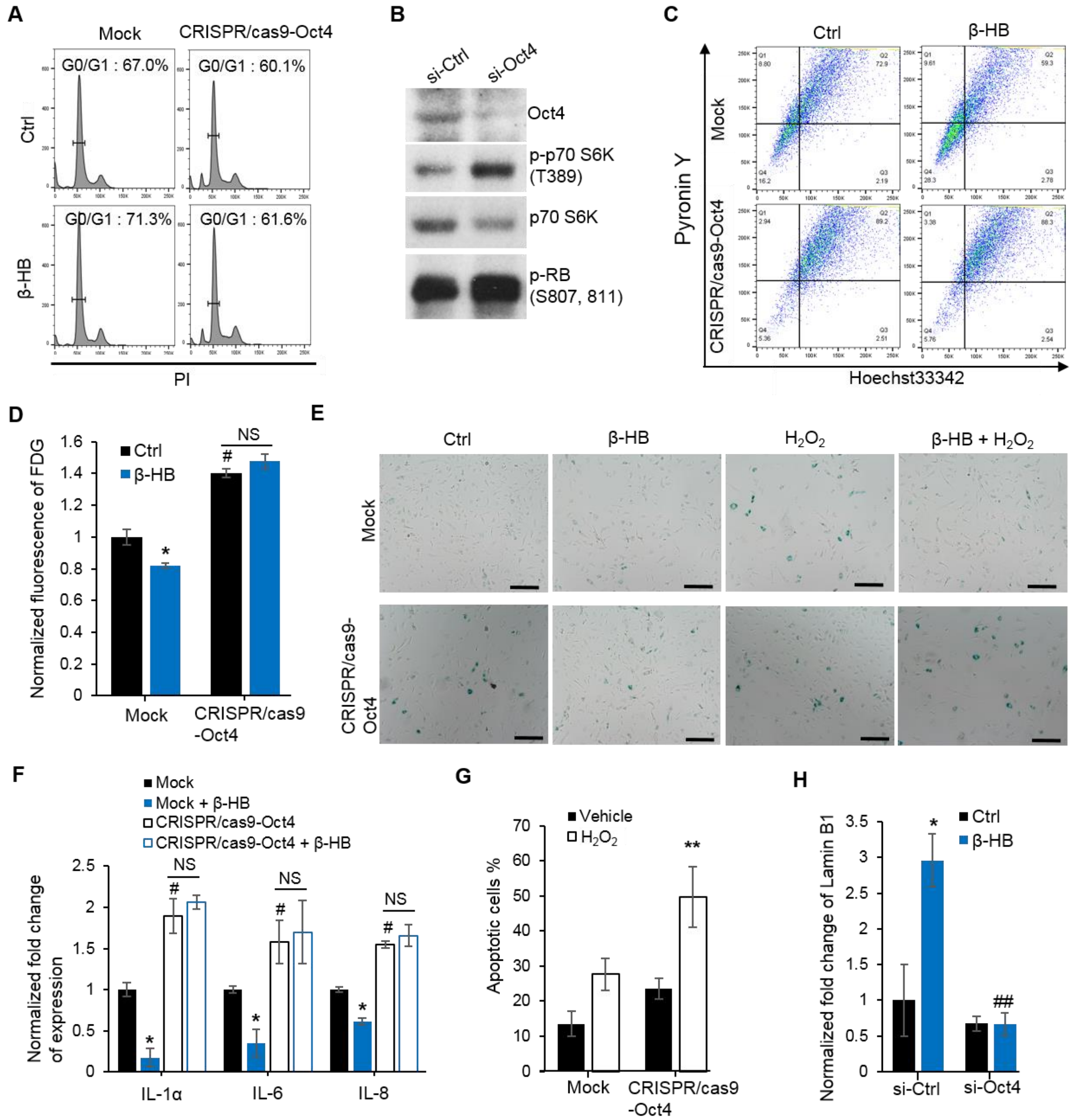
(C) Representative Western blots for subcellular localization of hnRNP A1. Upper panel represents localization of hnRNP A1 in cytoplasm and nuclear fraction of HUVECs with β-HB treatment (4 mM). Lower panel represents total level of hnRNP A1 after β-HB treatment for indicated time periods. n = 3.

(D) Representative images of immunofluorescence staining of hnRNP A1 (green) and TIA-1 (red) after β-HB treatment (4 mM) at indicated time points. Linear trajectories (yellow line) crossing the cell with the two signals were presented on the top of images. Arrows indicate colocalization of TIA-1 and hnRNP A1. n = 3, Scale bar, 20 μm.

(E) Quantification of TIA-1 and hnRNP A1 colocalization in Figure S4D. $^{*}p < 0.05$, $^{**}p < 0.01$ control *vs.* β-HB treatment. $^{\#\#\#}p < 0.01$ 15 h *vs.* Washout.

Data are presented as mean ± standard error of mean (SEM).

Figure S5 (related to Figure 5).



Supplemental Figure 5 (related to Figure 5). Oct4 knockdown and ectopic expression in HUVECs.

(A) Cell cycle distribution analysis of HUVECs, transfected with or without CRISPR/cas9-Oct4 plasmid to knockdown Oct4 gene expression. After transfection, β-HB (4 mM, 15 h) was treated to Mock and Oct4 knockdown group, then cells were assessed by flow cytometry analysis with PI staining. n = 3.

(B) Western blot analysis of Oct4, p-p70 S6K and p-RB in HUVECs after silencing Oct4 by siRNA.

(C) Cell cycle distribution of quiescent HUVECs (G0 phage) transfected with or without CRISPR/cas9-Oct4. After 2 days of transfection, β-HB (4 mM, 15 h) was added, then quiescent cells were assessed using flow cytometry based on 10,000 cells by staining of Pyronin Y and Hoechst 33342. n = 3.

(D) Fluorescein Di-β-D-galactopyranoside (FDG) assay for quantification of senescence-associated β-galatosidase activity in Mock and Oct4 knockdown HUVECs, transfected with CRISPR/cas9-Oct4. Excitation /emission = 490 /514 (nm). n = 3. $^{*}p < 0.05$ control (Ctrl) *vs*. β-HB, $^{\#}p < 0.05$ Mock *vs.* CRISPR/cas9-Oct4.

(E) Representative images of SA β-gal assay in Mock and Oct4 knockdown HUVECs (CRISPR/cas9-Oct4), stimulated by H_2O_2 (150 µM, 15 h) with or without β-HB treatment (4 mM, 15 h), Scale bar, 100 µm.

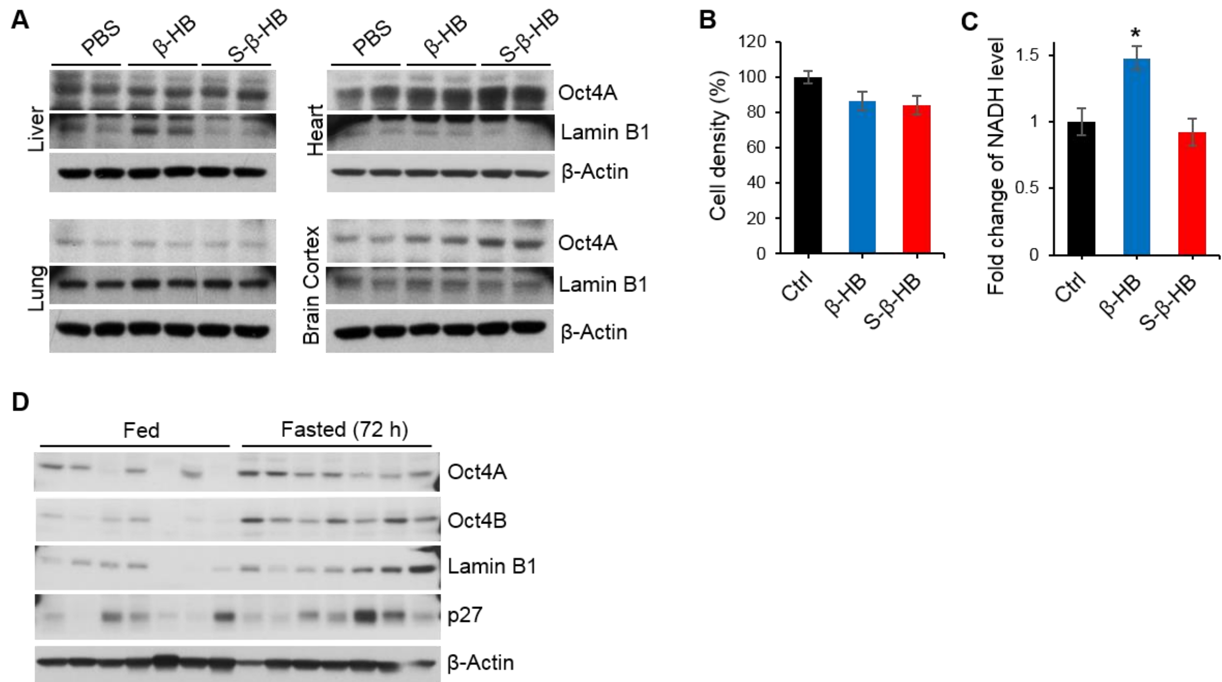
(F) q-RT PCR analysis of SASP (IL-1α, IL-6 and IL-8) in Mock and Oct4 knockdown group of HUVECs, transfected with CRISPR/cas9-Oct4, and the comparison of β-HB treatment (4 mM, 15 h). $^{*}p < 0.05$ vehicle vs. β-HB, $^{\#}p < 0.05$ Mock *vs.* CRISPR/cas9-Oct4, NS, non-significant.

(G) Quantitative analysis of Figure 5F. $^{**}p < 0.01$ vehicle *vs.* H_2O_2. n = 3.

(H) qRT-PCR analysis of Lamin B1 in HUVECs. After silencing Oct4 by siRNA, β-HB (4 mM, 15 h) was added to control siRNA-treated or Oct4-silenced cells. n = 3, $^{*}p < 0.05$ control (Ctrl) *vs.* β-HB, $^{\#\#}p < 0.01$ si-Ctrl + β-HB *vs.* si-Oct4 + β-HB.

Data are presented as mean ± standard error of mean (SEM). NS, non-significant; Ctrl, control; si-Ctrl, control si-RNA.

Figure S6 (related to Figure 6).



Supplemental Figure 6 (related to Figure 6). Oct4A upregulation in vascular tissues by β-HB injection or fasting.

(A) Western blot analysis of Oct4A and Lamin B1 in C57BL/6J mice organs (Liver, Lung, Heart and Brain Cortex) after intraperitonial injection with PBS, β-HB salt (1.5 g/kg) and S-β-HB (1.5 g/kg) dissolved in PBS. n = 6 mice/group.

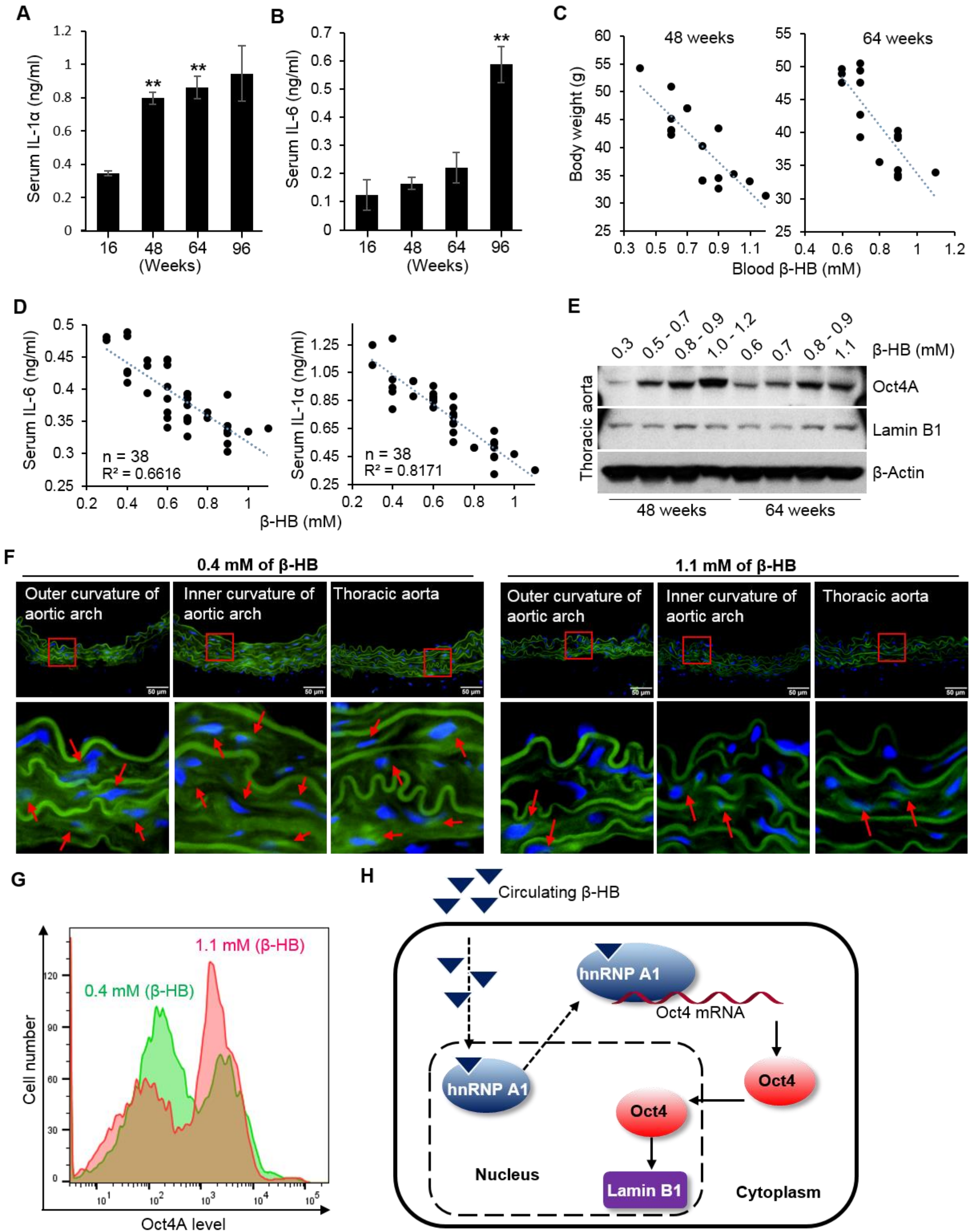
(B) Cell density analysis of HUVECs treated with β-HB (4 mM, 24 h) or S-β-HB (4 mM, 24 h). Cells were stained with cristal violet solution to measure the cell density on the plate. n = 3, non-significant.

(C) WST-8 assay to measure NADH level produced by mitochondria in HUVECs treated with β-HB (4 mM, 24 h) and S-β-HB (4 mM, 24 h). n = 3, $^{*}p < 0.05$ control (Ctrl) *vs.* β-HB.

(D) Western blot analysis of Oct4A, Oct4B, Lamin B1 and p27 in fed or fasted mice (72 h). n = 7 mice/group.

Data are presented as mean ± standard error of mean (SEM).

Figure S7 (related to Figure 7).



Supplemental Figure 7 (related to Figure 7). Negative correlation of senescence and circulating β-HB.

(A) Enzyme-linked immunosorbent assay (ELISA) to measure IL-1α in the different aged-mice. (n = 6-10 mice per group), $^{**}p < 0.01$ 16 weeks-old *vs.* 48 weeks- and 64 weeks-old.

(B) ELISA assay to measure IL-6 in the different aged-mice. (n = 6-10 mice per group), $^{**}p < 0.01$ 16 weeks-old *vs.* 96 weeks-old.

(C) Negative correlation of body weight and blood β-HB concentration in 48-week-old (n = 14, $R^2 = 0.6794$) and 64-week-old (n = 16, $R^2 = 0.6779$) mice.

(D) ELISA showed negative correlations of β-HB with IL-6 ($R^2 = 0.6616$) and IL-1α ($R^2 = 0.8171$) in aged C57BL/6J mice (80 weeks old, n = 38).

(E) Western blotting analyses of Oct4A and Lamin B1 in thoracic aorta from middle-aged C57BL/6J mice (48 and 64 weeks old, n =12 mice/group) stratified by blood β-HB level.

(F) Fluorogenic β-gal activity assays of aged mice aorta (inner and outer curvatures of arch and thoracic aorta). Mice were grouped according to blood β-HB level (0.4 and 1.1 mM), then SPiDer β-gal assays was performed to evaluate the site-specific effect of blood β-HB. Red arrow indicates SA β-gal positive cells. n = 5 mice/group

(G) Flow cytometry analysis of mouse primary aortic smooth muscle cells. Aortic smooth muscle cells were isolated from mice stratified by blood β-HB concentration (1.1 mM and 0.4 mM), then fixed and stained with Oct4 antibody.

(H) Schematic summary showing hnRNP A1/β-HB-mediated Oct4 upregulation.

Data are presented as mean ± standard error of mean (SEM).