SUPPLEMENTAL INFORMATION

CRISPR/Cas9-mediated mutations in both a cAMP response element and an ETS-binding site suppress *FLT1* gene expression

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Figure S1

mRNA and protein expression levels of tmFLT1, sFLT1-15a, and sFLT1-i13 in HUVECs (passage 5) and AG1-G1 cells.

Figure S2

Characterization of mutant HEK293 cell clones other than clone #1 cells.

Figure S3

Comparison of *FLT1* promoter activity using pFLT1-Luc (–1629/+8) in parental wild-type HEK293 cells and mutant clone #1 cells.

Figure S4

CREB1 (cAMP response element binding protein 1) and ELF1 (E74-like factor 1) might not be major players in *FLT1* expression in HTR-8/SVneo, HEK293, and AG1-G1 cells.

Figure S5

Uncropped images of the western blots and gels shown in the main and supplementary figures.

Table S1

Oligonucleotide primer sequences for qRT-PCR.

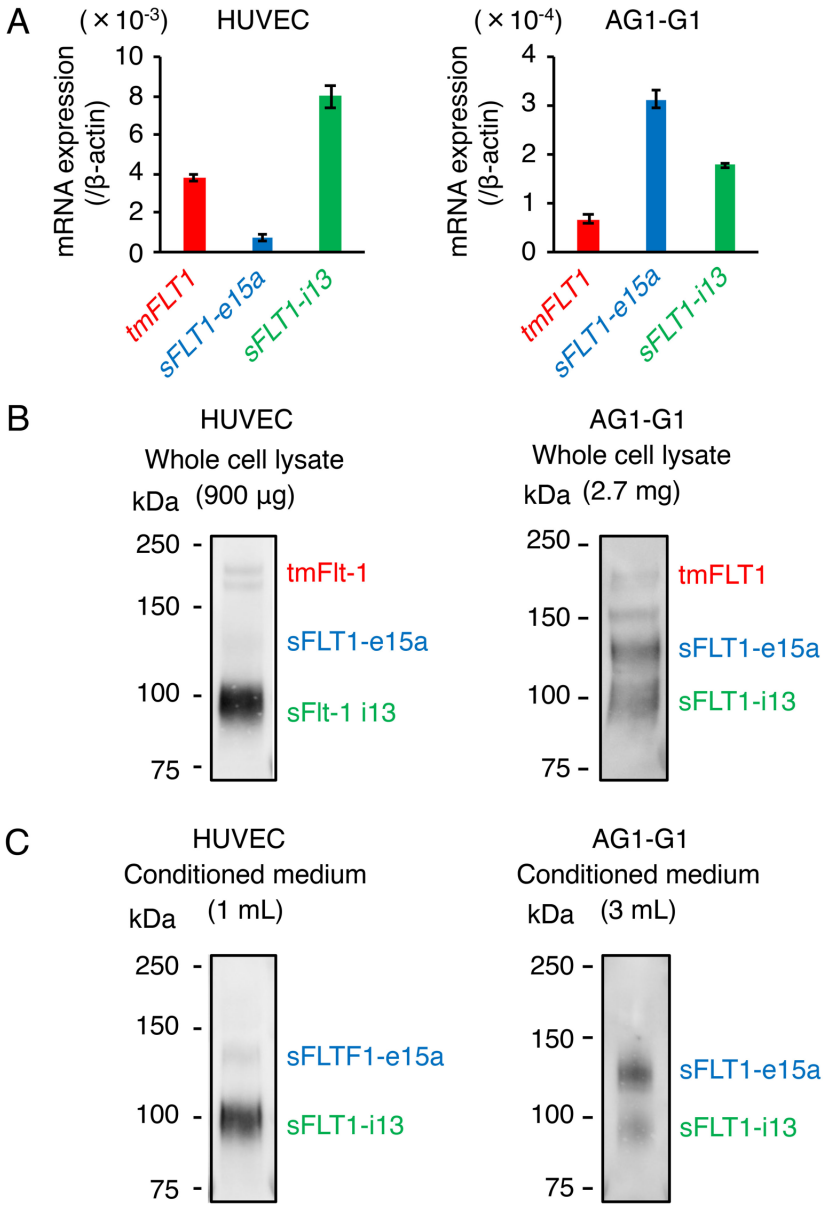


Figure S1. mRNA and protein expression levels of tmFLT1, sFLT1-15a, and sFLT1-i13 in HUVECs (passage 5) and AG1-G1 cells.

(A) The mRNA expression levels of three FLT1 splice variants. Results are expressed as a ratio relative to the expression of β-actin mRNA. (B) Immunoprecipitation of FLT1 isoforms from the cell lysates. The immunoprecipitates were subjected to western blot analysis. Numbers show the amount of protein subjected to immunoprecipitation. (C) Western blot of sFLT1 proteins secreted into conditioned media. Numbers show the volume of media subjected to heparin-affinity pull-down. Uncropped images of western blots are presented in in Supplementary Fig. S5. All values are represented as the mean ± SD (n =3).

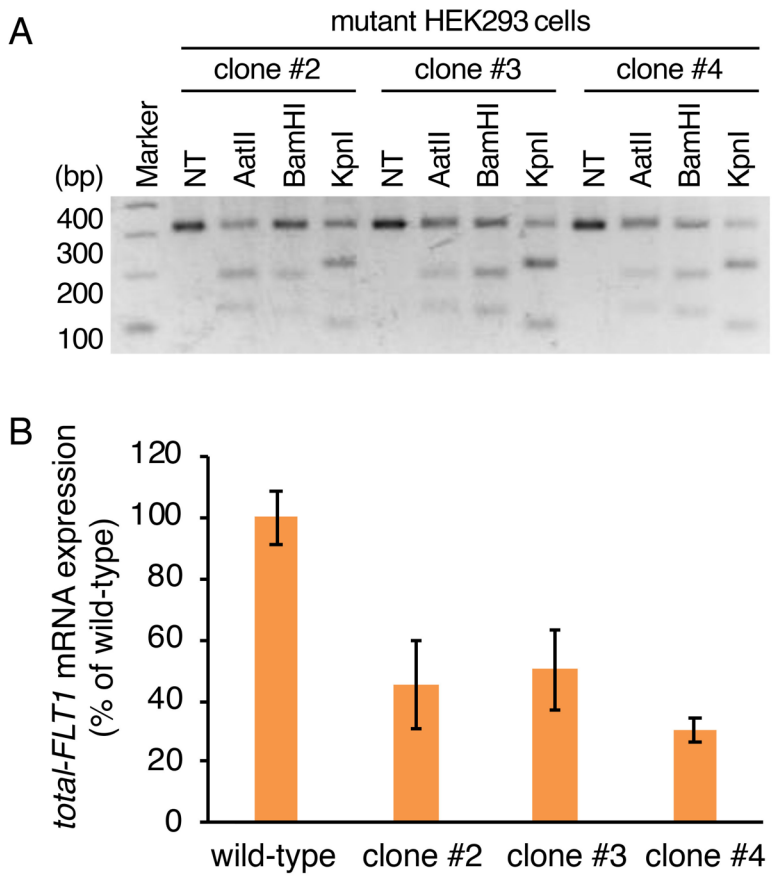


Figure S2. Characterization of three mutated HEK293 cell clones other than clone #1 cells.

(A) Genotyping of mutated HEK293 cell clones by RFLP assay. NT: non-treated. An uncropped image of the gel is presented in Supplementary Fig. S5. (B) The mRNA expression levels of all *FLT1* transcript variants (*total-FLT1*) were measured by qRT-PCR using *β-actin* mRNA as a reference. Results are expressed as a percentage relative to the unedited wild-type cells. All values are represented as the mean ± SD (n =3).

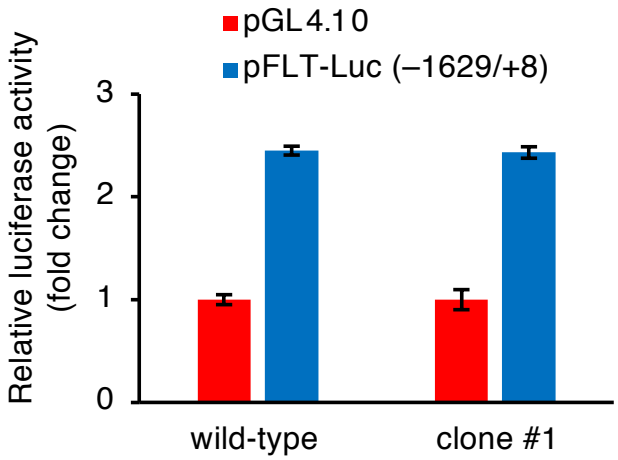


Figure S3. Comparison of *FLT1* promoter activity using pFLT1-Luc (−1629/+8) in parental wild-type HEK293 cells and mutant clone #1 cells.

Each cell was transiently co-transfected with 1 μg of the promoter-less plasmid pGL4.10, pFLT1-Luc (−1629/+8), and 20 ng of the control *Renilla* luciferase vector pGL4.74 (hRuc/TK). After 24 h of transfection, cells were subjected to a dual-luciferase assay. The results are expressed as fold-change relative to the promoter-less plasmid pGL4.10-transfected cells. All values are represented as the mean ± SD (n = 3).

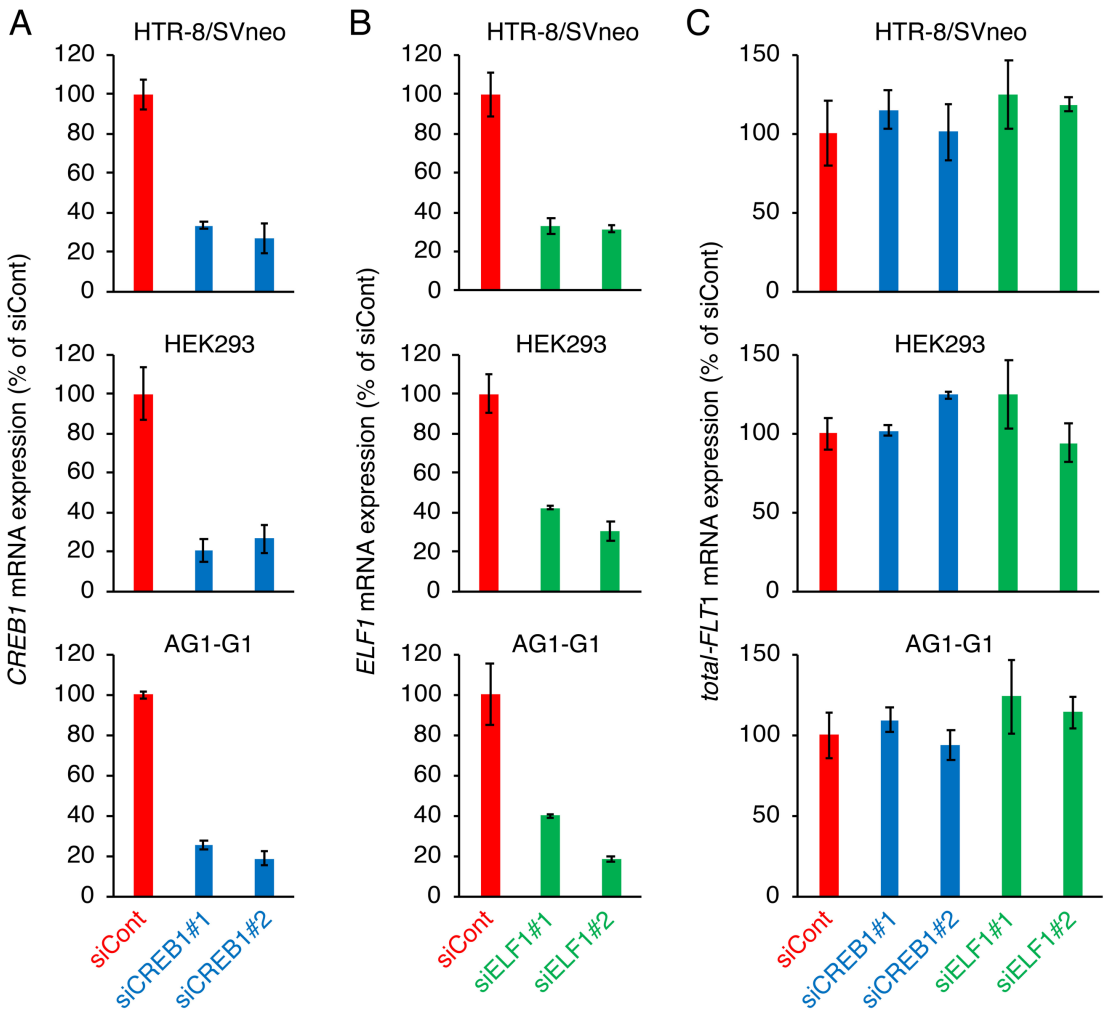


Figure S4. CREB1 (cAMP response element binding protein 1) and ELF1 (E74-like factor 1) might not be major players in the *FLT1* expression in HTR-8/SVneo, HEK293, and AG1-G1 cells.

HTR-8/SVneo, HEK293, and AG1-G1 cells were transfected with 10 nM siRNA for 48 h. The mRNA expression levels of *CREB1* (A), *ELF1* (B), and all *FLT1* transcript variants (*total-FLT1*) (C) were measured by qRT-PCR using *β-actin* mRNA as a reference. The results are expressed as a percentage relative to control siRNA (siCont)-transfected cells. All values are represented as the mean ± SD (n = 3).

Figure 3-B

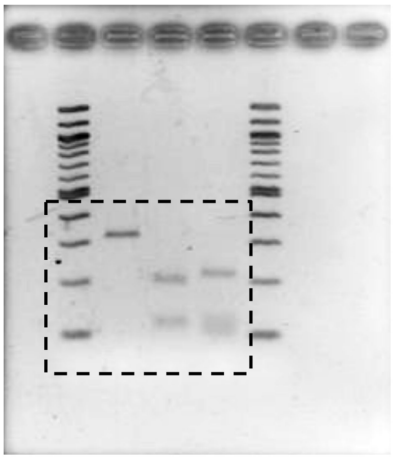


Figure 3-C

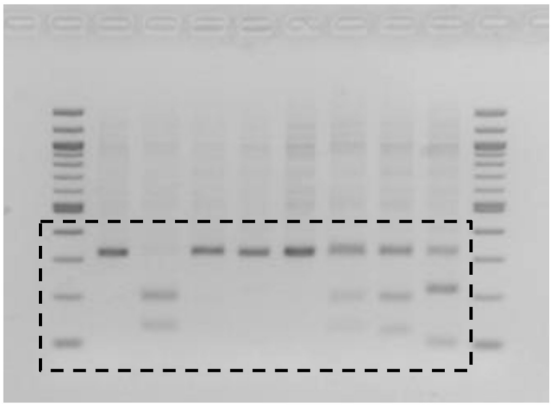
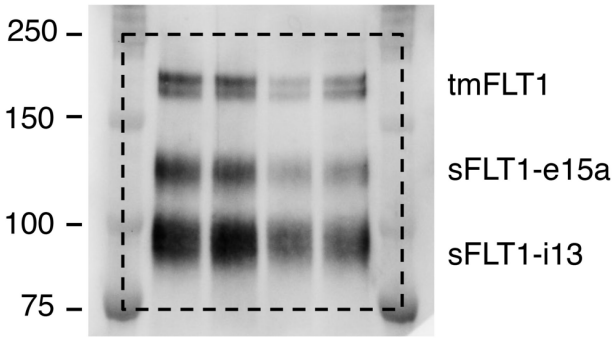


Figure 4-B : upper



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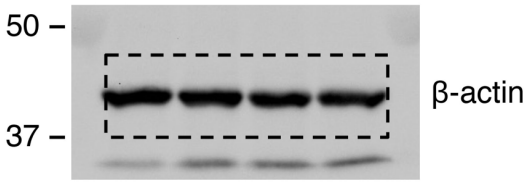


Figure S1-B : left

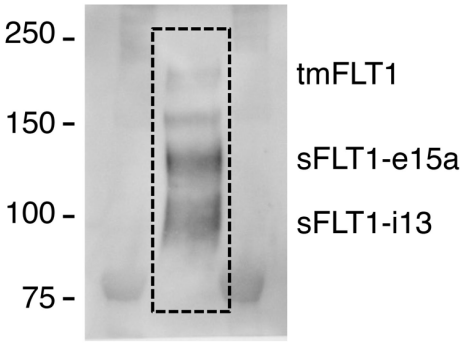
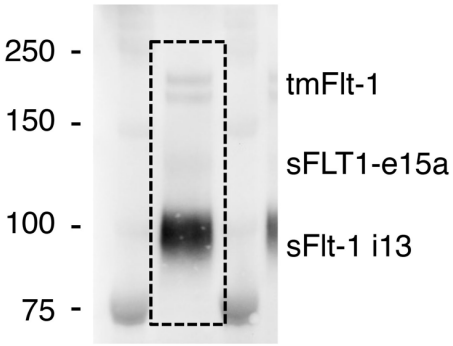


Figure S1-B : right



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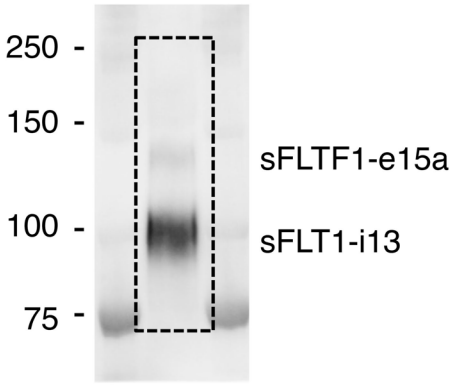

Figure S1-C : right

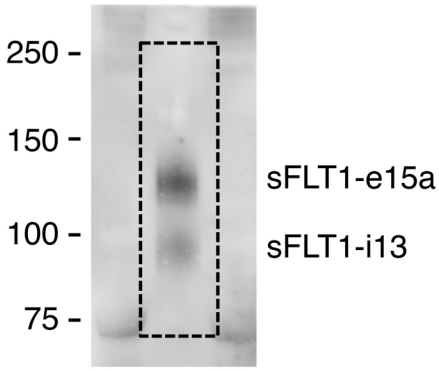

Figure S2-A

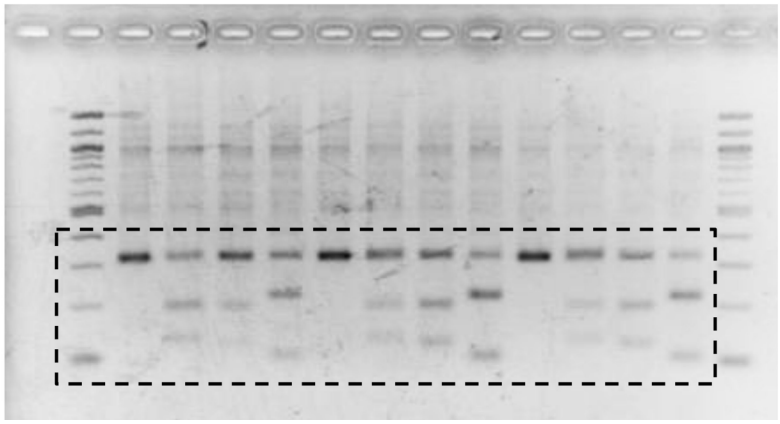

Figure S5. Uncropped images of the western blots and gels shown in the main and supplementary figures.

Boxed areas indicate cropped regions.

Table S1. Oligonucleotide primer sequences for qRT-PCR.

Gene		Primer sequence
total-FLT1	Forward	5′-CCCTGTAACCATAATCATTCCGAAG-3′
	Reverse	5′-TCAGCCACAACCAAGGTGCTA-3′
tmFLT1	Forward	5′-AGAGATGGGACCGTCATCAG-3′
	Reverse	5′-CTGGCTCTAGCCTGCTTTTG-3′
sFLT1-i13	Forward	5′-ACTTGGTGCACGTTTGGATT-3′
	Reverse	5′-AGAGGTTGGCATCAAAATGG-3′
sFLT1-e15a	Forward	5′-AGTTGGAGAGCCAAGACAATC-3′
	Reverse	5′-CAGCATTTCACCATCTTGGTC-3′
CREB1	Forward	5′-TGTTGTTCAAGCTGCCTCTG-3′
	Reverse	5′-TCTCTCTTTCGTGCTGCTTC-3′
ELF1	Forward	5′-GGGTATTCTGGCAAAAGTGG-3′
	Reverse	5′-ATGCTGGAACTTGGATCCTC-3′
β-actin	Forward	5′-AAATCTGGCACCACACCTTC-3′
	Reverse	5′-TGATCTGGGTCATCTTCTCG-3′

SUPPLEMENTARY METHODS

Transient transfection with reporter plasmids

HTR-8/SVneo cells were seeded into type I collagen-coated 12-well culture plates (Sumitomo Bakelite Co., Ltd., Tokyo, Japan) at a density of 3.80×10^4 cells/cm^2 and incubated for 24 h. The cells were transfected with 1 μg of each pFLT1-Luc reporter plasmid and 20 ng of the control *Renilla* luciferase vector pGL4.74 (hRuc/TK) (Promega, Madison, WI, USA) using 3 μL of ViaFect transfection reagent (Promega) for 24 h, and then cell lysates were prepared.

BeWo, JAR, and JEG-3 cells were seeded into 12-well cell culture plates (Sumitomo Bakelite Co., Ltd.) at densities of 4.35×10^4, 4.89×10^4, and 4.35×10^4 cells/cm^2, respectively. After 24 h of pre-incubation, the cells were transfected with 1 μg of each pFLT1-Luc reporter plasmid and 20 ng of the pGL4.74 (hRuc/TK) using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) for 6 h. At 42 h after transfection, the cells were harvested, and cell lysates were prepared.

HEK293 cells were seeded into 12-well cell culture plates at a density of 7.61×10^4 cells/cm^2 and incubated for 24 h. The cells were transfected using the same procedure as above for 24 h to prepare cell lysates.

AG1-G1 cells were seeded in type I collagen-coated 12-well culture plates at a density of 3.80×10^4 cells/cm^2 and incubated for 24 h. The cells were transfected with 1 μg of each pFLT1-Luc reporter plasmid and 20 ng of the pGL4.74 (hRuc/TK) using 3 μL of ViaFect transfection reagent for 4 h. At 24 h after transfection, the cells were harvested, and cell lysates were prepared.

HUVECs were seeded into 12-well cell culture plates at a density of 3.80×10^4 cells/cm^2 and incubated for 24 h. The cells were transfected with 1 μg of each pFLT1-Luc reporter plasmid and 20 ng of the pGL4.74 (hRuc/TK) using *Trans*IT-2020 transfection reagent (Mirus Bio LLC, Madison, WI, USA) for 1 h. At 27 h after transfection, the cells were harvested, and cell lysates were prepared.

Heparin-affinity pull-down for the concentration of sFLT1 proteins

The secreted sFLT1 isoforms in the conditioned medium were concentrated using heparin-immobilized beads (Heparin Sepharose 6 Fast Flow; GE Healthcare, Uppsala, Sweden). Briefly, 20 μL of bead slurry was pre-equilibrated with PBS and incubated with the appropriate volume of conditioned medium at 4 °C overnight. The protein/bead complexes were washed three times with 1 mL of PBS, and the bound proteins were eluted from the beads by boiling in 15 μL of elution buffer (62.5 mM Tris-HCl pH 6.8, 10% glycerol, 5% β-mercaptoethanol, 2.5% SDS, and 0.01% bromophenol blue). The eluted proteins were subjected to western blot analysis.

Transfection with small interfering RNA (siRNA)

Silencer Select Negative Control No.1 siRNA (4390843) and Silencer Select siRNAs targeting *CREB1* (#1; s3489 and #2; s3490) and *ELF1* (#1; s4617 and #2; s4619) were purchased from Ambion (Austin, TX, USA). The cells were transfected with siRNAs for 48 h using Lipofectamine RNAiMAX transfection reagent (Invitrogen), according to the manufacturer's instructions. The culture medium containing the siRNA and transfection reagent was changed daily during transfection.