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Supplementary Materials for

Cystathione β-synthase regulates HIF-1α stability through persulfidation of PHD2

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Supplementary Figures:

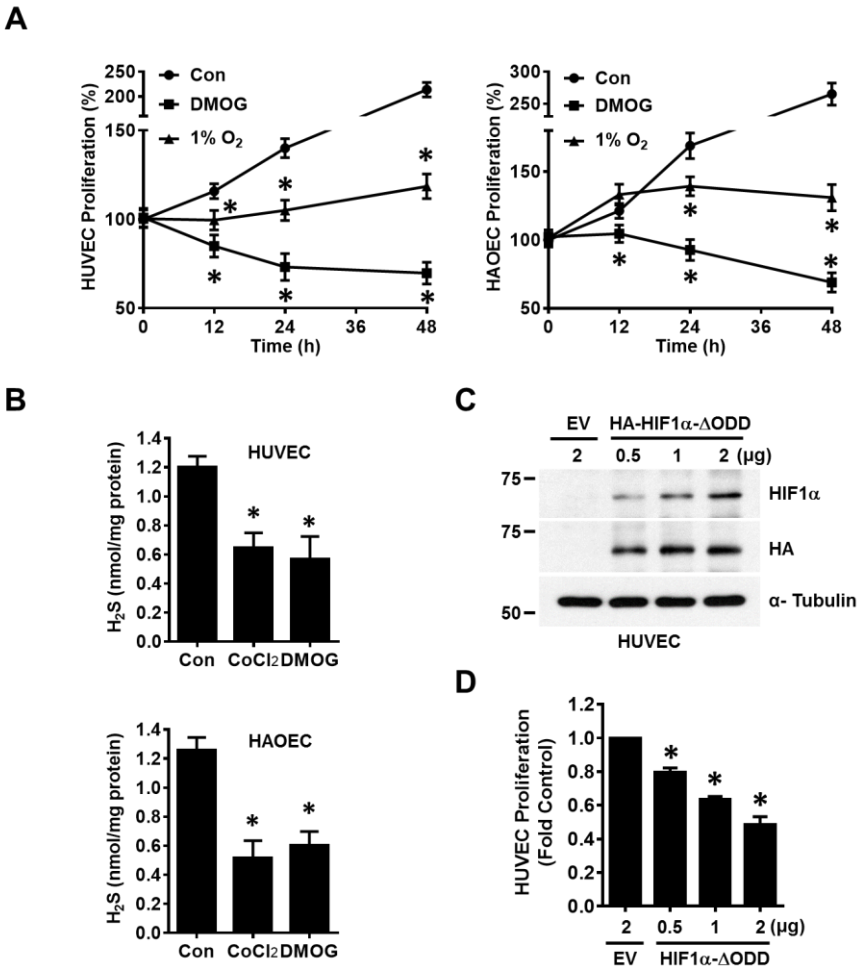

Figure S1

Figure S1: Inhibition in EC proliferation is modulated via HIF1α: (A) HUVEC and HAOEC were exposed to normoxia (Con) or hypoxia (1% O2) or treated with 1mM DMOG for 48h. Using the CyQUANT assay, EC proliferation was evaluated at different time intervals and presented in a single graph for the comparison of relative growth rates. Normoxic untreated control cells were set to 100%. Data are the mean ± SD of three independent experiments performed in triplicate. *P<.05 when comparing with respective normoxic controls by a two-way ANOVA. **(B)** HUVEC and HAOEC were trteated with either vehicle (Con), 100μM $CoCl_2$ or 1mM DMOG for 48h and H_2S levels were determined by methylene blue assay. Data represent mean ± SD of three independent experiments performed in triplicate. *P<.05 when compared with respective controls by a one-way ANOVA. **(C-D)** HUVEC were transfected with either empty vector (EV) or the HA tagged HIF1α with deleted oxygen-dependent degradation (ΔODD- note smaller size) domain at increasing concentrations. **(C)** Expression was confirmed by immunoblotting for HA and HIF-1α while α-Tubulin was used as loading control. **(D)** The effect of HIF1α-ΔODD overexpression in proliferation was evaluated by the CyQUANT assay. *P<.05 when comparing with EV controls by a one-way ANOVA.

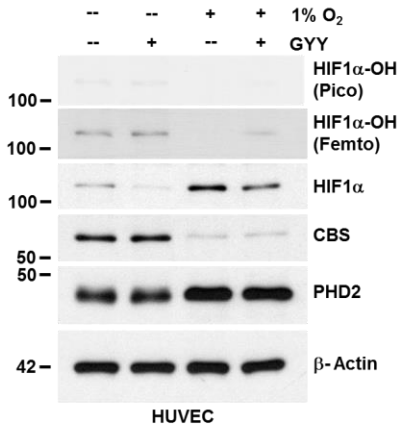

Figure S2

Figure S2. Hydrogen sulfide supplementation in normoxic and hypoxic conditions: HUVEC were exposed to normoxia or 1% O_2 in the presence or absence of 0.5 mM GYY for 48h and immunoblots were performed with the respective indicated antibodies. The hydroxylated HIF-1α bands using the regular chemiluminescence reagents (Pico) detected weak however, with the highest sensitive femto-molar reagents (Femto) and prolonged exposure more prominent bands were detected.

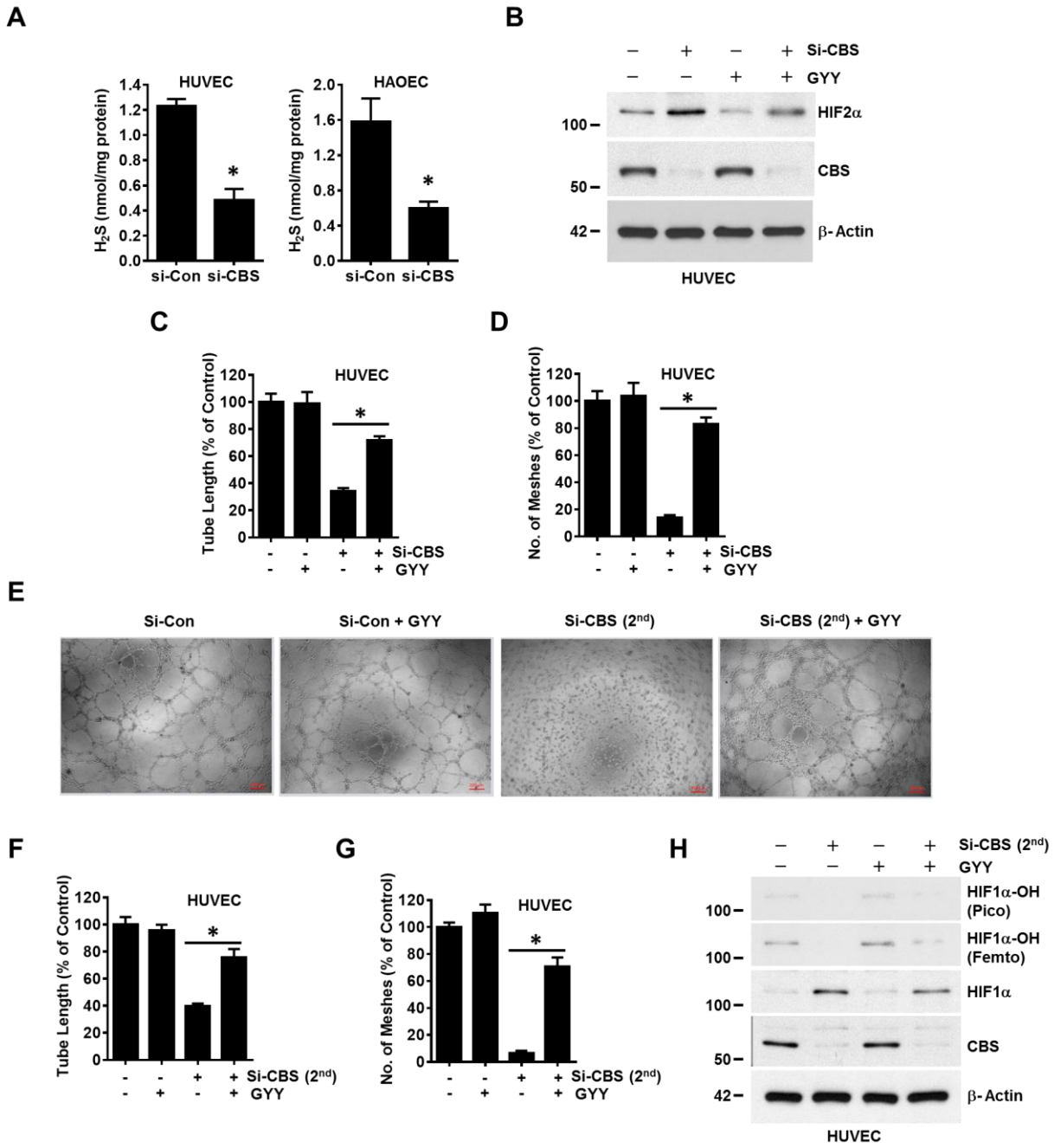

Figure S3

Figure S3. Inhibition of CBS stabilized HIFs in normoxia, decreased H_2S production and impaired hydroxylation of HIF1α: (A) HUVEC and HAOEC were transfected with either scrambled (si-Con) or CBS siRNA (si-CBS) for 48h and H_2S levels were determined by methylene blue assay. Data represent mean ± SD of three independent experiments performed in triplicate. *P<.05 when compared with the respective controls by Student's t test. **(B)** Both si-Con and si-CBS transfected (for 48h) HUVEC were supplemented with 0.5 mM GYY and immunoblots were performed with the indicated antibodies. **(C-D)** Quantitative analysis of tube formation results presented in Figure 3C. Total tube length **(C)** and number of meshes **(D)** was measured using NIH Image J software. Histograms represent the average (±SD) tube length or number of meshes (percent

of respective untreated si-control). *P<.05 when compared with respective controls by a one-way ANOVA. **(E)** Tube formation assay was repeated as in Fig.3C using a second CBS siRNA from Qiagen [si-CBS$^{(2nd)}$] transfected (48h) in HUVEC and supplemented with or without 0.5 mM GYY on 2 mg/ml growth-factor reduced Matrigel. The images were acquired 4h after plating HUVECs on matrigel in complete EBM medium and further quantified to represent total tube length **(F)** and number of meshes **(G).** Histograms represent the average (±SD) tube length or number of meshes (percent of respective untreated si-control). *P<.05 when compared with respective controls by a one-way ANOVA. **(H)** Both si-Con and si-CBS transfected (for 48h) HUVEC were supplemented with 0.5 mM GYY and immunoblotting was performed with the indicated antibodies. The hydroxylated HIF-1α bands using the regular chemiluminescence reagents (Pico) detected very weak however, with the highest sensitive femto-molar reagents (Femto) and prolonged exposure more prominent bands were detected.

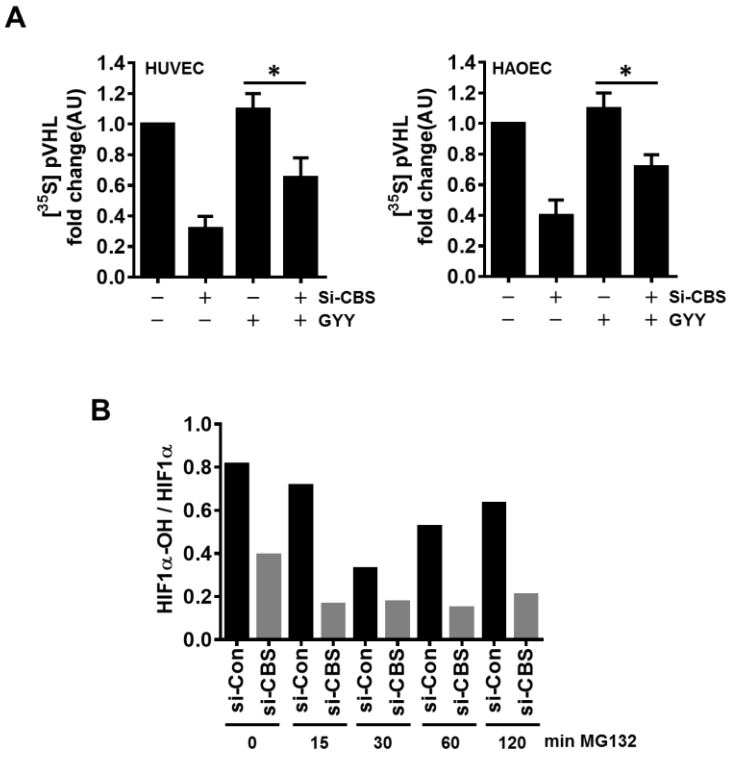

Figure S4

Figure S4. Inhibition of CBS stabilized HIFs in normoxia: (A) Quantitative analysis of [^{35}S] pVHL capture assay result as showed in Figure 3D as a measurement PHD2 activity. Relative integrated density values are normalized to the values of non-treated si-Control are quantified using NIH Image J software. *P<.05 when compared with respective controls by a one-way ANOVA. **(B)** Quantification of band intensities from Figure 3F.

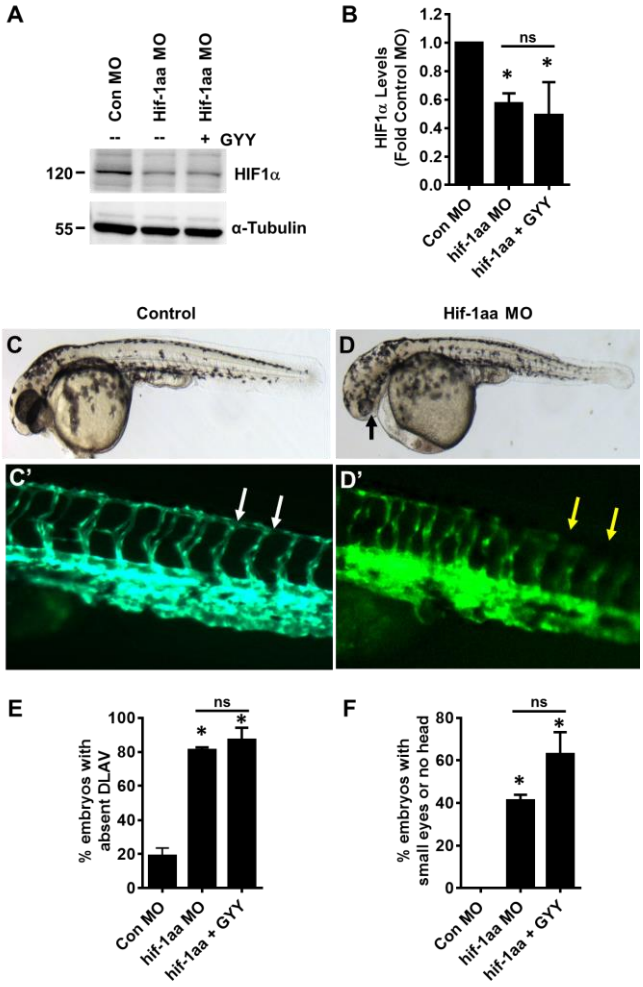

Figure S5

Figure S5. Knocking down of HIF1α in zebrafish embryos caused severe DLAV phenotype which was not rescued by exogenous H2S supplementation. (A) Zebrafish embryos were injected with either control morpholino (ConMO) or a single hif-1aa MO, which was tolerated by the embryos. Successful knockdown of HIF1α was confirmed by immunoblotting analysis. The lysates were generated from the surviving embryos as per procedure described in the materials and methods section. n=47 for hif-1aa morpholino (MO) + DMSO, n=22 for hif-1aa MO + GYY4137 (GYY), n=112 for control MO. **(B)** Quantification of the HIF1α protein using Image J and normalized to α-tubulin. Graph shows HIF1α levels relative to control MO injected embryos, the values are plotted as an average of three independent experiments. Error bars represent standard error of the mean. *P<.05 when compared with respective controls by a one-way ANOVA. **(C)** control MO and **(D)** hif-1aa MO are phase contrast images of 52 hpf Tg(flk1:EGFP) embryos. Black arrow in D shows the small eye phenotype, which was observed in embryos with severe DLAV phenotype. C' and D' are corresponding fluorescent images of the trunk vasculature. White arrows in C' and yellow arrows in D' are gaps in dorsal longitudinal anastomotic vessel (DLAV) and show the DLAV in a control MO-injected embryo. **(E-F)** represent compiled data from three independent experiments performed at separate times. (**E)** Shows quantification of percent embryos with an absent DLAV. Panel **(F)** shows the quantification of percent embryos with small eyes or no head. The quantification was performed on the surviving embryos which are shown on top of each bar for each sample. p<0.01

for comparison between hif-1aa morpholino (MO) + DMSO and control groups, hif-1aa MO + GYY4137 and control MO groups for both phenotypes.

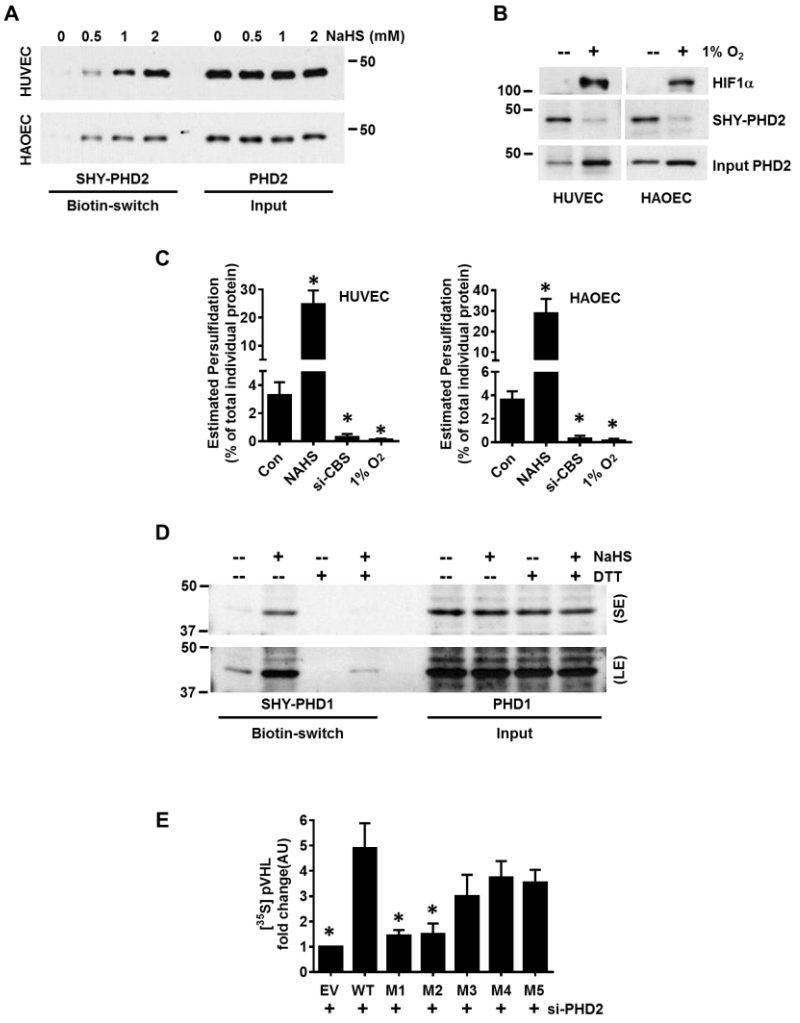

Figure S6

Figure S6: Persulfidation of PHDs and determination of hydroxylated HIF-1α by pVHL capture: (A) Modified biotin switch assay for detection of persulfidated PHD2; HUVECs or HAOECs were incubated with various concentrations of NaHS, or in media alone, 2 h prior to processing for detection of persulfidated PHD2. **(B)** Modified biotin switch assay for determination of the persulfidation status of PHD2 in normoxic and hypoxic (1% O_2) HUVEC and HAOECs. **(C)** Densitometric analysis quantitating amount of protein persulfidation in ECs. Data are the mean ± SD for three to five independent experiments with representative data shown in **(A), (B)** and in Fig. 5A and 5B. **(D)** Modified biotin switch assay for detecting persulfidation of PHD1 in untreated and NaHS (1mM) treated HUVEC and HAOECs. DTT treatment (1mM) for 30 min reversed PHD2 persulfidation. SE and LE represent as shorter or longer exposure. **(E)** Quantitative analysis of [^{35}S] pVHL capture assay result as showed in Figure 5E as a measurement PHD2 activity. Relative integrated density values are normalized to the values of EV are quantified using NIH Image J software. *P<.05 when compared with respective controls by a one-way ANOVA.

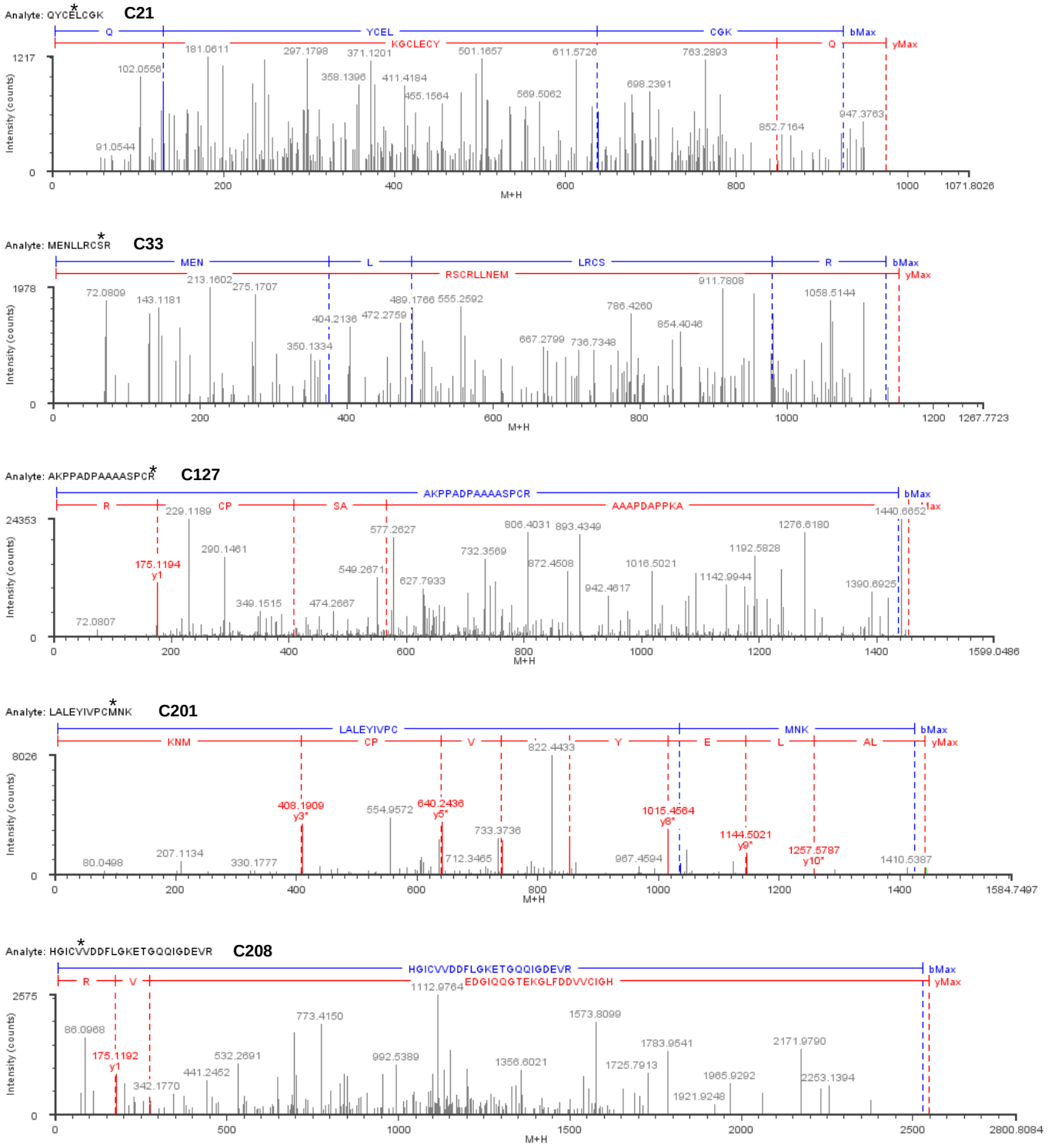

Figure S7: Mass spectrometric analysis of the cysteine persulfidation status of human PHD2: Charge-reduced, isotope-deconvoluted MSE spectra (fragment ion matched profiles) of full-length human PHD2 protein. * site of persulfidation.

Supplementary Method:

Mass spectrometric analysis of PHD2:

HA-PHD2 was overexpressed in HUVECs for 36h and was immunoprecipitated by PHD2 Antibody (Santacruz Biotechnology, Dallas, TX, USA #sc-271835 and further eluted using Pierce™ Crosslink Magnetic IP/Co-IP Kit (#88805) was protected with iodoacetamide in the absence of reductant and digested with trypsin. The ensuing peptides were analyzed by LC-MS/MS on a Synapt G2S (Waters, Milford, MA, USA) in data independent mode. The peptides and their cysteine persulfidation modification sites were identified using Biopharmalynx 1.3 (Waters).