

Identification of cyclins A1, E1 and vimentin as downstream targets of heme oxygenase-1 in vascular endothelial growth factor-mediated angiogenesis

Andrea Bauer¹, Hayley Mylroie^{1*}, C Clare Thornton^{1*}, Damien Calay^{1*}, Graeme M Birdsey¹,
Allan P Kiprianos¹, Garrick K Wilson¹, Miguel P Soares², Xiaoke Yin³, Manuel Mayr³,
Anna M Randi¹, Justin C Mason¹.

Supplementary Material

Supplementary Figure Legends

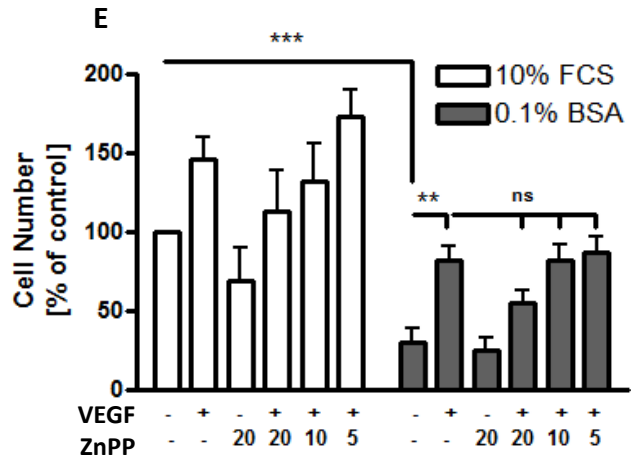
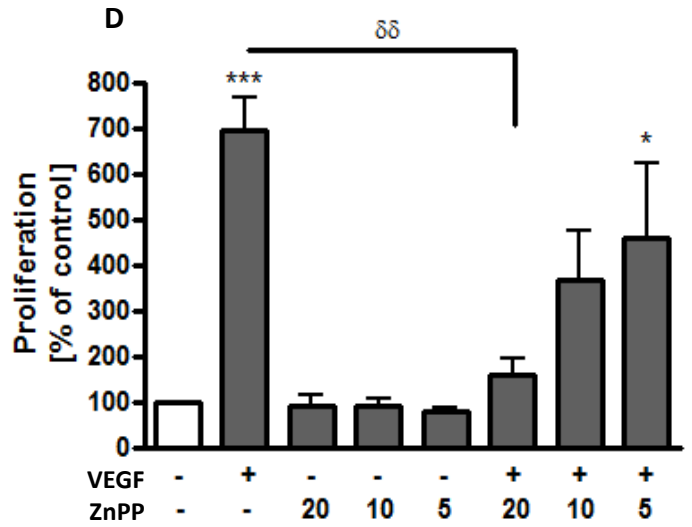
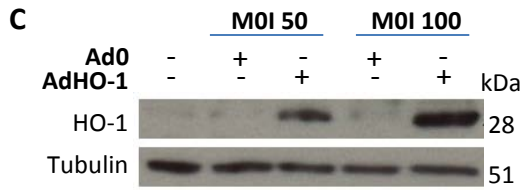
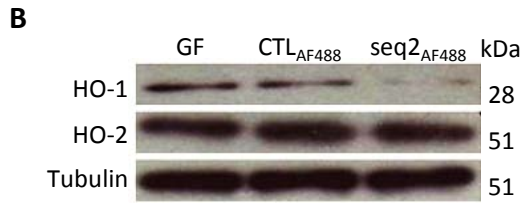
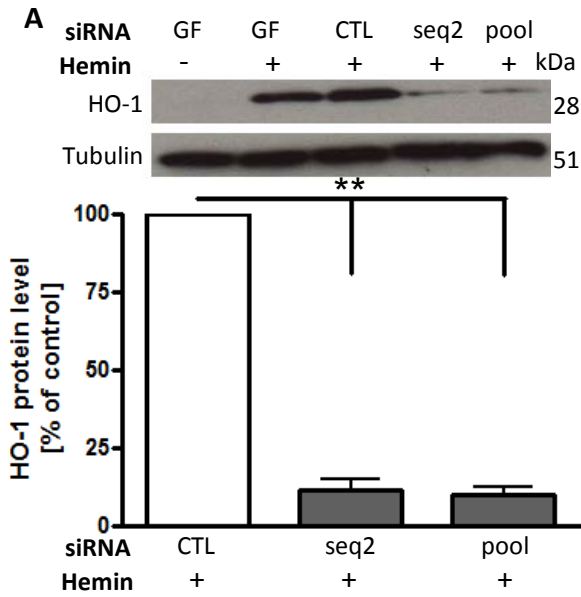
Supplementary Figure 1. Effect of HO-1 depletion on VEGF-induced EC proliferation and cytoprotection. HUVEC were transfected with geneFECTOR (GF) alone, control siRNA (CTL), or HO-1 siRNAs (seq2, pool) prior to treatment with hemin (0.2 μ M) or vehicle for 6h. (A) immunoblotting of HO-1 and tubulin with densitometric quantification of HO-1 relative to hemin-treated positive control EC. (C) HUVEC were transfected with CTLAF₄₈₈ or HO-1 siRNA seq2AF₄₈₈ prior to immunoblotting for HO-1, HO-2 and tubulin. (C) HUVEC were left uninfected or infected with Ad-HO-1 or Ad0 for 24 h prior to immunoblotting of HO-1 and tubulin. (D and E) HUVEC were left untreated or treated with VEGF (25 ng/ml), ZnPP (5-20 μ M), or VEGF and ZnPP in combination. (D) EC proliferation was quantified by BrdU incorporation after 48h, and (E) cell number by MTT assay after 24 h. Data are presented as mean \pm SEM (n=3 experiments). * p <0.05, ** p <0.01, *** p <0.001 versus UT control. $\delta\delta$ p <0.01 and ns = non-significant versus VEGF-treated positive control.

Supplementary Figure 2. HO-1 inhibition impairs microtubule formation. Human microvascular EC (HMEC-1) were transfected with CTLAF₄₈₈ or HO-1 siRNAs (seq2AF₄₈₈). (A) confluent EC monolayers were scratched and migration distance in the absence or presence of VEGF (25 ng/ml) for 16h was assessed by live cell imaging. (B) HMEC-1 were transfected with control or HO-1 siRNA (seq2, pool) for 24h prior to harvesting and re-seeding onto Matrigel in the absence or presence of VEGF (25 ng/ml) for 6 h and visualised using phase-contrast microscopy. Images from five fields of view were captured and tube length quantified using ImageJ (NeuronJ). Data are presented as mean \pm SEM (n=3 experiments), ** p <0.01, *** p <0.001.

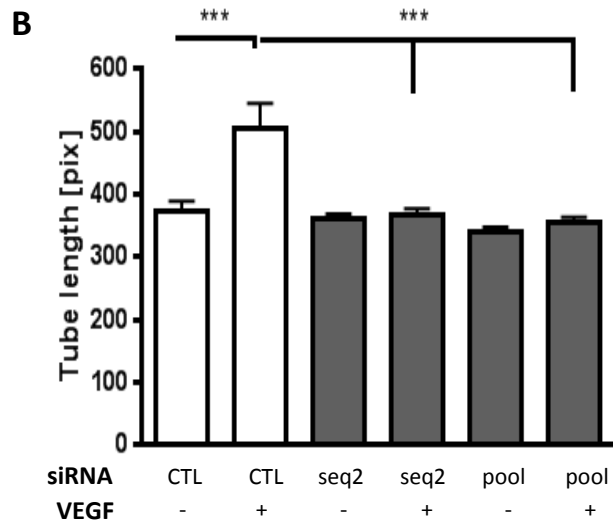
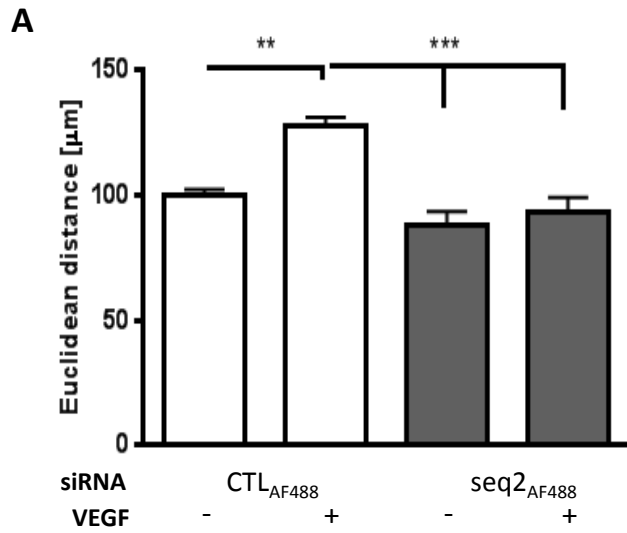
Supplementary Figure 3. HO-1 inhibition senescence and cyclin regulation. (A) HUVEC transfected with CTL or HO-1 siRNAs (seq2 or pool) were left untreated or treated with VEGF (25 ng/ml) for 48 h. Senescent cells were identified by SA- β -gal staining. HUVEC cultured for 12 passages (P12) acted as a positive control. (B-C) HUVEC were treated with geneFECTOR (GF) alone or transfected with CTL or HO-1 siRNAs prior to treatment with VEGF (25 ng/ml) or vehicle. (B) cyclin B1 and (C) cyclin D1 mRNAs were quantified by qRT-PCR. (D-E) Murine EC were treated with GF alone or transfected with CTL, cyclin A1 or HO-1 siRNAs and after 48 h (D) HO-1 and (E) cyclin A1 mRNAs were quantified by qRT-PCR. Data are presented as mean \pm SEM (n=3-5 experiments). ns = not significant and δ p <0.05 versus untreated CTL-transfected cells.

Supplementary Figure 4. Difference in gel electrophoresis. A representative DIGE fluorescent image showing the overlay of Cy5-labelled Ctrl siRNA + VEGF (red) and Cy3-labelled HO-1 siRNA + VEGF (green).

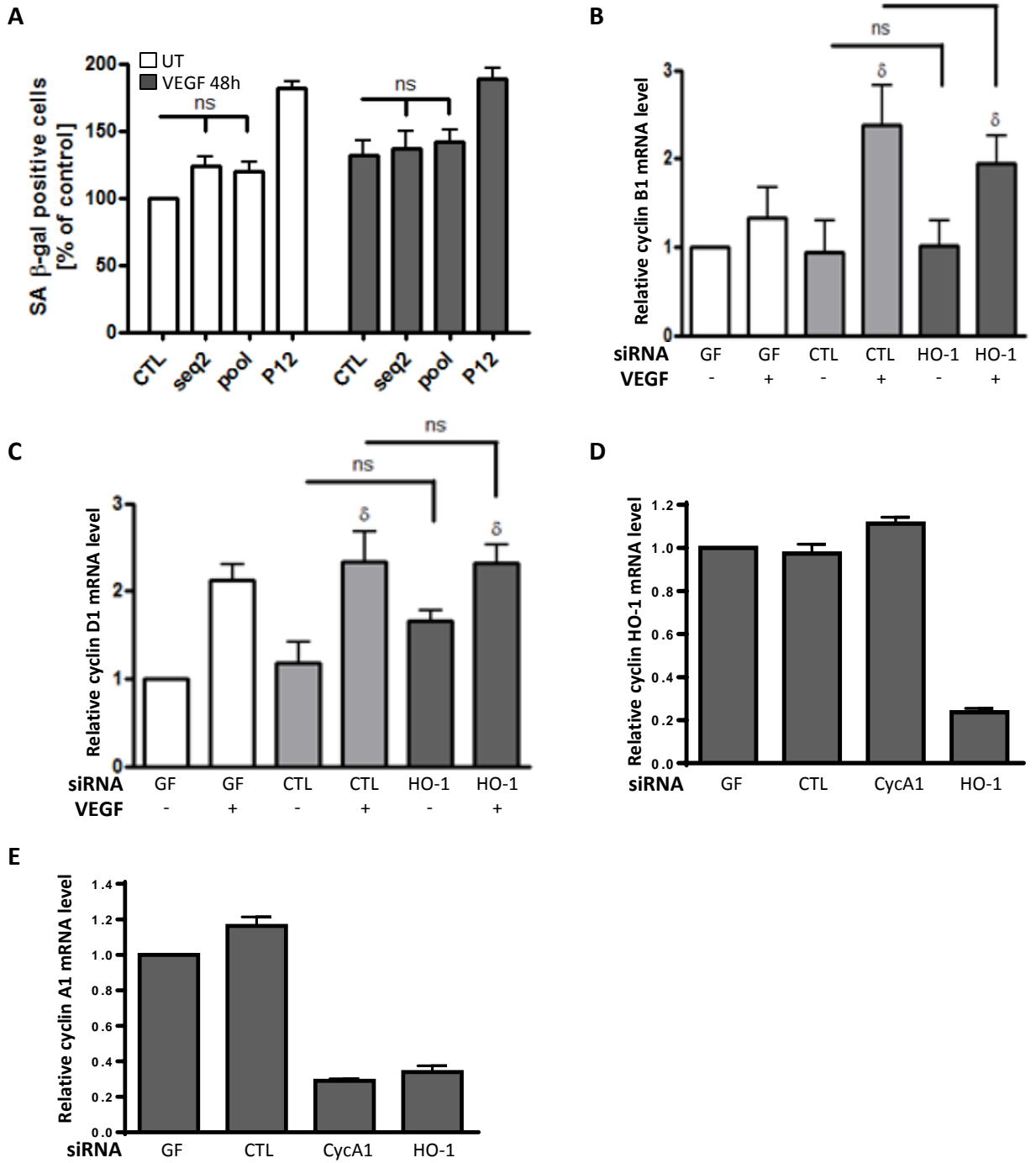
Supplementary Figure 5. Vimentin band phosphorylation and $\alpha_v\beta_3$ expression. (A) HUVEC were treated with geneFECTOR (GF) alone or transfected with CTL or HO-1 siRNAs prior to treatment with VEGF (25 ng/ml) or vehicle for 16h. Protein lysates were treated with lambda phosphatase (λ PP, 200 units). Vimentin and GAPDH were analysed by Phos-tagTM SDS-PAGE and immunoblotting. A representative blot from two separate experiments is shown. (B) HUVEC were transfected as above prior to treatment with VEGF or vehicle for 40 minutes. Integrin $\alpha_v\beta_3$ expression was analysed by flow-cytometry and presented as relative fluorescent intensity, normalized to EC treated with GF alone. Data are shown as mean \pm SEM (n=4 experiments), ns=not significant.



Supplementary Figure 1



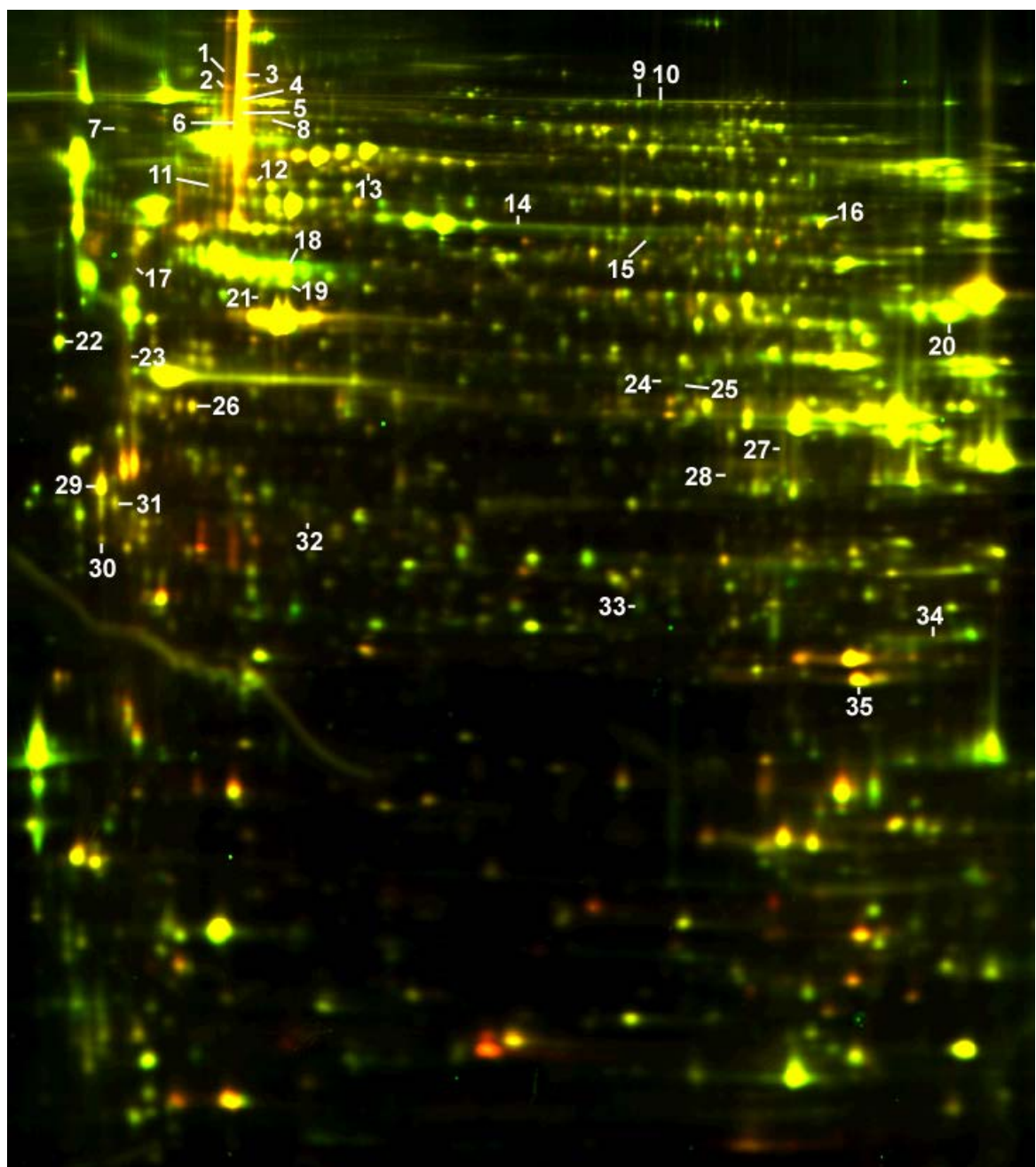
Supplementary Figure 2



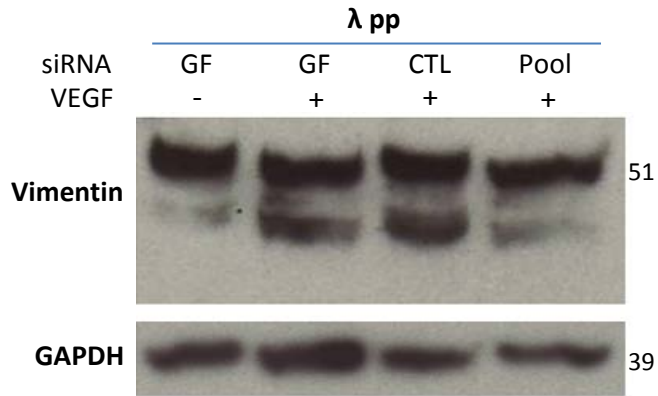
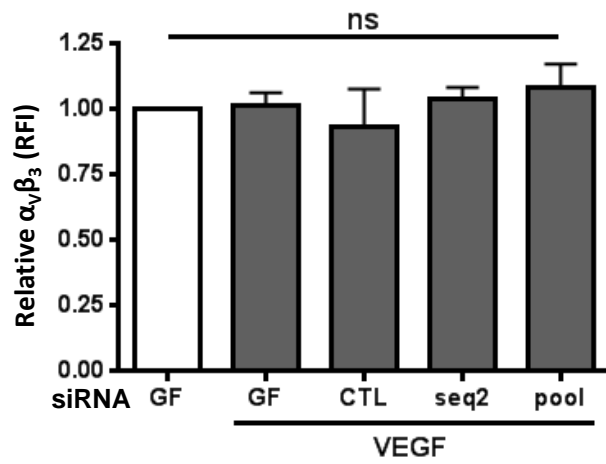
Supplementary Figure 3

Mw (kD)

80
60
40
30
20
10



Supplementary Figure 4

A**B**

Supplementary Figure 5

Supplementary Table 1: Cytoskeleton Regulator PCR array results. Changes in mRNA expression of siCTL- or siHO-1 siRNA-transfected HUVEC in response to VEGF were assessed using a PCR Array. Upregulation of gene expression ≥ 2.00 fold and downregulation ≤ 0.50 fold were considered significant changes in gene expression.

Gene Symbol	Gene Name	Fold Up- or Down-Regulation
ACTR2	ARP2 actin-related protein 2 homolog (yeast)	1.01
ACTR3	ARP3 actin-related protein 3 homolog(yeast)	0.77
ARFIP2	ADP-ribosylation factor interacting protein 2	1.34
ARHGAP6	Rho GTPase activating protein 6	1.16
ARHGDIB	Rho GDP dissociation inhibitor (GDI) beta	0.82
ARHGEF11	Rho guanine nucleotide exchange factor (GEF) 11	1.43
ARPC1B	Actin related protein 2/3 complex, subunit 1B, 41kDa	1.34
ARPC2	Actin related protein 2/3 complex, subunit 2, 34kDa	2.17
ARPC3	Actin related protein 2/3 complex, subunit 3, 21kDa	0.82
ARPC4	Actin related protein 2/3 complex, subunit 4, 20kDa	1.54
ARPC5	Actin related protein 2/3 complex, subunit 5, 16kDa	0.58
AURKA	Aurora kinase A	1.43
AURKB	Aurora kinase B	1.34
AURKC	Aurora kinase C	1.65
BAIAP2	BAI1-associated protein 2	1.09
CALD1	Caldesmon 1	0.88
CALM1	Calmodulin 1 (phosphorylase kinase, delta)	0.95
CASK	Calcium/calmodulin-dependent serine protein kinase (MAGUK family)	1.09
CCNA1	Cyclin A1	0.44
CCNB2	Cyclin B2	1.43
CDC42	Cell division cycle 42 (GTP binding protein, 25kDa)	0.95
CDC42BPA	CDC42 binding protein kinase alpha (DMPK-like)	1.25
CDC42EP2	CDC42 effector protein (Rho GTPase binding) 2	1.01
CDC42EP3	CDC42 effector protein (Rho GTPase binding) 3	1.16
CDK5	Cyclin-dependent kinase 5	1.43
CDK5R1	Cyclin-dependent kinase 5, regulatory subunit 1 (p35)	1.43
ARAP1	ArfGAP with RhoGAP domain, ankyrin repeat and PH domain 1	1.25
CFL1	Cofilin 1 (non-muscle)	1.01
CIT	Citron (rho-interacting, serine/threonine kinase 21)	2.03
CLASP1	Cytoplasmic linker associated protein 1	0.88
CLASP2	Cytoplasmic linker associated protein 2	0.88
CLIP1	CAP-GLY domain containing linker protein 1	1.09
CLIP2	CAP-GLY domain containing linker protein 2	1.25
CRK	V-crk sarcoma virus CT10 oncogene homolog (avian)	0.82
CTTN	Cortactin	1.01
CYFIP1	Cytoplasmic FMR1 interacting protein 1	0.95
CYFIP2	Cytoplasmic FMR1 interacting protein 2	1.77
DIAPH1	Diaphanous homolog 1 (Drosophila)	0.95
DSTN	Destrin (actin depolymerizing factor)	1.01
EZR	Ezrin	1.65
FNBP1L	Formin binding protein 1-like	1.16
FSCN2	Fascin homolog 2, actin-bundling protein, retinal (Strongylocentrotus purpuratus)	2.17
Supplementary Figure 4 (continued)		
GSN	Gelsolin (amyloidosis, Finnish type)	1.09
IQGAP1	IQ motif containing GTPase activating protein 1	0.88

IQGAP2	IQ motif containing GTPase activating protein 2	1.09
LIMK1	LIM domain kinase 1	1.09
LIMK2	LIM domain kinase 2	0.82
LLGL1	Lethal giant larvae homolog 1 (Drosophila)	1.25
MACF1	Microtubule-actin crosslinking factor 1	0.82
MAP3K11	Mitogen-activated protein kinase kinase kinase 11	1.54
MAP4	Microtubule-associated protein 4	0.72
MAPK13	Mitogen-activated protein kinase 13	1.16
MAPRE1	Microtubule-associated protein, RP/EB family, member 1	0.82
MAPRE2	Microtubule-associated protein, RP/EB family, member 2	0.58
MAPT	Microtubule-associated protein tau	0.72
MARK2	MAP/microtubule affinity-regulating kinase 2	1.09
MID1	Midline 1 (Opitz/BBB syndrome)	0.88
MSN	Moesin	0.62
MYLK	Myosin light chain kinase	1.16
MYLK2	Myosin light chain kinase 2	1.34
NCK1	NCK adaptor protein 1	1.01
NCK2	NCK adaptor protein 2	1.01
PAK1	P21 protein (Cdc42/Rac)-activated kinase 1	1.43
PAK4	P21 protein (Cdc42/Rac)-activated kinase 4	1.09
PFN2	Profilin 2	1.09
PHLDB2	Pleckstrin homology-like domain, family B, member 2	0.95
PIKFYVE	Phosphoinositide kinase, FYVE finger containing	1.16
PPP1R12A	Protein phosphatase 1, regulatory (inhibitor) subunit 12A	1.43
PPP1R12B	Protein phosphatase 1, regulatory (inhibitor) subunit 12B	2.03
PPP3CA	Protein phosphatase 3 (formerly 2B), catalytic subunit, alpha isoform	0.82
PPP3CB	Protein phosphatase 3 (formerly 2B), catalytic subunit, beta isoform	0.51
RAC1	Ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac1)	0.88
RACGAP1	Rac GTPase activating protein 1	1.25
RDX	Radixin	0.82
RHOA	Ras homolog gene family, member A	0.82
ROCK1	Rho-associated, coiled-coil containing protein kinase 1	0.95
SSH1	Slingshot homolog 1 (Drosophila)	1.16
SSH2	Slingshot homolog 2 (Drosophila)	0.77
STMN1	Stathmin 1	1.09
TIAM1	T-cell lymphoma invasion and metastasis 1	1.65
VASP	Vasodilator-stimulated phosphoprotein	1.01
WAS	Wiskott-Aldrich syndrome (eczema-thrombocytopenia)	0.51
WASF1	WAS protein family, member 1	1.01
WASL	Wiskott-Aldrich syndrome-like	1.16

Supplementary Table 2. Significantly altered protein spots identified by DIGE LC-MS/MS.

No	Protein name	Accession No.	Mw (Da)	p value	Average Ratio*	# unique peptides	# unique spectra	# total spectra	Sequence coverage
1	Vimentin	VIME_HUMAN	53,635	0.013	-2.03	16	18	30	36.30%
2	Vimentin	VIME_HUMAN	53,635	0.016	-1.71	21	25	43	48.30%
3	Vimentin	VIME_HUMAN	53,635	0.0024	-1.69	35	48	90	68.70%
4	Vimentin	VIME_HUMAN	53,635	0.0067	-1.65	38	44	81	79.20%
5	Vimentin	VIME_HUMAN	53,635	0.0068	-1.63	34	40	69	71.00%
6	Vimentin	VIME_HUMAN	53,635	0.0047	-1.66	34	40	76	72.50%
7	Periodic tryptophan protein 1 homolog	PWP1_HUMAN	55,811	0.017	-1.2	3	3	5	6.39%
8	Lamin-B1	LMNB1_HUMAN	66,392	0.0054	-1.22	4	4	5	7.85%
8	Ran GTPase-activating protein 1	RAGP1_HUMAN	63,525	0.0054	-1.22	4	4	6	7.84%
9	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 2	PLOD2_HUMAN	84,669	0.0046	1.2	8	9	17	13.00%
10	Nucleolin	NUCL_HUMAN	76,598	0.00056	1.21	3	3	6	4.37%
10	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 2	PLOD2_HUMAN	84,669	0.00056	1.21	9	9	14	13.70%
11	Vimentin	VIME_HUMAN	53,635	0.0018	-1.91	10	10	16	24.00%
12	Heterogeneous nuclear ribonucleoprotein K	HNRPK_HUMAN	50,961	0.028	-1.24	7	11	19	17.90%
13	Ras GTPase-activating protein-binding protein 1	G3BP1_HUMAN	52,145	0.02	-1.26	3	3	5	6.22%
14	Protein disulfide-isomerase A3	PDIA3_HUMAN	56,767	0.026	1.25	11	14	23	23.40%
15	Succinyl-CoA:3-ketoacid-coenzyme A transferase 1, mitochondrial	SCOT1_HUMAN	56,141	0.01	1.38	5	6	9	10.40%
16	Pyruvate kinase isozymes M1/M2	KPYM_HUMAN	57,920	0.043	1.23	10	10	19	17.50%
17	Vimentin	VIME_HUMAN	53,635	0.03	-1.52	9	9	15	15.50%
18	Thioredoxin domain-containing protein 5	TXND5_HUMAN	47,611	0.044	1.36	16	18	31	35.20%
19	Heterogeneous nuclear ribonucleoprotein F	HNRPF_HUMAN	45,654	0.012	1.21	8	11	20	23.40%
20	Serpin H1	SERPH_HUMAN	46,424	0.012	1.37	17	22	44	40.90%
21	Eukaryotic translation initiation factor 3 subunit F	EIF3F_HUMAN	37,546	0.039	-1.28	6	7	13	21.60%
21	Perilipin-3	PLIN3_HUMAN	47,028	0.039	-1.28	5	5	9	18.40%
22	Protein SET	SET_HUMAN	33,471	0.037	1.23	7	11	18	22.40%

23	Tropomyosin alpha-1 chain	TPM1_HUMAN	32,692	0.043	-1.29	5	5	7	15.50%
23	Vimentin	VIME_HUMAN	53,635	0.043	-1.29	4	4	7	7.51%
24	Ubiquitin fusion degradation protein 1 homolog	UFD1_HUMAN	34,483	0.047	1.25	3	4	7	7.82%
25	Heterogeneous nuclear ribonucleoproteins C1/C2	HNRPC_HUMAN	33,653	0.02	1.36	5	5	7	13.40%
26	Elongation factor 1-delta	EF1D_HUMAN	31,104	0.018	-1.24	13	15	25	47.00%
27	Voltage-dependent anion-selective channel protein 2	VDAC2_HUMAN	31,549	0.018	-1.24	6	6	10	25.50%
28	Voltage-dependent anion-selective channel protein 2	VDAC2_HUMAN	31,549	0.026	-1.31	2	2	3	6.80%
29	Elongation factor 1-delta	EF1D_HUMAN	31,104	0.027	-1.23	3	3	4	7.12%
30	Elongation factor 1-beta	EF1B_HUMAN	24,746	0.027	-1.23	4	5	7	23.10%
30	Eukaryotic translation initiation factor 6	IF6_HUMAN	26,580	0.05	-1.2	6	7	13	29.40%
31	14-3-3 protein epsilon	1433E_HUMAN	29,157	0.018	-1.29	12	16	30	35.30%
32	Prohibitin	PHB_HUMAN	29,787	9.40E-06	-1.24	7	7	13	22.80%
33	LDLR chaperone MESD	MESD_HUMAN	26,060	0.0098	1.41	7	8	16	32.50%
34	Cysteine and glycine-rich protein 1	CSRP1_HUMAN	20,549	0.038	1.27	9	10	17	50.80%
35	Transgelin-2	TAGL2_HUMAN	22,374	0.047	-1.43	9	13	23	44.20%

* Average Ratio is HO-1 siRNA + VEGF / Control siRNA + VEGF. Positive value indicates increased in HO-1 siRNA + VEGF group. Negative value indicates decreased in HO-1 siRNA + VEGF group.

Supplementary Table 2. Proteins differentially expressed between HO-1 deficient and control human EC. 29 identified proteins were analysed and grouped according to gene ontology (GO) using DAVID Bioinformatics Resources 6.7.

Cellular Component GO Term	Count	Gene Name
intracellular organelle lumen	12	SCOT1 SET IF6 HNRPF HNPRK LMNB1 NUCL PHB PDIA3 SERPH TXNDC5 VDAC2
organelle lumen	12	SCOT1 SET IF6 HNRPF HNPRK LMNB1 NUCL PHB PDIA3 SERPH TXNDC5 VDAC2
membrane-enclosed lumen	12	SCOT1 SET IF6 HNRPF HNPRK LMNB1 NUCL PHB PDIA3 SERPH TXNDC5 VDAC2
nuclear lumen	7	SET IF6 HNRPF HNPRK LMNB1 NUCL PHB
spliceosome	3	HNRPC HNRPF HNPRK
ribonucleoprotein complex	4	HNRPC HNRPF HNPRK NUCL
nucleoplasm	5	SET HNRPF HNPRK NUCL PHB
nucleolus	4	IF6 HNRPF HNPRK NUCL
organelle envelope	6	RAGP1 IF6 LMNB1 PHB TAGL2 VDAC2

Supplementary Table 2. (cont)

envelope	6	RAGP1 IF6 LMNB1 PHB TAGL2 VDAC2
nuclear envelope	4	RAGP1 IF6 LMNB1 TAGL2
nuclear membrane	3	IF6 LMNB1 TAGL2
organelle inner membrane	3	IF6 LMNB1 PHB
organelle membrane	5	IF6 LMNB1 PHB TAGL2 VDAC2
endomembrane system	4	RAGP1 IF6 LMNB1 TAGL2
endoplasmic reticulum lumen	3	PDIA3 SERPH TXNDC5
endoplasmic reticulum	6	SET MESD PLOD2 PDIA3 SERPH TXNDC5
endoplasmic reticulum part	3	SET MESD PDIA3 SERPH TXNDC5
integral to membrane	3	RAGP1 PHB VDAC2
intrinsic to membrane	3	RAGP1 PHB VDAC2
intermediate filament	3	IF6 LMNB1 VIME
intermediate filament cytoskeleton	3	IF6 LMNB1 VIME
non-membrane-bound organelle	9	RAGP1 HNRPK HNRPF IF6 LMNB1 NUCL TPM1 VDAC2 VIME
intracellular non-membrane bound organelle	9	RAGP1 HNRPK HNRPF IF6 LMNB1 NUCL TPM1 VDAC2 VIME

Supplementary Table 2. (cont)

cytoskeletal part	4	IF6 LMNB1 TPM1 VIME
cytoskeleton	4	IF6 LMNB1 TPM1 VIME
mitochondrial part	3	SCOT1 PHB VDAC2
mitochondrion	4	SCOT1 PHB 1433E VDAC2
cytosol	9	G3BP1 SET EF1B EF1D EIF3F 1433E KPYM UFD1 VIME
plasma membrane	7	G3BP1 RAGP1 PLIN3 PHB TAGL2 TPM1 VIME
plasma membrane part	4	RAGP1 PLIN3 PHB TPM1

Molecular Function

GO Term	Count	Gene Name
translation factor activity, nucleic acid binding		
RNA binding	5	G3BP1 HNRPC HNRPK HNRPF NUCL
nucleotide binding	5	G3BP1 HNRPC HNRPF NUCL VDAC2
DNA binding	3	G3BP1 HNRPK NUCL
structural molecule activity	3	LMNB1 TPM1 VIME
metal ion binding	3	CSRP1 PLOD2 KPYM
cation binding	3	CSRP1 PLOD2 KPYM
ion binding	3	CSRP1 PLOD2 KPYM

Supplementary Table 2. (cont)

Biological Process GO Term	Count	Gene Name
translation	4	EF1B EF1D EIF3F IF6
RNA splicing, via transesterification reactions	3	HNRPC HNRPF HNPRK
nuclear mRNA splicing, via spliceosome	3	HNRPC HNRPF HNPRK
RNA splicing	3	HNRPC HNRPF HNPRK
mRNA processing	3	HNRPC HNRPF HNPRK
mRNA metabolic process	3	HNRPC HNRPF HNPRK
RNA processing	3	HNRPC HNRPF HNPRK
intracellular transport	5	SET PDIA3 1433E TXDN5 TPM1
regulation of apoptosis	4	PHB PDIA3 1433E TXDN5
regulation of programmed cell death	4	PHB PDIA3 1433E TXDN5
regulation of cell death	4	PHB PDIA3 1433E TXDN5
cell motion	3	1433E TPM1 VIME
negative regulation of macromolecule metabolic process	3	SET PHB 1433E