Ubiquitination of basal VEGFR2 regulates signal transduction and endothelial function

Gina A. Smith1, Gareth W. Fearnley1, Izma Abdul-Zani1, Stephen B. Wheatcroft2, Darren C. Tomlinson1, Michael A. Harrison3 and Sreenivasan Ponnambalam1,*

ABSTRACT
Cell surface receptors can undergo recycling or proteolysis but the cellular decision-making events that sort between these pathways remain poorly defined. Vascular endothelial growth factor A (VEGF-A) and vascular endothelial growth factor receptor 2 (VEGFR2) regulate signal transduction and angiogenesis, but how signaling and proteolysis is regulated is not well understood. Here, we provide evidence that a pathway requiring the E1 ubiquitin-activating enzyme UBA1 controls basal VEGFR2 levels, hence metering plasma membrane receptor availability for the VEGF-A-regulated endothelial cell response. VEGFR2 undergoes VEGF-A-independent constitutive degradation via a UBA1-dependent ubiquitin-linked pathway. Depletion of UBA1 increased VEGFR2 recycling from endosome-to-plasma membrane and decreased proteolysis. Increased membrane receptor availability after UBA1 depletion elevated VEGF-A-stimulated activation of key signaling enzymes such as PLCγ1 and ERK1/2. Although UBA1 depletion caused an overall decrease in endothelial cell proliferation, surviving cells showed greater VEGF-A-stimulated responses such as cell migration and tubulogenesis. Our study now suggests that a ubiquitin-linked pathway regulates the balance between receptor recycling and degradation which in turn impacts on the intensity and duration of VEGF-A-stimulated signal transduction and the endothelial response.

KEY WORDS: Endothelial, VEGF-A, VEGFR2, UBA1, Ubiquitination, Signal transduction, Angiogenesis

INTRODUCTION
Vascular endothelial growth factor A (VEGF-A) is an important regulator of animal health and disease (Ferrara, 1999). VEGF-A-stimulated pathological angiogenesis is an important player in chronic inflammatory diseases, cancer and retinopathy (Carmeliet, 2005; Coultas et al., 2005; Ferrara and Kerbel, 2005), whilst insufficient angiogenesis leads to damaged blood vessels, causing tissue ischaemia and heart disease (Ungvari et al., 2010). VEGF binding to a vascular endothelial growth factor receptor (VEGFR) can trigger multiple signal transduction pathways and cellular responses in vascular and non-vascular cells and tissues. In particular, VEGF-A binding to VEGFR2 on endothelial cells causes a diverse range of pro-angiogenic responses (Olsson et al., 2006; Shibuya, 2010). Although highly studied, it is not well understood how the endothelial cell integrates multiple pathways to direct the sprouting of new blood vessels upon encountering ligands such as VEGF-A.

It is well-established that VEGF-A binding to plasma membrane VEGFR2 causes tyrosine kinase activation and post-translational modifications such as tyrosine trans-autophosphorylation and ubiquitination (Ewan et al., 2006a; Koch and Claesson-Welsh, 2012). Ligand-activated VEGFR2 can undergo ubiquitin-linked proteolysis (Bruns et al., 2010; Ewan et al., 2006) which is regulated by E3 ubiquitin ligases such as the proto-oncogene c-Cbl and β-transducin repeat-containing protein (β-TrCP1) (Duval et al., 2003; Shaik et al., 2012; Singh et al., 2007). However, it is unclear how the endothelial cell regulates resting or basal VEGFR2 levels. One possibility is that non-modified, basal VEGFR2 located at the plasma membrane undergoes constitutive endocytosis and delivery to lysosomes for proteolysis. An alternative explanation is that a ubiquitination-dependent mechanism targets basal VEGFR2 for trafficking to degradative compartments such as late endosomes and lysosomes. A recent study has suggested that basal VEGFR2 turnover is regulated by an endosome-associated de-ubiquitinase, USP8 (Smith et al., 2016). Furthermore, the E3 ubiquitin ligase RNF121 controls turnover of newly synthesized VEGFR2 in the secretory pathway (Maghsoudlou et al., 2016). Hence there is an emerging body of evidence that ubiquitination of newly synthesized or basal VEGFR2 trafficking and turnover.

Ubiquitination is a covalent modification involving the formation of an isopeptide bond between the amino terminus of lysine side chains with the free carboxyl terminus of ubiquitin monomers or polymers. The addition of these ubiquitin moieties to a specific protein can alter degradation, intracellular localization and modulate protein activity. Adding such a modification first requires activity of an E1 ubiquitin-activating enzyme, followed by an E2 ubiquitin-conjugating enzyme working in concert with an E3 ubiquitin ligase (Hershko and Ciechanover, 1992). Nine loci within the human genome encode E1-related enzymes which initiate activation and conjugation of a variety of ubiquitin and ubiquitin-like proteins (e.g. SUMO, Nedd8) to target substrates (Pickart, 2001). This study reveals the existence of a novel pathway that programs E1 ubiquitin ligase-dependent modification of basal VEGFR2 to regulate membrane trafficking and proteolysis. Such regulation is important in controlling the endothelial response to VEGF-A by integrating signal transduction, membrane trafficking and cellular responses.

RESULTS
UBA1 regulates basal VEGFR2 levels in endothelial cells
Ligand-stimulated ubiquitination of VEGFR2 facilitates trafficking and degradation in the endosome-lysosome system (Bruns et al., 2010).
UBA1 regulates constitutive ubiquitination and degradation of VEGFR2

Blocking new protein synthesis using cycloheximide (CHX) enables the monitoring of mature VEGFR2 degradation (Shaik et al., 2012). In these experiments, we combined CHX treatment and RNAi-mediated UBA1 depletion to evaluate UBA1 contribution to VEGFR2 turnover (Fig. 2). Immunoblotting confirmed that basal VEGFR2 levels were elevated upon UBA1 depletion, in the absence of VEGFR2 tyrosine phosphorylation (Fig. 2A). In comparing VEGFR2 turnover to other membrane receptors, UBA1 depletion did not affect basal levels of other cell surface receptors such as fibroblast growth factor receptor 1 (FGFR1) or transferrin receptor (TfR) (Fig. 2A). Quantification of relative protein levels upon CHX treatment revealed that ∼60% of mature VEGFR2 underwent constitutive degradation over an 80 min period (Fig. 2B). In contrast, UBA1-depleted endothelial cells displayed a ∼40% increase in basal VEGFR2 levels prior to CHX addition (t=0 min; Fig. 2B). Upon subjecting UBA1-depleted cells to CHX treatment for different time periods there was a gradual decrease in VEGFR2 levels, however these VEGFR2 levels were still higher (1.6-fold) than in controls with normal UBA1 levels (Fig. 2B). Depletion of UBA1 thus increases steady-state levels of mature VEGFR2 but this is still subject to degradation with similar kinetics to controls (Fig. 2B).

UBA1 regulates basal VEGFR2 recycling

Ubiquitination at the plasma membrane frequently precedes receptor tyrosine kinase (RTK) endocytosis, delivery to early endosomes and further trafficking to lysosomes for terminal degradation (Clague and Urbé, 2001; Ewan et al., 2006; Haglund and Dikic, 2012). However, RTK de-ubiquitination in early or late endosomes could also enable recycling from endosome-to-plasma membrane (Clague and Urbé, 2006). Such features have previously been observed in this system with VEGF-A-stimulated VEGFR2 ubiquitination promoting trafficking to late endosomes, linked to terminal degradation in lysosomes (Bruns et al., 2010; Ewan et al., 2006). Furthermore, VEGFR2 can also undergo substantial constitutive ligand-independent recycling via endosomes (Jopling et al., 2011). Another RTK such as FGFR1 undergoes similar constitutive recycling (Hausott et al., 2012). One possibility is that upon UBA1 depletion, VEGFR2 undergoes decreased basal ubiquitination that in turn permits increased endosome-to-plasma membrane recycling. To test this idea, we used a VEGFR2 recycling assay (Jopling et al., 2011) in which control and UBA1-depleted endothelial cells were incubated with antibodies specific for the extracellular domains of VEGFR2 or FGFR1. Constitutive RTK endocytosis and recycling was then monitored using accessibility of VEGFR2-antibody and FGFR1-antibody complexes to a pulse of
labeled secondary antibody. Only VEGFR2-antibody or FGFR1-antibody complexes that underwent endocytosis followed by endosome-to-plasma membrane recycling were detected in this assay (Fig. 3A). Compared to control cells, UBA1-depleted endothelial cells displayed a twofold ($P<0.01$) increase in endosome-to-plasma membrane recycling of non-activated VEGFR2 (Fig. 3B). In contrast, FGFR1 recycling was not significantly affected by UBA1 depletion (Fig. 3C).

One possibility is that increased endosome-to-plasma membrane recycling after UBA1 depletion caused an overall net increase in plasma membrane VEGFR2 levels. To test this idea, new protein synthesis was blocked with CHX and a cell surface biotinylation assay was performed to monitor the plasma membrane pool. Immunoblot analysis showed that basal VEGFR2 plasma membrane levels in UBA1-depleted cells were $\sim$25% higher than in control cells ($t=0$ min, Fig. 3D,E). Another cell surface receptor, transferrin receptor, was not significantly affected (Fig. 3D). In control cells treated with CHX, there was a $\sim$55% decrease in levels of plasma membrane VEGFR2 (Fig. 3D). In contrast, under the same CHX treatment of UBA1-depleted cells there was a less marked ($\sim$24%) decrease in plasma membrane VEGFR2 levels (Fig. 3E). These data suggest that loss of UBA1 causes an increase
in plasma membrane VEGFR2 levels and is also consistent with increased VEGFR2 recycling from endosome-to-plasma membrane.

**UBA1 regulates VEGFR2 trafficking to endosomes and lysosomes**

VEGFR2 undergoes endocytosis, delivery to endosomes and recycling back to the plasma membrane or commitment for terminal degradation in late endosomes and lysosomes (Ewan et al., 2006; Jopling et al., 2014). If UBA1 depletion affects VEGFR2 membrane dynamics, loss of UBA1 would be expected to alter VEGFR2 distribution within these compartments. To ascertain this, we compared VEGFR2 co-distribution with EEA1, CD63 or LAMP2 (Fig. 4). UBA1-depleted endothelial cells exhibited a ∼50% increase in VEGFR2 co-distribution with the early endosome marker EEA1 compared to controls (Fig. 4A,B). There was a similar increase in VEGFR2 co-distribution with the late endosome marker CD63 (Fig. 4B). In contrast, UBA1-depleted cells exhibited
a ~34% decrease in VEGFR2 co-distribution with the lysosome marker LAMP2 compared to control (Fig. 4B). These data suggest that decreased UBA1 levels alter VEGFR2 distribution within the endosome-lysosome network.

**UBA1 regulates VEGF-A-stimulated signal transduction**

VEGF-A binding to plasma membrane VEGFR2 stimulates multiple signal transduction pathways (Koch et al., 2011; Zhang et al., 2008). Our experiments now show that UBA1 depletion leads to a net increase in plasma membrane VEGFR2; this could modulate VEGF-A-stimulated signal transduction. To test this idea, control and UBA1-depleted endothelial cells were stimulated with VEGF-A before probing downstream signaling events using quantitative immunoblotting (Fig. 5). VEGFR2 activation is exemplified by phosphorylation on cytoplasmic residue Y1175 (Koch et al., 2011); this was clearly evident in both control and UBA1-depleted cells (Fig. 5A). However, UBA1 depletion caused a significant ~30% increase in VEGFR2-pY1175 levels (Fig. 5B). Plasma membrane VEGFR2 activation is also linked to recruitment of phospholipase Cγ1 (PLCγ1) followed by tyrosine phosphorylation on residue Y783 and increased phospholipase activity (Koch et al., 2011). UBA1-depleted cells exhibited enhanced PLCγ1 phosphorylation (Fig. 5A) with ~43% increase in PLCγ1-pY783 levels (Fig. 5C). A key feature of VEGF-A-stimulated signaling is activation of the canonical mitogen-activated protein kinase (MAPK) pathway leading to phosphorylation and activation of extracellular signal-regulated kinase enzymes 1 and 2 (ERK1/2) (Koch and Claesson-Welsh, 2012). VEGF-A stimulation caused a 3.7-fold (P<0.05) increase in ERK1/2 phosphorylation in UBA1-depleted endothelial cells compared to controls (Fig. 5A,D). UBA1-depleted cells contained ~40% higher basal VEGFR2 levels. Surprisingly, the kinetics of VEGF-A-stimulated VEGFR2 degradation were not significantly affected by UBA1 depletion (Fig. 5A,E). Thus, UBA1 is not required for VEGF-A-stimulated VEGFR2 degradation.

These data suggest that increased VEGFR2 phosphorylation in UBA1-depleted cells (Fig. 5B) was due to an overall net increase in plasma membrane VEGFR2 levels rather than effects on VEGFR2 activation at the individual receptor level (Fig. 5F). To test whether UBA1 depletion affects other RTK signal transduction events, UBA1-depleted cells were subjected to a time-course of bFGF which is known to activate FGFR1-regulated MAPK signal transduction in endothelial cells. Both control and UBA1-depleted cells exhibited similar responses to bFGF stimulation such as ERK1/2 activation (Fig. 5G). Thus UBA1 regulates signal transduction by VEGFR2 but not FGFR1.

**Basal VEGFR2 turnover regulates VEGF-A-dependent endothelial cell tubulogenesis**

UBA1 is the principal E1 enzyme in human cells and is likely to be involved in many cellular processes (Groen and Gillingwater, 2015). Depletion of UBA1 caused 2.4-fold (P<0.001) decrease in endothelial cell proliferation in the absence of VEGF-A (Fig. 6A). However, remaining viable endothelial cells showed a 2.3-fold (~30%) increase in VEGF-A-stimulated proliferation compared to a 1.7-fold increase in control cells (P<0.01) (Fig. 6B). Signaling transduction...
by VEGF-A-activated VEGFR2 promotes new vascular tube formation by endothelial cells, an essential feature in angiogenesis (Ferrara, 1999). Immunoblotting confirmed that UBA1-specific siRNA transfection was effective at depleting endothelial UBA1 levels for extended periods (Fig. 6C), corresponding to the 7-day duration of the tubulogenesis assay. UBA1-depleted cells exhibited lower tubule length (Fig. 6D) and number of tubule branch points (Fig. 6E) in absolute numbers compared to non-transfected or control siRNA-transfected controls. However, VEGF-A stimulation of UBA1-depleted cells increased tubule length 4.4-fold (Fig. 6F) and branch point number 22.3-fold when compared to UBA1-depleted cells in the absence of VEGF-A (Fig. 6F). These effects were substantially higher than the VEGF-A-stimulated 2.6-fold \((P<0.01)\) increase in tubule length (Fig. 6F) and 4.6-fold \((P<0.001)\) increase in branch point number (Fig. 6G) for control siRNA-transfected controls. These findings show that UBA1 has functional impact on VEGF-A-stimulated endothelial tubulogenesis.

**VEGF-A-stimulated endothelial cell migration is elevated by decreased UBA1 levels**

VEGF-A-dependent signaling also stimulates endothelial cell migration (Fearnley et al., 2014; Smith et al., 2015a,b). To test the role of UBA1, we analyzed control and UBA1-depleted endothelial cells for migration towards VEGF-A (Fig. 7A). Quantification of these images showed that UBA1 depletion caused an overall decrease in non-stimulated and VEGF-A-stimulated endothelial cell migration compared to non-transfected or control siRNA-transfected cells (Fig. 7B). However, comparison of non-stimulated versus VEGF-A-stimulated migration of UBA1-depleted cells showed 14.6-fold increase in VEGF-A-stimulated endothelial cell migration (Fig. 7C). This effect was 3.7-fold \((P<0.001)\) higher than the VEGF-A-stimulated migration exhibited by control siRNA-transfected cells (Fig. 7C). We also tested the VEGF-A-stimulated closure of a wounded endothelial cell monolayer which represents both cell proliferation and migration. There was a significant VEGF-A-stimulated re-occupation of the wounded area by UBA1-depleted cells in comparison to non-transfected and control

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**Fig. 4. UBA1 depletion perturbs VEGFR2 endosome-lysosome dynamics.** (A) Endothelial cells transfected with non-targeting or UBA1 siRNA were processed for immunofluorescence microscopy using antibodies to VEGFR2 (green), EEA1 (red), CD63 (red) or LAMP2 (red) followed by fluorescent species-specific secondary antibodies. Nuclei were stained with DNA-binding dye, DAPI (blue). Scale bar: 70 μm. (B) Quantification of VEGFR2 co-distribution with early endosome (EEA1), late endosome (CD63) and lysosome (LAMP2) markers in endothelial cells transfected with non-targeting control siRNA or UBA1-specific siRNA. Error bars denote mean±s.e.m. \((n≥3)\), with significance denoted as \(*P<0.05;\) analysed using two-way ANOVA.
siRNA-treated cells (Fig. 7D). This experiment showed a ∼31% increase in VEGF-A-stimulated wound closure in UBA1-depleted cells compared to control siRNA-treated cells (Fig. 7E). These data show that loss of UBA1 elevates the endothelial cell response to VEGF-A which is reflected by endothelial cell migration and monolayer wound closure.

Fig. 5. UBA1 depletion causes an increase in VEGF-A-stimulated signal transduction. (A) Endothelial cells transfected with non-targeting control siRNA or UBA1-specific siRNA were stimulated with 25 ng/ml VEGF-A for specific time periods (0-60 min), lysed and immunoblotted for phospho-VEGFR2 (Y1175), phospho-PLCγ1 (Y783), phospho-ERK1/2 (T202/Y204), VEGFR2, ERK1/2, UBA1 and tubulin. Quantification of (B) phospho-VEGFR2, (C) phospho-PLCγ1, (D) phospho-ERK1/2, and (E) VEGFR2 levels in endothelial cells transfected with non-targeting control siRNA or UBA1-specific siRNA and treated with 25 ng/ml VEGF-A for the time periods indicated. Relative levels for each signal were normalized against tubulin. (F) Quantification of phospho-VEGFR2 (Y1175) levels after 5 min VEGF-A stimulation and normalized against total VEGFR2 levels in control or UBA1-depleted endothelial cells. (G) Endothelial cells transfected with non-targeting control siRNA or UBA1-specific siRNA were treated with 25 ng/ml basic FGF (bFGF), lysed and immunoblotted for phospho-ERK1/2 (T202/Y204), VEGFR2, FGFR1, UBA1 and tubulin. In panels B-F, error bars denote mean±s.e.m. (n≥3), with significance denoted as *P<0.05, ***P<0.001; analysed using two-way ANOVA.
DISCUSSION

Our study now provides compelling evidence for a ubiquitin-linked pathway which regulates basal VEGFR2 levels and impacts on VEGF-A-stimulated signal transduction and multiple cellular responses. Our findings support the existence of a mechanism whereby cells adjust the net pool of plasma membrane VEGFR2,
thus controlling RTK-mediated signal transduction and the cellular response to extracellular ligands such as VEGF-A. This ligand-independent regulatory pathway mediates VEGFR2 availability at the plasma membrane for VEGF-A-stimulated signal transduction. The E1 enzyme, UBA1, regulates basal plasma membrane VEGFR2 levels which influence VEGF-A-stimulated activation of PLC\(\gamma\)1 and ERK1/2 signal transduction pathways. A key point is that UBA1 influences the pool of plasma membrane VEGFR2 which in turn dictates net VEGFR2 activation.

VEGFR2 ubiquitination plays key roles in membrane trafficking and degradation but previous work has focused on VEGF-stimulated responses (Bruns et al., 2010; Ewan et al., 2006; Smith et al., 2015a,b). Our study now highlights a mechanism involving UBA1 which controls basal VEGFR2 levels and VEGF-A-stimulated cellular
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E3 ubiquitin protein ligase (β-transducin repeat containing 1 (β-TrCP1)) in VEGF-A-stimulated proteolysis of VEGFR2 (Bruns et al., 2010; Duval et al., 2003; Murdaca et al., 2004; Shaik et al., 2012; Singh et al., 2007). Furthermore, differences in VEGFR1 and VEGFR2 proteolysis under either resting (Mittar et al., 2009) or hypoxic (Ulyatt et al., 2011) conditions suggest that endothelial cells exploit VEGFR availability to fine-tune the cellular response to VEGF-A. Recent studies have also highlighted ligand-independent VEGFR2 de-ubiquitination linked to the de-ubiquitinase USP8 that controls membrane trafficking, recycling and proteolysis (Smith et al., 2015a,b). Interestingly, kinase-independent regulation of RTK function is highlighted by the discovery that constitutive binding of cytosolic adaptors such as growth factor receptor-binding protein 2 (Grb2) to basal FGFR2 regulates ligand-independent activation of downstream signaling pathways (Lin et al., 2012). In addition, ligand-independent ubiquitination and endocytosis of EGFR involves the Hrs endocytic adaptor protein (Katz et al., 2002) that is found on a subset of early endosomes. There is also a new kinase-independent autophagic role for EGFR (Tan et al., 2015). These diverse studies emphasize how ligand-independent control of RTK turnover and function can impact on ligand-stimulated cellular responses.

UBA1 is an essential cellular enzyme expressed by many cells and tissues and is functionally implicated in multiple pathways including DNA replication. Notably, suppression of UBA1 activity in Schwann cells is linked to spinal muscular atrophy (Aghamaleky Sarvestany et al., 2014; Sugaya et al., 2015). Other studies have identified UBA1 as a novel target for the treatment of hematological malignancies (Xu et al., 2010; Yang et al., 2007). UBA1-mediated surveillance of disease-linked responses could thus be utilized for controlling RTK levels and cellular responses in different tissues. The potential for UBA1 in cell proliferation and disease is highlighted in the profiling of certain cancers (e.g. prostate cancer) which show reduced UBA1 expression (www.proteinatlas.org). One mechanism employed by cancerous cells could be down-regulation of UBA1 expression to stimulate tumor angiogenesis. By providing a UBA1-regulated mechanism to control basal VEGFR2 availability which impacts on signal transduction and cellular responses, our study provides a non-canonical pathway that is unique to the established model for ligand-stimulated RTK ubiquitination, trafficking and proteolysis. Our findings provide a new understanding of ubiquitin-linked regulation of VEGF-regulated outcomes and could be of use to new strategies that target angiogenesis in diverse disease states.

**MATERIALS AND METHODS**

**Cell culture and materials**

Primary HUVECs were cultured as previously described (Fearnley et al., 2014; Howell et al., 2004), HDMECs and appropriate growth media were from PromoCell (Heidelberg, Germany). Purified primary and secondary antibodies were typically used at 1 µg/ml for microscopy and at 0.1 µg/ml for immunoblotting. These antibodies were goat anti-VEGFR2 (R&D Systems, Minneapolis, USA), rabbit anti-phospho-VEGFR2 (Y1175), rabbit anti-UBA1 (Cell Signaling Technologies, Danvers, USA), rabbit antibodies to native and phosphorylated PLCγ1 (Y783), rabbit anti-ERK1/2, mouse anti-phospho-ERK1/2 (T202, Y204), mouse anti-α-tubulin (Santa Cruz Biotechnology, USA), mouse anti-transferrin receptor (TIR), mouse anti-β-actin (Abcam, Cambridge, UK), mouse anti-LAMP2 (Santa Cruz, USA), HRP-conjugated secondary antibodies (Thermo Fisher, Loughborough, UK) and Alexa Fluor-conjugated secondary antibodies (Thermo Fisher). Endothelial cell growth medium (PromoCell), non-targeting and UBA1 siRNA duplexes (GE Dharmacon, USA) and recombinant human VEGF-A165 (Genentech Inc., San Francisco, USA) were obtained as stated. Chemicals were obtained from Sigma-Aldrich (Poole, UK) or Thermofisher (Loughborough, UK).

**Immunoblotting and immunofluorescence microscopy**

Endothelial cells were serum starved in MCDB131 (Thermo Fisher) for 2 h prior to treatment with 25 ng/ml VEGF-A165 (0-60 min), 20 µg/ml CHX (0-80 min) or 10 µM PYR41 (1 h) and lysed for immunoblotting or immunoprecipitation studies. Cells were lysed in 2% (w/v) SDS and run on a 10% SDS-PAGE gel at 120 V for 90 min. Proteins were transferred onto nitrocellulose membrane at 300 mA for 3 h and incubated in primary antibodies overnight prior to incubation in HRP-conjugated secondary antibodies for 1 h and detection using enhanced chemiluminescence. Immunoblots were quantified, normalized against tubulin and made relative to the control (i.e. the control siRNA 0 min condition) for representation on graphs. For immunofluorescence analysis, HUVEC's were seeded in 96-well plates or on cover-slips before fixation, permeabilization in 0.1% (w/v) Triton X-100, incubation with primary antibodies and visualization by incubation in Alexa Fluor488- or 594-conjugated secondary antibodies and DAPI. Images were acquired using an EVOS-fl inverted digital microscope (Thermo Fisher) at 20× magnification or a wide-field deconvolution microscope DeltaVision (Applied Precision Inc., Issaquah, USA) at 60× magnification at room temperature. Fluorescence intensity (integrated density) and colocalization (co-localization threshold plugin) were quantified using NIH ImageJ (https://imagej.nih.gov/ij/download.html).

**Immunoprecipitation analysis**

HUVECs were serum starved for 2 h prior to CHX treatment or VEGF-A stimulation, lysed in buffer [150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 0.1% (w/v) SDS, 0.5% (w/v) sodium deoxycholate, 2 mM EDTA, 1% (v/v) NP-40, 50 mM NaF, 1 mM PMSF, 10 mM iodoacetamide], incubated with 1 µg/ml goat anti-VEGFR2 for 2 h and immuno-isolated with protein G-agarose beads before SDS-PAGE and immunoblot analysis.

**Plasma membrane protein recycling assay**

HUVECs were incubated in primary antibody to VEGFR2 or FGFR1 for 30 min at 37°C. Cell surface-bound primary antibody was stripped by washing in acidic MCDB131 medium (pH 2.0) at 4°C. Cells were incubated in secondary antibody (anti-sheep Alexa Fluor488) for 30 min at 37°C and fixed for 5 min at 37°C before incubation with 1 µg/ml DAPI to visualize nuclear DNA. Only cell surface VEGFR2 that had bound primary antibody and undergone internalization and subsequent recycling would be available to bind secondary antibody after acid-washing. Thus, only VEGFR2 that recycled one or more times was visualized. Images were acquired using an EVOS-fl inverted digital microscope at 20× magnification at room temperature. Fluorescence intensity was quantified using NIH ImageJ (https://imagej.nih.gov/ij/download.html).
**Cell surface biotinylation**

HUVECs were serum starved for 2 h prior to CHX treatment, washed in ice-cold PBS, cell surface biotinylation by incubation with 0.25 mg/ml biotin in buffer (2 mM CaCl₂, 2 mM MgCl₂, PBS) for 45 min, washed in TBS to quench biotinylation and lysed in NP-40 lysis buffer [1% (v/v) NP-40, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM PMSF]. Cell surface proteins were isolated using NeutraAvidin agarose beads before SDS-PAGE and immunoblotting.

**Protein depletion using RNAi**

Endothelial cells were reverse transfected in 6- or 96-well plates with 4 pooled siRNA duplexes (SMARTpool siRNA, GE Dharmacon) as follows. 20 nM non-targeting control siRNA: 5'-UUGGUUUAACGUAUAGCAU-3'; 5'-UUGGUUUAACGUAUAGCAU-3'; 5'-UUGGUUUAACGUAUAGCAU-3'; 5'-UUGGUUUAACGUAUAGCAU-3'; 20 nM UBA1 siRNA: 5'-CCUUAUACUUUAGCAGUU-3'; 5'-CCUUAUACUUUAGCAGUU-3'; 5'-GCAGAAUAAUCUGUAGCU-3'. All siRNA duplexes were used according to the manufacturer’s instructions (GE Dharmacon). Endothelial cells were incubated for 6 h with siRNA duplexes using a previously described lipid-based transfection protocol (Fearnley et al., 2014). After 72 h, cells were processed for lysis and immunoblotting as previously described.

**Cell migration and proliferation assays**

For the cell migration assay, 48 h after transfection with control or UBA1 siRNA, HUVECs were seeded in starvation media (MCD131) at 3x10⁴ cells per well in an 8 µm pore size Transwell filter inserted into a 24-well companion plate (BD Biosciences, Oxford, UK). MCD131 containing 25 ng/ml VEGF-A was added to the lower chambers to set up a chemotactic gradient for cells to migrate towards. Cells were incubated for 24 h before being fixed and stained with 0.2% (w/v) crystal violet in 20% (v/v) methanol. Non-migrated cells were removed from the upper chamber. 3-5 random fields were imaged per Transwell filter.

For the cell proliferation assay, 48 h after transfection with control or UBA1 siRNA, HUVECs were seeded at 2x10⁵ cells per well in 96-well plates in complete growth media. After 24 h cells were serum starved in MCD131 for 2 h prior to stimulation with 25 ng/ml VEGF-A for 24 h. At the 20 h time point, 10 µM bromodeoxyuridine (BrdU) was added and a cell proliferation ELISA performed according to manufacturer’s instructions (Roche Diagnostics, Burgess Hill, UK). Color change was developed using 3,3′,5,5′-tetramethylbenzidine solution and the reaction quenched with 1 M H₂SO₄. Absorbance was measured at 450 nm using a variable wavelength 96-well Tecan Sunrise plate reader (Mannedorf, Switzerland).

**Endothelial tubulogenesis and monolayer wound assays**

For the tubulogenesis assay, HUVECs transfected with siRNA were seeded onto a bed of confluent primary human fibroblasts and stimulated with 25 ng/ml VEGF-A for 24 h. At the 20 h time point, 10 µM bromodeoxyuridine (BrdU) was added and a cell proliferation ELISA performed according to manufacturer’s instructions (Roche Diagnostics, Burgess Hill, UK). Color change was developed using 3,3′,5,5′-tetramethylbenzidine solution and the reaction quenched with 1 M H₂SO₄. Absorbance was measured at 450 nm using a variable wavelength 96-well Tecan Sunrise plate reader (Mannedorf, Switzerland).

For the monolayer wound assay, endothelial cells were transfected with control or UBA1 siRNA and grown to a confluent monolayer in ECGM for 48 h. The cell monolayer was scratched using a 1 ml blue plastic pipette tip at the 0 h time point and stimulated with 25 ng/ml VEGF-A. After 24 h, wound closure was captured using an Evos-Fl inverted digital microscope and scratch width was quantified using ImageJ.

**Statistical analysis**

This was performed using a one-way analysis of variance (ANOVA) and Tukey’s post-test analysis for multiple comparisons or two-way ANOVA followed by the Bonferroni multiple comparison test using GraphPad Prism software (La Jolla, USA). Significant differences between control and test groups were evaluated at *P*<0.05, **P**<0.01, ***P***<0.001 and ****P***<0.0001 indicated on the graphs. Error bars in graphs denote mean±s.e.m. of results from at least three independent experiments.

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