Supplementary information, Data S1 EXPERIMENTAL PROCEDURES

Cell Culture and Transfection: 293T, DLD1, DLD1 FBW7-/-, and human umbilical vascular endothelial cells (HUVECs) were cultured as described [1,2]. Transfections were performed using the calcium phosphate-DNA co-precipitation method for HEK293T cells and Lipofectamine 2000 for HUVECs following the manufacturer's protocol. CGR8 embryonic stem cells (ESCs) were grown under feeder-free conditions as previously described [3]. For the generation of TTRE-shFbw7 and TTRE-shKlf2 stable cell lines, CGR8 ES cells were transfected with the indicated plasmids using Lipofectamine 2000 (Invitrogen). Transfected cells were selected with 1 μg/ml puromycin (Sigma) for 10 days. Colonies were selected and analyzed using Western blotting and qRT-PCR to monitor the knockdown efficiency.

Antibodies and Reagents: The anti-KLF2 monoclonal antibody was generously provided by Dr. Takashi Minami (The University of Tokyo). The rabbit polyclonal anti-phospho-KLF2 (T243) antibody was generated using a KLH conjugated peptide (CPPATRGLLpTPPAS) (Abmax Biotechnology). The rabbit polyclonal anti-KLF2 antibody was generated using purified GST-KLF2 recombinant protein. Anti-Flag, anti-HA, and secondary antibodies were purchased from Sigma (St. Louis, MO). The anti-GFP antibody was obtained from Santa Cruz Biotechnology, Inc. The anti-FBW7 antibody was obtained from Abcam (Ab12292) and Bethyl (A301-721). Cycloheximide, 4, 6-diamidino-2-phenylindole (DAPI), and MG132 were purchased from Sigma. The kinase inhibitors were obtained from the Beyotime Institute of Biotechnology.

Plasmids and virus: The HA-ubiquitin, GSK3, FBW2, 5, 7, 8, β-TrCP1 expression plasmids and their mutants were described previously [2]. Mouse KLF2 and its mutants were cloned into pCDNA3.1 vector with Flag, HA, or GFP tags at the N terminus using standard cloning methods. GST-KLF2 CPD and GST-FBW7 WD40 were cloned into pGEX-4T-1. Zebrafish Fbw7s and their probes were cloned into PCS2+. Dr. Feng Liu provided zebrafish Klf2a plasmid [4]. Human FBW7, KLF2, and their mutants were cloned into pLVX-Flag-IRES-ZsGreen1 lentiviral vector. To generate the shRNA-resistant mutant of FBW7, Lenti-FBW7γ containing synonymous mutations of T520C and G523A was made using KOD plus (Takara) according the manufacturer's instruction. The primers for generating the construct: Sense: 5'-CTAAAGAGTTGGCACTCTACGTACTTTCATTC-3'; Antisense: 5'-GGTTCCAGGAATGAAAGTACGTAGAGTGCC-3'. All the vectors were confirmed using DNA sequencing.

RT-PCR： Total mRNA was isolated using TRIzol (Invitrogen) and 1 μg RNA was used to synthesize cDNA using the Prime ScriptTM RT reagent kit (Takara, DRR037A) according to the manufacturer's protocol. The primers for detecting FBW7: 5'-GATACTAACTGGAGGCGAG-3'; 5'-GATGACCATACTCCACCTG-3'. The PCR was performed using a standard protocol. FBW7α：Sense: 5'-GGAGATGGACCAGGAGAGTG-3'; Antisense: 5'-GTTGGTGTTGCTGAACATGG-3'; FBW7β：Sense: 5'-TTGTCAGAGACTGCCA-3'; Antisense: 5'-GTTGGTGTTGCTGAACATGG-3'; FBW7γ: Sense: 5'-ATGGCTTGGTTCCTGTTGAT-3'; Antisense: 5'-GTTGGTGTTGCTGAACATGG-3'.

Immunoprecipitation and Western Blotting: Cells were transfected, treated with 10 μM MG132 for 6 hours, and lysed in lysis buffer (50 mM Tris.HCl (pH7.4), 150 mM NaCl, 10% glycerol, 1 mM EDTA, 1% Trixon X-100 and a cocktail of proteinase inhibitors). For immunoprecipitation (IP), the cell lysates were cleared using centrifugation and incubated with 1 μg anti-HA or anti-Flag antibody and 16 μl protein A/G beads for 2-3 hours. The beads were boiled after extensive washing; the proteins were resolved using SDS-PAGE gel electrophoreses; and the proteins were transferred onto nitrocellulose membranes (Millipore) followed by Western blotting. The proteins were detected using the Odyssey system (LI-COR Biosciences-Biotechnology), and the quantifications were performed using the Image J software.

Ubiquitination Assay: An in vivo ubiquitination assay was performed as described in previous study [5]. Briefly, transfected 293T cells were treated with 10 μM MG132 for 5 hours. 36 hours post transfection, cells were lysed using denaturing buffer (6 M guanidine-HCl，0.1 M Na_2HPO_4/NaH_2PO_4, 10 mM imidazole, pH8.0). The lysates were sonicated and subjected to immunoprecipitation with Ni-NTA-agarose for 3 hours. Then the agarose were washed with the denaturing buffer (25 mMTris.HCl, 20 mM imidazolel, pH6.8), and subjected to Western blotting analysis. Polyubiquitinated KLF2 was detected using an anti-HA antibody. The in vitro ubiquitination was performed as previously described [6]. To detect the in vitro ubiquitination of KLF2, Flag-KLF2 was expressed in HEK293T cells, and purified using 1 μg anti-Flag antibody and 16 μl of protein A/G beads at 4°C. After extensive washing, the KLF2 proteins were eluted using Flag peptide (100 μg/ml, Sigma). The $SCF^{FBW7\alpha}$ E3

complex (5xHA-FBW7α, HA-CUL1, HA-SKP1, and HA-RBX1) were purified from HEK293T cells using an immunoprecipitation assay. The in vitro ubiquitination reactions were performed with purified E1, E2 (UbcH5c), ubiquitin (Boston Biochem), KLF2, E3 ($SCF^{FBW7\alpha}$), and 2 mM ATP in a final volume of 30 µl at 30°C for 2 hours. The reactions were terminated by adding 180 µl of denaturing lysis buffer (0.1% Triton X-100, 50 mMTris.HCl, pH7.5, 150 mM NaCl, 1 mM EDTA containing 1% SDS and 1% deoxycholate) and heating for 20 min at 70 °C, the samples were transferred to a fresh tube and diluted 10 times with the lysis buffer without SDS and deoxycholate. Then the KLF2 proteins were immunoprecipitated using an anti-Flag antibody and protein A/G beads for 2 hours at 4 °C. The beads were washed four times with the lysis buffer without detergent and then boiled in 30 µl 2XLaemmli SDS loading buffer. The ubiquitinated KLF2 proteins were detected using Western blotting with an anti-Ubiquitin antibody.

In vitro phosphorylation assay: Wild-type and mutant His-KLF2 fragments (residues 110-267) were purified from *E.coli* using a standard protocol. The kinase assay was performed at 30°C for 1 hour in kinase buffer containing 200 µM ATP with 5 µCi $\gamma[^{32}P]$-ATP. The reactions were terminated upon the addition of 8 µl 4X Laemmli SDS sample buffer. The phosphorylation of KLF2 was detected using the phospho-KLF2 antibody or autoradiography.

GST-Pull down assay: HEK293T cells transiently expressing Flag-KLF2 were lysed and cleared using centrifugation. The KLF2 cell lysates were incubated with 10 µg of purified GST or GST-WD40 protein. The GST proteins were purified using glutathione

sepharose 4B (Amersham Biosciences), and the bound KLF2 was detected using Western blotting.

Biotinylated Peptides-Pull down assay: The biotinylated peptides were synthesized by GL Biochem (Shanghai) Ltd and dissolved in PBS solution. 2 μg of peptides were preloaded onto 10 μl of Neutravidin agarose (Thermo). 293T cells were transfected with Flag-FBW7. 24 hours post transfection, cells were collected, lysed, and the lysates were incubated with the peptide-preloaded agarose for 3 hour at 4°C in binding buffer (20 mM HEPES (pH7.9), 150 mM KCl, 1 mM DTT, 1 mM PMSF, 10% glycerol, 0.1% NP-40 and proteinase inhibitors). After five washes with washing buffer (20 mM HEPES (pH7.9), 150 mM KCl, 1 mM DTT, 1 mM PMSF, 0.1% NP-40, and proteinase inhibitors), the bound proteins were eluted by boiling in 2XLaemmli SDS loading buffer, resolved by SDS-PAGE, transferred onto nitrocellulose membranes (Millipore), followed by Western blotting analysis.

siRNA Knockdown: HUVECs were transfected with siRNA oligonucleotides using Lipofectamine. Two FBW7 siRNA were used: siRNA1: 5'-ACCUUCUCUGGAGAGAGAAAUGCTT-3' and siRNA2: 5'-GUGUGGAAUGCAGAGACUGGAGATT-3', which have been described [2]. FBW7α siRNA: 5'-GTGAAGTTGTTGGAGTAGA-3'; FBW7β siRNA: 5'-TATGGGTTTCTACGGCACA-3'; FBW7γ siRNA: 5'-CTACTCTAAACCATGGCTT-3'; and KLF2 siRNA: 5'-UGCUGGAGGCCAAGCCAAAUU-3'. To knockdown the expression of FBW7 and KLF2 using shRNA: double stranded DNAs for human and mouse shFBW7:

5’-GGCACTCTATGTGCTTTCATTC-3’ and mouse shKLF2: 5’-CGCGGACAGCCTGGGAACAGAG-3’ were cloned into pLVX-ZsGreen1 Lentiviral vector. The Lentivirus-mediated delivery of shRNA was performed using a standard protocol.

Mouse aortic ring assay: A mouse aortic ring assay was performed as described in previous study [7]. In brief, aortas were harvested from 6-week-old C57BL/6 wild-type (WT) mice cleaned of periadventitial fat and connective tissues in cold serum-free Minimum Essential Medium (MEM, Life Technologies Ltd., Paisley, Scotland). After extensively washed in MEM, aortas were cut into rings of 1~1.5 mm in circumference. Afterwards, the aortic segments were incubated in a 6-well plate with supernatant of lentivirus ($1x10^9$ pfu) of empty vector or shFbw7 as described previously [8]. 36 hours post infection, the rings were placed in a Matrigel-coated plate and sealed with a 100 µl of Matrigel. The Matrigel containing the aortic rings were kept in triplicate at 37°C in 48-well plates. Each dish contained 500 µl of serum-free ECGM. The cultures were kept in a 95%: 5% (v/v) mixture of air and CO2 for 6 days and the fresh medium was exchanged for every 2 days. After 6 days, microvessel sprouting was fixed and photographed using an inverted microscope (Olympus, Center Valley, PA; magnification ×100). The assay was scored from 0 (least positive) to 5 (most positive) in a double-blind manner. Each data point was assayed 6 times.

In vitro wound healing assay: Confluent HUVEC monolayers grown in 24-well plates precoated with 5 µg/ml collagen were treated with ECGM containing mitomycin (10 µg/ml) for 3 hours to inactivate cell proliferation. The cells were wounded with 200

μl pipette tips and washed with phosphate-buffered saline (PBS) to remove the debris. Complete culture media was subsequently added to allow wound healing. Images of the cells were captured after 8–10 hours of incubation at 37°C in a 95%: 5% (v/v) mixture of air and CO_2. The migrated cells were counted manually, and the percentage of inhibition was expressed using untreated wells at 100%. Three independent experiments were performed.

Endothelial cell Transwell migration assay: The chemotactic motility of the HUVECs was assayed using Transwell chambers (Millipore). In brief, the lower surface of the filter was coated with collagen. The bottom of the chambers was filled with 500 μl of ECGM containing 0.5% FBS supplemented with 50 ng/ml VEGF. The inactivated HUVECs were loaded into the upper wells and allowed to migrate for 4 hours. The migrated cells were fixed and stained with 1% crystal violet. Images were captured using an inverted microscope (Olympus), and the migrated cells were counted manually. The percentage of migrated cells infected with empty vector was expressed on the basis of the untreated control wells.

Endothelial cell capillary-like tube formation assay: Growth factor–reduced Matrigel (BD Biosciences) was pipetted into prechilled 96-well plates and polymerized at 37°C. The HUVECs were collected and placed onto the Matrigel layer. After 8 to 10 hours, the endothelial cells were photographed using an inverted microscope. Three independent experiments were performed. The tube network was quantified using Image J (National Institutes of Health).

Measurement of Endothelial Leakage: A commercially available kit (Millipore, Temecula, Calif) was used to measure the EC monolayer leakage. Briefly, ECs were seeded in a Transwell insert coated with collagen. The inserts were placed into 24-well plates overnight. To measure agonist-induced EC leakage, 200 μl FITC-dextran (50 μg/ml) was added into the insert and incubated for 2 hours. The insert was then removed and 50 μl medium was collected from the bottom chamber. The fluorescence density of the samples was analyzed on a microplate fluorometer. In leakage assays involving siRNA transfection, the HUVECs transfected with non-specific siRNA were used as controls.

Monocyte Adhesion Assay: Transfected HUVECs were cultured and starved in EGCM containing 1% FBS for 2 hours. The cells were prestimulated with vehicle or 50 ng/ml VEGF for 4 hours. U937 cells labeled with CFSE were added to the confluent HUVEC monolayers and incubated for 90 min. The cells were then washed extensively and fixed with 4% formaldehyde. Phase contrast and CFSE fluorescent images were captured using a fluorescence microscope. The adherent U937 cells were quantified by measuring fluorescent intensity at five randomly selected fields in each well using Image J.

In Vitro Flow Assays: HUVECs transfected with siFBW7 or control siRNA were cultured on the collagen-coated coverslips. HUVECs were placed on a parallel-plate flow chamber and subjected to a shear stress of 15 dyn/cm^2 for 16 hours. Then the cells were collected for Western blotting analysis.

Proliferation assay and cell cycle analysis

Cell proliferation was measured using a MTT assay and EdU labeling. HUVECs were seeded in a 96-well plate. Cells were incubated for 72 hours and then the MTT solution was added for 4 hours. The reaction was stopped by addition of DMSO solution for 20 mins, and the samples were measured at 490 nm. EdU labeling were performed using the EdU Apollo®567 DNA Kit (Cat.#C10031 Guangzhou Ribobio) according to the instruction. Apoptosis analysis was performed using the flow cytometry (FACSCalibur; BD Biosciences). HUVECs were harvested, washed with PBS, and suspended in DNA staining buffer (50 mg/ml propidium iodide, 100 mg/ml RNase A, 0.2% Triton X-100).

Teratoma formation and Embryoid body (EB) differentiation: For teratoma generation, 10^6 embryonic stem cells (ESCs) were harvested and injected subcutaneously into nude mice. Five-week-old male BALB/cA nude mice (National Rodent Laboratory Animal Resources, Shanghai, China) weighing about 20 g each were randomly divided into each group of 5. The body weight of each mouse was recorded every 7 days. At the same time, solid tumor volume was determined using Vernier caliper measurements and the formula $A \times B^2 \times 0.52$, where A is the longest diameter of the tumor and B is the shortest diameter of the tumor. Approximately 5 weeks later, teratomas were harvested and processed with HE staining and CD31 staining. For the generation of EBs, the ESCs were trypsinized and cultured in petri dishes. The EBs were collected at the indicated time. For the isolation of Vegfr2-positive cells, the EBs were stained and sorted using an anti–murine Vegfr2-PE antibody by FACS. IOD (Integrated Optical Density): Mice were sacrificed

and tumors were removed, fixed with formaldehyde and embedded in paraffin. Specific blood vessel staining was performed with an anti-CD31 antibody according to the standard IHC protocol. Images were recorded using a Leica DM 4000B photomicroscope. Using Image-Pro Plus 6.0 software, we analyzed the mean integrated optical density (mean IOD) of blood vessels in tumor sections according to the following formula: mean IOD = IOD/area of the tumor section.

Purification of kdrl:GFP positive cells from zebrafish: Fbw7(605) MO-injected or uninjected kdrl:GFP transgenic embryos at 30 hours post fertilization (hpf) were dissected in DPBS (Invitrogen) after dechorionation and deyolking [PMID:16412219] , and digested with 1Xtrypsin/EDTA (Life Technologies) for 30 min at 28.5 °C. Single cell suspension was obtained by centrifugation at 400 g for 5 min, washed twice with 0.9XDPBS/5% FBS, and passed through a 40 μm nylon mesh filter. Fluorescence-activated cell sorting was performed with FACSAria (BD Biosciences).

Reference

1 Bhattacharya R, Senbanerjee S, Lin Z *et al.* Inhibition of vascular permeability factor/vascular endothelial growth factor-mediated angiogenesis by the Kruppel-like factor KLF2. *J Biol Chem* 2005; **280**:28848-28851.
2 Liu N, Li H, Li S *et al.* The Fbw7/human CDC4 tumor suppressor targets proproliferative factor KLF5 for ubiquitination and degradation through multiple phosphodegron motifs. *J Biol Chem* 2010; **285**:18858-18867.
3 Li L, Sun L, Gao F *et al.* Stk40 links the pluripotency factor Oct4 to the Erk/MAPK pathway and controls extraembryonic endoderm differentiation. *Proc Natl Acad Sci U S A* 2010; **107**:1402-1407.
4 Wang L, Zhang P, Wei Y, Gao Y, Patient R, Liu F. A blood flow-dependent klf2a-NO signaling cascade is required for stabilization of hematopoietic stem cell programming in zebrafish embryos. *Blood* 2011; **118**:4102-4110.
5 Bloom J, Pagano M. Experimental tests to definitively determine ubiquitylation of a substrate. *Methods Enzymol* 2005; **399**:249-266.
6 Wang HR, Ogunjimi AA, Zhang Y, Ozdamar B, Bose R, Wrana JL. Degradation of RhoA by Smurf1 ubiquitin ligase. *Methods Enzymol* 2006; **406**:437-447.

7 Masson VV, Devy L, Grignet-Debrus C *et al.* Mouse Aortic Ring Assay: A New Approach of the Molecular Genetics of Angiogenesis. *Biological Proced Online* 2002; **4**:24-31.
8 Abele-Ohl S, Leis M, Wollin M *et al.* Human cytomegalovirus infection leads to elevated levels of transplant arteriosclerosis in a humanized mouse aortic xenograft model. *Am J Transplant* 2012; **12**:1720-1729.