Materials and Methods

Cell culture and shear experiments. Human umbilical vein ECs within passages 4-7 were used in the current study. An *in vitro* circulating flow chamber system was used to impose fluid shear stress to the cultured ECs [1]. The ECs cultured on collagen I coated slides were exposed to shear stress with PS (12 ± 4 dyn/cm^2, 1 Hz) or OS (0.5 ± 4 dyn/cm^2, 1 Hz), or kept as static control (ST).

Antibodies and reagents. Antibodies against CCNA, CCND1, CCNH, CCNE, CD45, CDK1, CDK2, CDK4, CDK7, p27, KLF2, vWF were purchased from Santa Cruz Biotechnology; antibodies against phospho-Pol II CTD (Ser5, H14; Ser2, H5) and Pol II (8WG16) were purchased from Covance; antibodies against Ki67 and pan-AGOs were purchased from Millipore; Antibody against β-actin was obtained from Sigma; fluorescence-labeled secondary antibodies and Digoxigenin (DIG) antibody were purchased from Jackson ImmunoResearch. KLF2 siRNA, Pre-miR-23b (PM23b) and Anti-miR (AM23b) of miR-23b and control oligonucleotides (siCtrl, PM-Ctrl, and AM-Ctrl) were purchased from Ambion. 3'-Digoxigenin (DIG) labeled miR-23b locked nucleic acid (LNA) detection probe was from Exiqon. The plasmid expressing HA-tagged human CCNH was obtained from Addgene. The plasmid expressing CCNH shRNA was purchased from ABM Inc. Adenoviruses carrying KLF2 and GFP cDNA were kindly provided by Dr. Mukesh Jain at Case Western Reserve University GST-CTD peptide was obtained from Jena Bioscience. The primer sets listed in Online Table I were synthesized by ValueGene, and the TaqMan primer sets for primary and mature miR-23b, RNU48, and U6 snRNA were purchased from Ambion.

Transfection. The oligonucleotides (siKLF2, AM23b, PM23b or the respective control molecule) were reversely transfected with siPort^{TM} NeoFx^{TM} (Ambion) reagent into ECs at the final concentration of 25-50 nM according to the manufacturer's protocol. The efficiencies of siKLF2, AM23b and PM23b transfections were examined with quantitative real-time PCR and shown in Online Figures XB and XIII, respectively. The plasmids expressing CCNH and HA-CCNH, pcDNA3, and the luciferase reporter constructs (10 μg DNA/10-cm dish) were transfected into ECs by using AMAXA Nucleofector^{TM} (Lonza). Overexpression of KLF2 in ECs was achieved by adenoviral transduction (Adv-KLF2) at M.O.I. of 100. Forty-eight hours post-transfection, ECs seeded on slides were subjected to shear experiment.

Quantitative real-time PCR (qRT-PCR). Total RNA was isolated using TRIzol® according to a manufacturer-suggested protocol (Invitrogen). The concentration and quality of RNA were determined by Nanodrop spectrophotometer (Thermo). Reverse transcription of mRNA was carried out at 42°C for 1 h, with 1 μg of total RNA using the MLV reverse transcriptase and oligo-dT primer (Invitrogen). The synthesized cDNA was used to perform qRT-PCR with the iQ SYBR Green supermix on the iCycler real-time PCR detection system (Bio-Rad). Primer sets used for qRT-PCR are listed in Online Table I. Quantification of miRNA was carried out with TaqMan miRNA assay (Ambion) on the iCycler according to the manufacturer's protocol.

Western blot. ECs or tissue samples were lysed in the ice-cold lysis buffer: 25 mM HEPES, pH 7.4, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 125 mM NaCl, 5 mM EDTA, 50 mM NaF containing 1 mM Na_3VO_4, 1 mM PMSF, 10 μg/ml leupeptin, and 2 mM glycerophosphate. Equal amounts of protein were loaded onto SDS-PAGE. After electrophoresis, the proteins were transferred to nitrocellulose membranes, blocked with 5% BSA-containing PBS, and incubated with the primary antibodies. The bound primary antibodies were detected using appropriate

secondary antibodies coupled to horseradish peroxidase (Santa Cruz Biotech) and the ECL detection system (Amersham).

Kinase assay. One hundred μg of EC lysate was incubated with 2 μg of CCNH antibody overnight at 4°C. Protein A/G-agarose was then added for 1 h. The immunoprecipitated complexes were wash three times in lysis buffer and once in kinase buffer, and then assayed for CAK kinase activity toward the substrate GST-CTD with ATP in kinase buffer. After 1h reaction at 30°C, the kinase assay samples were subjected to Western blot analysis.

Reporter construction and luciferase assay. To examine whether miR-23b transcription is through KLF2, the putative promoter region of miR-23b (-600 to +100) was amplified by PCR from human genomic DNA. The PCR product was isolated and cloned into pGL2 Basic vector (Promega). Mutation was introduced into KLF2 binding site using Q5®Site-Directed Mutagenesis Kit (New England Biolabs). ECs were transfected with each reporter construct and then transduced with Adv-KLF2 or Adv-Ctrl. To test whether CCNH is a direct target of miR-23b, the wild-type and mutated 3'UTR sequences of CCNH mRNA were synthesized and cloned into the pMir-Report luciferase vector (Ambion). For luciferase assay, ECs were transfected with CCNH 3'UTR-wt or CCNH 3'UTR-mt together with PM23b or PM-Ctrl. The plasmid expressing either β-galactosidase or renilla luciferase was co-transfected as a normalizer. Seventy-two h post-transfection, cell lysates were collected, and luciferase activity of each sample was measured with GloMax 96 Microplate Luminometer (Promega). The luciferase activities were normalized to the activities of β-gal/renilla luciferase and the respective control.

Bromodeoxyuridine incorporation assay. ECs were pulse-labeled with 10 mM Bromodeoxyuridine (BrdU) during the last 4 h of shearing and static incubation. After labeling, the ECs were assayed using the FITC BrdU Flow kit (BD Bioscience) according to the manufacturer's manual. The cells were then subjected to flow cytometirc analysis with FACScan (BD Bioscience).

Chromatin immuno-precipitation (ChIP) and RNA immune-precipitation. Chromatin was harvested from ~$1.5x10^6$ of ECs after shearing or being kept under static condition, as well as untreated controls. The cells were crosslinked with 1% formaldehyde for 10 min at room temperature with rocking. The crosslinking procedure was quenched with the addition of 0.125 M glycine for 10 min at room temperature followed by four washes with ice-cold PBS. The cell pellets were collected, and the nuclear fractions were separated from cytoplasmic proteins with NE-PER kit (Thermo). The nuclear fractions were subjected to 10-sec sonication pulses on ice on a 60 Sonic Dismembranator (Fisher Scientific). Optimal sheared chromatin fragment sizes were targeted to approximately 250-300 bp. Aliquot chromatin was incubated overnight with Pol II phospho-serine 5 antibody (H14), Pol II antibody (8WG16), or isotype control, and then pulled down with anti-IgM IgG-conjugated protein A/G agarose beads, pre-blocked with salmon sperm DNA. The cross-linking of immune-precipitated complex was reversed overnight, and proteins were digested with proteinase K treatment. Input DNA and DNA associated with specific immunoprecipitates or with negative control were purified (QIAquick PCR Purification Kit, Qiagen, Valencia, CA), and 2 μl were used as a template for qRT-PCR to amplify transcription start sites (TSS) of CDK1, CDK2, and CDK4 with primer sets listed in Online Table I. For RNA immune-precipitation (RIP), ECs subjected to different flow patterns were lysed in polysome lysis buffer (100 mM KCl, 5 mM MgCl2, 10 mM HEPES pH7.0, 0.5% NP40, 1mM DTT) supplemented with RNaseOut and protease inhibitor cocktail. After centrifugation, supernatant was collected and subjected to immuno-precipitation as previously described with anti-pan Ago (clone 2A8, Millipore) against AGO family proteins in NT2 buffer (50 mM Tris-HCl, pH7.4, 150 mM NaCl, 1mM MgCl2, 0.05% IGEPAL) at 4°C overnight. The pull-down mRNAs and miRNAs

in miRISC complexes (immunoprecipitated pellet) were recovered with mirVana miRNA isolation kit (Ambion) and subjected to qRT-PCR analysis.

Animal model. The animal experiments were performed in accordance with NIH guidelines and approved by the Animal Care Committee of UC San Diego. Adult male Sprague-Dawley rats weighing 300-350 g were subjected to partial carotid ligation as described [2, 3]. Sprague-Dawley rats (300-350g) purchased from Harlan Laboratories were used in this study. The animals were anesthetized with intraperitoneal ketamine (100 mg/kg body weight) and xylazine (10 mg/kg body weight). The general health conditions of animals (including body temperature, pulse rate, respiratory rate, mucous membrane color, shivering, jawtone, and gag) were monitored. The rat was laid supine, and its ventral side of the neck was de-haired and disinfected with Betadine. A ventral mid-line incision (~2 cm) was made in the neck, and the carotid bifurcation was exposed by blunt dissection. The partial carotid ligation was performed by ligating three branches (left external artery, left internal artery, and occipital artery) of the left carotid artery (LCA) with 6-0 silk suture, while the superior thyroid artery was left intact. 12 rats were subjected to partial carotid ligation (PL), and another 12 rats received a sham surgery (sham). The wound was closed and the rat was returned to the cage for recovery. Post-operative rats were monitored until sacrificed. Buprenophine was given at 0.05 mg/kg to each rat every 12 h to prevent post-operation pain.
To locally deliver the PM23b or control oligonucleotide (PM-Ctrl) into the partially ligated (PL) carotid artery, we applied an established local oligonucleotide delivery method using F-127 pluronic gel. Briefly, immediately after partial ligation of the left carotid artery, transfection solutions (6 μl TurboFect *in vivo* transfection reagent in 40 μl 5% glucose solution) was mixed with PM23b or PM-Ctrl (1 nmol). Then, the transfection solutions were mixed with equal volume 30% pluronic F-127 gel in DEPC-treated phosphate buffer saline (PBS) at 4 °C and applied locally to the adventitia around the segments of partially-ligated LCA. In this part of study, partial carotid ligation was performed on 24 rats. 12 of those rats received the transfection mixture with PM23b (PM23b/PL), and the rest of those received PM-Ctrl (PM-Ctrl/PL).

Ultrasound study. Doppler imaging was performed on the rat LCA at the Shared Animal Imagine Resource in UCSD Moores Cancer Center to visualize the flow alternations at/around the surgery sites. Two days post-operation, the animal was anesthetized and placed in either supine or lateral recumbent position, and the neck was shaved to facilitate the ultrasound Doppler measurement. The ultrasound measurement was taken using the VisualSonics Vevo 770 ultrasound imaging system. Color Doppler mode was used to identify the location of LCA, and Pulse Wave (PW) Doppler was used for measuring blood flow velocity in LCA.

Vessel Preparation. At the end of each experiment, the rat was euthanized with an overdose of Ketamine-Xylazine cocktail and perfused with DEPC-treated phosphate-buffered saline (PBS) at a pressure of 120-140 mm Hg. For intima RNA extraction, LCA was carefully dissected from surrounding tissues. Five hundred microliter of TRIzol was injected intraluminally into proximal end of LCA and then collected from the other end. The mRNA levels of vWF and SMA indicating the purity of intima RNA were determined by qRT-PCR (Online Figure XIV). For intima protein extraction, the segment of LCA was cut longitudinally through the vessel wall. Intima cells were scraped off in PBS, centrifuged, and lysed in ice-cold lysis buffer. For immunohistochemistry, the vessels were perfusion-fixed with 4% paraformaldehyde (PFA) in PBS at a pressure of 120-140 mm Hg. The LCA was dissected out, and adventitia was carefully removed. The artery was further fixed by immersion in 1 ml of 4% PFA solution for 16 h before being subjected to frozen-section.

Frozen section and Immunohistochemistry. The PFA-fixed carotid artery was embedded with Tissue-Tek OCT compound in a tissue base mold and slowly submerged into pre-chilled 2-methyl butane until frozen completely. The frozen tissue block was then immediately stored at -80°C until sectioning. Serial sectioning (7 μm/section) was performed with Cyrotome cryostat machine (Leica) in UCSD Histology Core facility, and the tissue sections were placed on poly-lysine-treated glass slides. Tissue sections on slides were dried at room temperature for 30 min before staining. For immunohistochemistry, frozen sections on slides were first washed with PBS to remove residual OCT medium and then blocked with 3% BSA in PBS for 1 h, followed by incubation with primary antibodies against vWF, Ki67 and CD45 (1:100) at 4°C for 12 h. Alexa Fluor 594-conjugated anti-rabbit and mouse antibodies were used (1:200) as secondary antibodies, and DAPI was used for counterstaining. After mounting, images were acquired with an epi-fluorescence microscope.

Fluorescence in situ hybridization (FISH). For the detection of miR-23b expression, a modified FISH protocol with miR-23b-LNA probe (Exiqon) was performed. ECs cultured and PFA-fixed on slides or frozen section samples were first washed, and then treated with acetylation solution. The hybridization was performed in 150 μl hybridization solution (50% formamide, 5x SSC, 5x Denhardt's Solution, 250 μg/ml yeast tRNA, 2.5 mM EDTA, 0.1% tween-20, 0.25% CHAPS) containing 1 μl DIG-labeled miR-23b-LNA probe at 60°C for 4 h. After stringent washes, the slides were incubated with Alexa Fluor 594-conjugated DIG antibody at room temperature for 4 h, and then counterstained with Hoechst 33258 for 10 min. The negative control images of FISH staining for both *in vitro* and *in vivo* samples are shown in Online Figure XV.

Statistical analysis. For comparisons between two groups, statistical analyses were performed using the two-sample independent-groups *t* test. Comparison of multiple mean values was made by one-way ANOVA, and statistical significance among multiple groups was determined by Tukey's post-hoc test (for pair-wise comparisons of means). All results are presented as mean ± SD from at least three independent experiments.

References

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