Figure S1

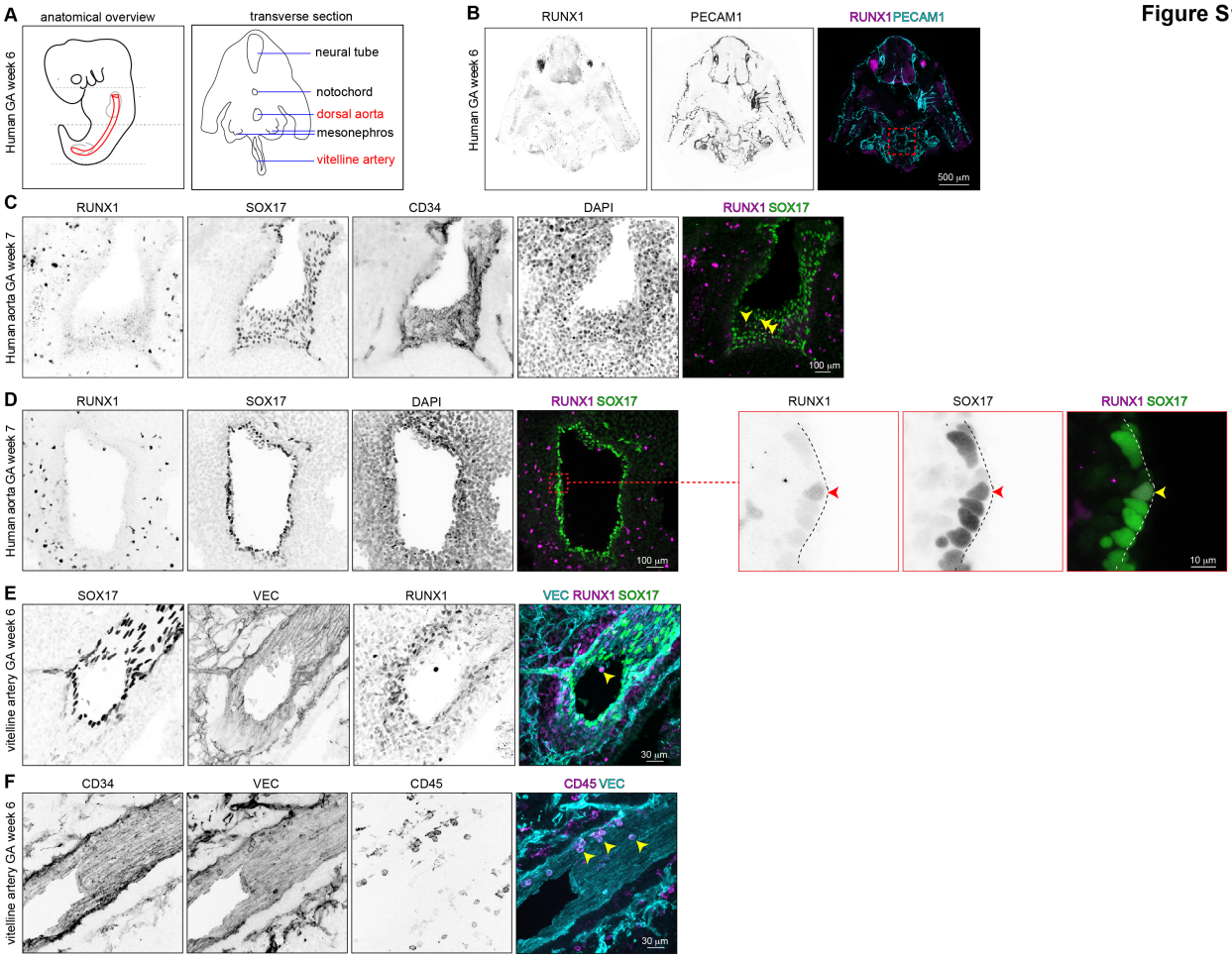

Figure S1. Hemogenic endothelium in human development.

A. Diagram of human embryo at GA week 6. Anatomical landmarks as indicated in transverse section.

B-**F**. Single channels in black and white. Scale bars as shown.

B. Transverse section of GA week 6 of Figure 1A immunostained with RUNX1 (magenta) and PECAM1 (cyan). PECAM1 labels the endothelium. The aorta is highlighted by dashed red box, with higher magnification of boxed area in Figure 1B.

C. Transverse section of GA week 7 immunostained with RUNX1, SOX17, and CD34, with DAPI fluorescence. RUNX1+ (magenta) cells embedded in SOX17+ (green) endothelium are denoted by arrowheads.

D. GA week 7 aorta immunostained with RUNX1 (magenta) and SOX17 (green), with DAPI fluorescence. Higher magnification of boxed region reveals RUNX1 (magenta) immunofluorescence detected at very low levels in a SOX17+ (green) endothelial cell (arrowhead).

E. GA week 6 vitelline artery immunostained with SOX17 (green), VEC (cyan), and RUNX1 (magenta). Arrowhead denotes a single RUNX1+ cell associated with the endothelium.

F. Vitelline artery (GA week 6) immunostained with CD34, VEC (cyan), and CD45 (magenta). Hematopoietic cells (CD45+) in the vitelline artery appear to be attached to endothelium as clusters and single cells (arrowheads).

Figure S2

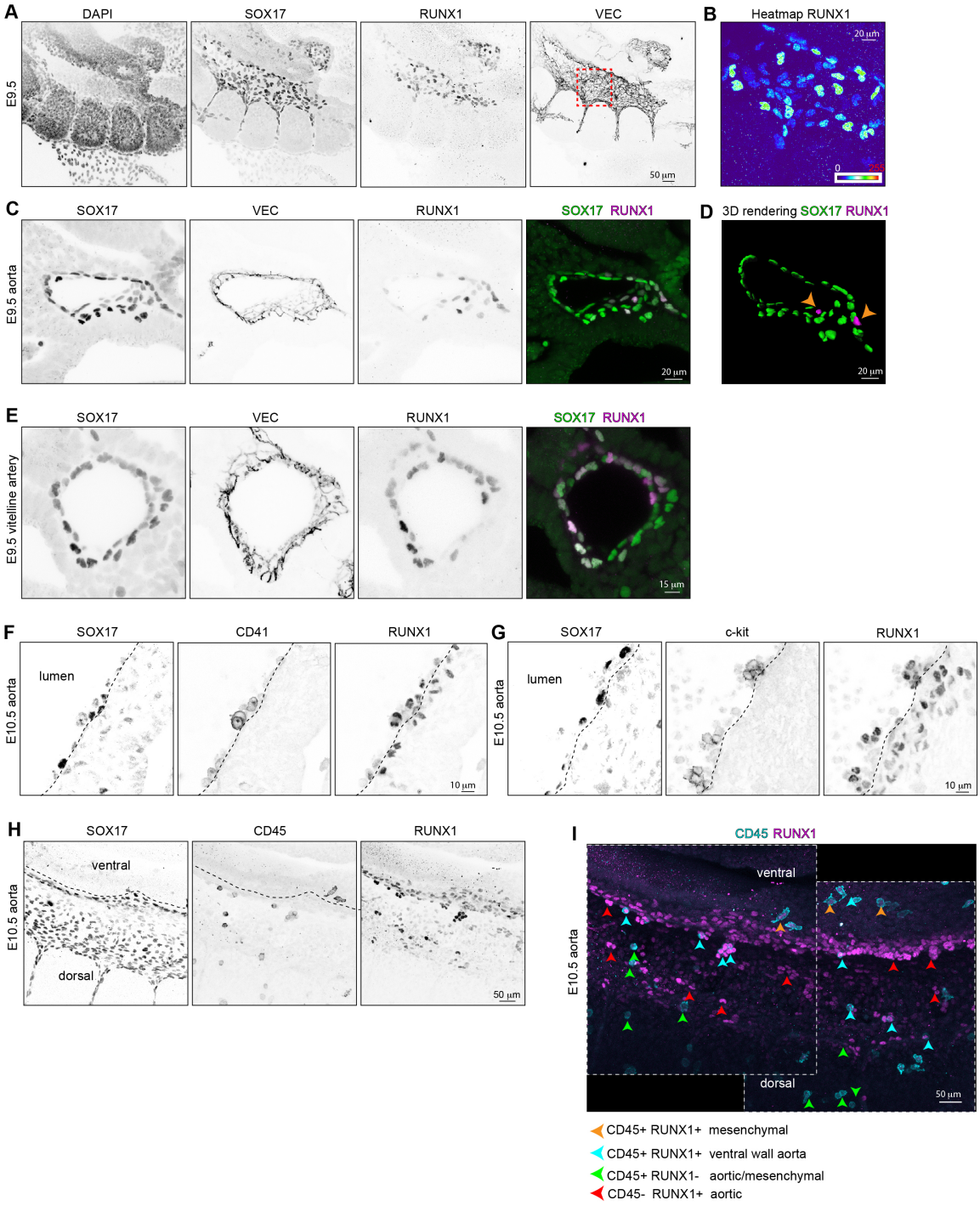
A
DAPI
SOX17
RUNX1
VEC
E9.5
50 µm
B
Heatmap RUNX1
20 µm
0
C
SOX17
VEC
RUNX1
SOX17 RUNX1
E9.5 aorta
20 µm
D
3D rendering SOX17 RUNX1
20 µm
E
SOX17
VEC
RUNX1
SOX17 RUNX1
E9.5 vitelline artery
15 µm
F
SOX17
CD41
RUNX1
lumen
E10.5 aorta
10 µm
G
SOX17
c-kit
RUNX1
lumen
E10.5 aorta
10 µm
H
SOX17
CD45
RUNX1
ventral
dorsal
E10.5 aorta
50 µm
I
CD45 RUNX1
ventral
dorsal
E10.5 aorta
50 µm
CD45+ RUNX1+ mesenchymal
CD45+ RUNX1+ ventral wall aorta
CD45+ RUNX1- aortic/mesenchymal
CD45- RUNX1+ aortic

Figure S2. RUNX1 and SOX17 in hemogenic endothelium and intra-aortic clusters.

A-H. Single channels in black and white. Scale bars as shown.

A. Sagittal section at E9.5 with DAPI fluorescence, immunostained for SOX17, RUNX1, and VEC. Area of interest depicted by red outlined box.

B. Heat map of RUNX1 immunofluorescence of boxed region in (**A**) indicating heterogeneity of RUNX1 levels in endothelium.

C. Transverse section of E9.5 embryo immunostained with SOX17 (green), VEC, and RUNX1 (magenta).

D. 3D rendering of DAPI nuclear volumes of cells in (**C**), with RUNX1 (magenta) and SOX17 (green) immunostaining. Arrowheads point to cells with high RUNX1 levels in aorta.

E. E9.5 vitelline artery with immunofluorescence for SOX17 (green), VEC, and RUNX1 (magenta) revealing heterogeneous RUNX1 expression.

F. E10.5 aorta depicting small hematopoietic cell clusters. Single channel immunofluorescence of SOX17, CD41, and RUNX1 from Figure 2H

G. Hematopoietic cell clusters in E10.5 aorta. Single channel immunofluorescence of SOX17, c-kit, and RUNX1 from Figure 2I.

H. Sagittal section of the aorta at E10.5. Immunofluorescence of SOX17, CD45, and RUNX1.

I. Sagittal section of E10.5 aorta. Merged panels (outlined by white dashed lines) depict immunofluorescence of CD45 (cyan) and RUNX1 (magenta). Multicolored arrows depict different populations of cells based on CD45 and RUNX1 expression.

Figure S3

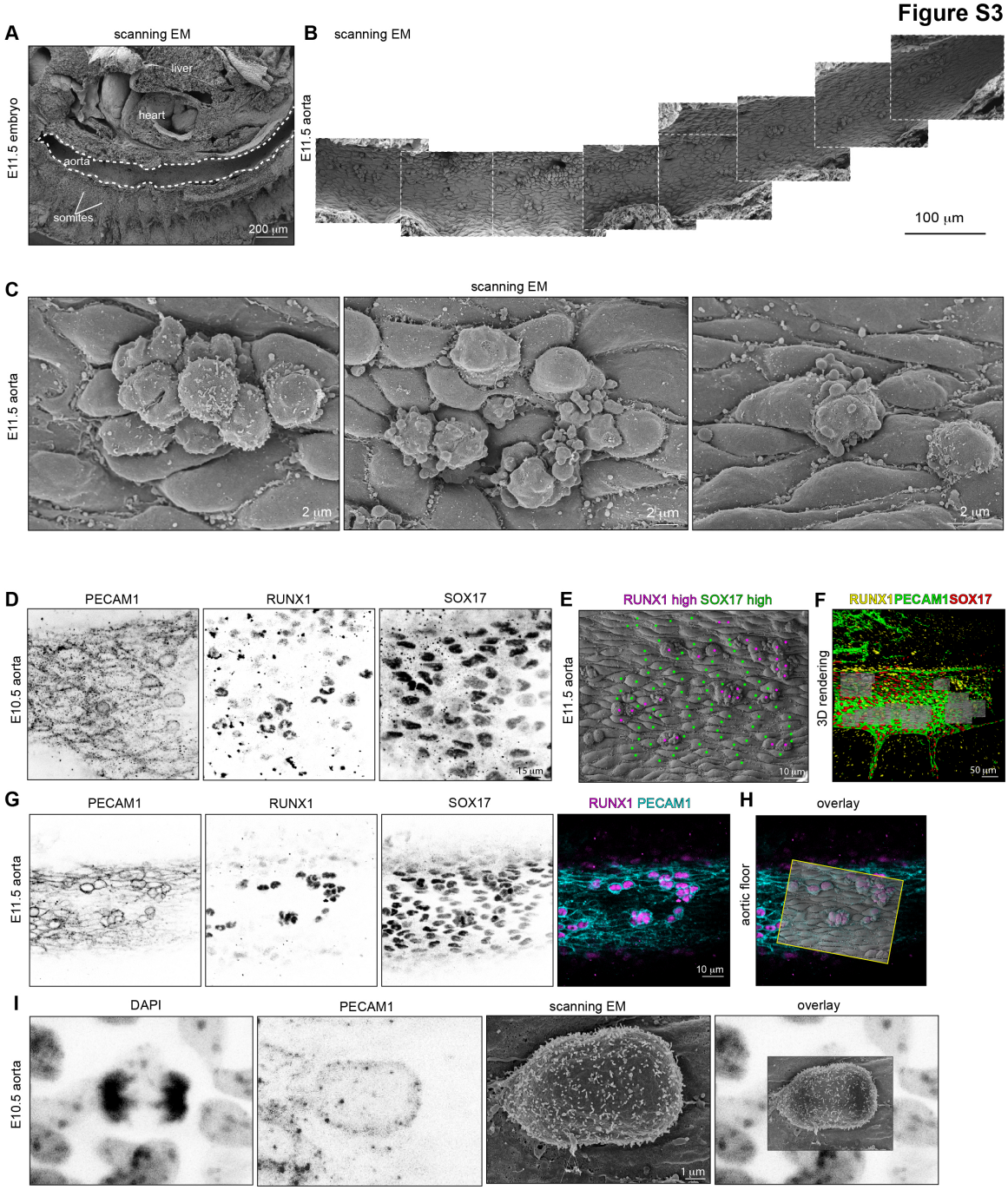

Figure S3. Correlative microscopy of aortic endothelium reveals hemogenic morphology.

A - **I.** Scale bars as noted.

A. Scanning EM (SEM) of an E11.5 embryo with landmarks as noted. Aorta is outlined by dashed white line.

B. Consecutive SEM images of the dorsal aorta (dashed outlines) are aligned to reveal the topography of the aortic endothelium.

C. Scanning EM images of three separate cell clusters in the aorta. Cells within the clusters exhibit various cell morphological attributes.

D. Dorsal aorta at E10.5 immunostained with PECAM1, RUNX1, and SOX17, single channels in black and white.

E. SEM of E11.5 aortic region evaluated for protrusions within RUNX1high (magenta) or SOX17high (green) cell populations.

F. 3D volume rendering of a complete aorta with scanning EM overlay. RUNX1 in yellow, PECAM1 in green and SOX17 in red.

G. Sagittal section of E11.5 aorta immunostained for PECAM1 (cyan), RUNX1 (magenta), and SOX17. RUNX1+ intra-aortic clusters and single cells embedded in endothelium are noted. Single channels in black and white.

H. Immunofluorescence and scanning EM overlay of section depicted in (**G**).

I. High magnification of a PECAM1+ cell in mitosis from Figure 3F (anaphase, marked by DAPI) with scanning EM overlay. Single channels in black and white.

Figure S4

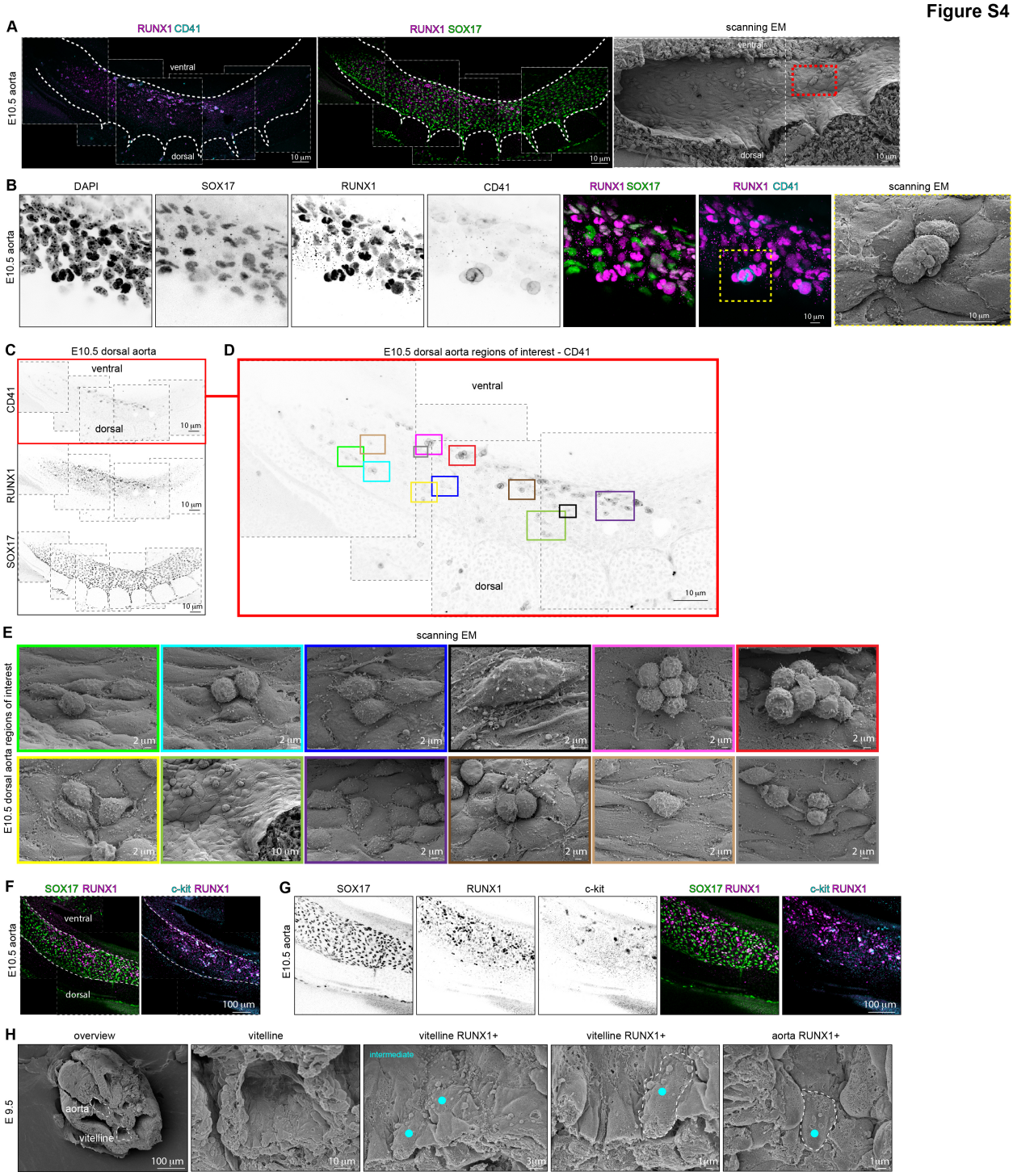
A
RUNX1 CD41
RUNX1 SOX17
scanning EM
E10.5 aorta
ventral
dorsal
B
DAPI
SOX17
RUNX1
CD41
RUNX1 SOX17
RUNX1 CD41
scanning EM
C
E10.5 dorsal aorta
CD41
RUNX1
SOX17
D
E10.5 dorsal aorta regions of interest - CD41
E
scanning EM
E10.5 dorsal aorta regions of interest
F
SOX17 RUNX1
c-kit RUNX1
G
SOX17
RUNX1
c-kit
H
overview
vitelline
vitelline RUNX1+
aorta RUNX1+
E 9.5
aorta
vitelline
intermediate

Figure S4. Correlative microscopy of aortic endothelium via RUNX1, CD41, and c-kit.

A - **H.** Scale bars as shown.

A. Merged panels (white dashed outlined boxes) of E10.5 aorta (white dashed outline) immunostained with RUNX1 (magenta), CD41 (cyan) and SOX17 (green). Merged images (left and middle panel) correspond to the scanning EM image (right panel). Area of interest is denoted by red dashed line/box.

B. Boxed region of interest in (**A**) with single channels in black and white. DAPI fluorescence with immunostaining for SOX17 (green), RUNX1 (magenta), and CD41 (cyan) labels a RUNX1+ CD41+ cell cluster (yellow dashed line/box). Corresponding scanning EM image of boxed area (far right).

C. Sagittal section of the aorta at E10.5 in (**A**) with CD41, RUNX1, and SOX17 single channels in black and white. Merged panels outlined by black dashed lines/boxes. Images were used for evaluation of SOX17 and RUNX1 MFIs depicted in Figure 2J-K.

D. Red outlined region in (**C**) depicts CD41+ cells in the E10.5 aorta. Different areas of interest containing CD41+ cells are highlighted with different colored boxes.

E. Scanning EM images of boxed areas in (**D**) demonstrate the variety of cell shape and surface morphology of CD41+ cells.

F. Sagittal section of the aorta at E10.5 immunostained for SOX17 (green), RUNX1 (magenta), and c-kit (cyan).

G. Higher magnification of aorta in (**F**). SOX17 (green), RUNX1 (magenta), and c-kit (cyan) with single channels in black and white. Images were used for evaluation of SOX17 and RUNX1 MFIs depicted in Figure 2J-K.

H. Scanning EM of E9.5 embryonic transverse section (left) with higher magnification of the vitelline artery (middle three panels) and aorta (right panel) revealing the morphology of RUNX1+ cells with intermediate RUNX1/SOX17 ratios (identified by cyan dots).

Supplementary Table S1. List of antibodies employed for immunofluorescence analyses.

Primary	Company	ID#	Species	Working Dilution
CD117 (c-kit)	BD Pharmingen	553352	Rat	1:100
PECAM1	BD Pharmingen	553370	Rat	1:100
CD41	BD Pharmingen	553847	Rat	1:100
RUNX1/2/3*	Abcam	AB92336	Rabbit	1:100
RUNX1	Abcam	AB35962	Rabbit	1:100
SOX17	R&D systems	AF1924	Goat	4µg/mL
CD143	Biolegend	344202	Mouse	1:100
Secondary	**Company**	**ID#**	**Species**	**Working Dilution**
Alexa 488 α goat	Invitrogen	A11055	Donkey	1:100
Alexa 488 α rabbit	Invitrogen	A21206	Donkey	1:100
Alexa 488 α rat	Invitrogen	A21208	Donkey	1:100
Alexa 594 α rabbit	Invitrogen	A21207	Donkey	1:100
Alexa 594 α rat	Invitrogen	A21209	Donkey	1:100
Alexa 647 α goat	Invitrogen	A21447	Donkey	1:100
Alexa 647 α rabbit	Invitrogen	A31573	Donkey	1:100

* Figure 2H,I,M

Conjugated	Company	ID#	Working Dilution
mouse α hCD45-APC	BD Pharmingen	555485	1:25
mouse α hVEC-PE	BD Pharmingen	560410	1:25
mouse α hCD34-FITC	BD Pharmingen	348053	1:25