Supplemental Information

Uncoupling VEGFA Functions in Arteriogenesis and Hematopoietic Stem Cell Specification

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Supplemental Information Inventory

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**Figure legends**

Supplemental Experimental Procedures

Supplemental References
Leung_Figure S2, related to Figures 2, 3

A

Cx37

Stage 34

WT

22/22

Eto2-MO

23/23

B

VE-cad

Stage 37

WT

24/24

Eto2-MO

16/19

Ami

Stage 37

WT

20/20

Eto2-MO

11/11

Fli1

Stage 34

WT

21/21

Eto2-MO

19/19

C

WT

AA4 - Stage 34

Eto2-MO

uncleared

3/3

8/8

Number of intersomitic vessels

WT

Eto2-MO

Number of embryos

D

WT

Eto2-MO

Flt1

Stage 26

25/25

21/24

Fli4

Stage 26

23/23

16/22

WT

Eto2-MO2

23/23

14/15
Leung Figure S6, related to Figure 6

A

| Gene   | WT | Vegfa hypomorph |
|--------|----|-----------------|
| Amh    |    |                 |
| uncleared |    |                 |
| Aa4    | 5/5| 5/5             |
| Cx37   | 19/19 | 15/22          |
| Scl    | 21/21 | 16/21          |
| Fli4   | 25/25 | 16/21          |

B

| Gene   | WT | Vegfa-MOi6e7 |
|--------|----|--------------|
| Gf1    | 21/21 | 11/11       |
| Gata2  | 21/21 | 19/21       |
| Fli1   | 24/24 | 16/19       |
| CD31   | 27/27 | 23/23       |
| Cx37   | 21/21 | 13/15       |
| Notch4 | 24/24 | 23/23       |
| Scl    | 20/20 | 24/24       |
| Fli1   | 22/22 | 23/23       |
Figure Legends

Figure S1, related to Figure 1. Design and testing of the Eto2 morpholinos (MOs)

(A) Xenopus laevis has a pseudo-tetraploid genome; genes are therefore present in two “pseudo-allele” forms. Sequence alignment of the beginning of the coding sequence of Eto2 pseudo-alleles A and B and Eto-related genes (top) and of the 5’ UTR of Eto2 pseudo-alleles A and B (bottom); the target regions for the “ATG” MO (ETO2-MO) and “5’UTR” MO (ETO2-MO2) are highlighted in yellow and blue respectively. The MOs were designed to have 100% homology to the mRNAs transcribed from both Eto2 pseudo-alleles. The nucleotides in red are conserved between Eto2 and the Eto-related transcripts in the region targeted by Eto2-MO.

The Eto-related transcripts (Eto, Mtgr1 and two transcripts with high similarity to Mtgr1, namely Mtgr1-like1 (MGC68858) and Mtgr1-like2 (IMAGE 5156021), accession numbers are in Supplemental Experimental Procedures, section Probes used for in situ hybridisation) are not targeted by the Eto2 MOs. For Mtgr1, Mtgr1-like1 and Mtgr1-like2, the sequences targeted by the MOs are sufficiently divergent to avoid unspecific targeting effects. Of note, 5 mismatches are recommended to avoid unspecific targeting effects (Eisen and Smith 2008). Given that only 3 nucleotides differ between the Eto sequence and the sequence recognised by Eto2-MO, the Eto transcripts could potentially be targeted. However, as detailed in Figure S3, they are not expressed in hematopoietic or somitic tissues and are therefore unlikely to have any function in hematopoiesis. The sequences targeted by Eto2-MO2 are too divergent to be aligned; this MO is therefore very unlikely to interfere with translation of any of the Eto-related mRNAs.

(B) A GFP-reporter mRNA tethered to the 5’ region of ETO2 containing the Eto2-MO target sequence (Eto2:GFP) was used to test the efficacy of the MO in vivo. Embryos co-injected with Eto2-MO and Eto2:GFP mRNA had no visible GFP expression (right panel), as opposed to mRNA alone (middle panel). Eto2-MO is therefore able to bind to its intended target sequence in vivo and to block translation.

(C) Expression analysis of the HSC markers Gfi1 and SpiB in Eto2-MO2 injected embryos. Red arrow; DA. Numbers at the bottom of the panels indicate the number of embryos with the given phenotype out of the total number examined. Whole mounts are shown with anterior to the left and dorsal to the top.
Figure S2, related to Figures 2,3. Analysis of arteriogenesis and vasculogenesis in Eto2-MO and Eto2-MO2 injected embryos

Expression analysis of the arterial marker Cx37 (A, stage 34), the endothelial genes VE-cadh, Ami, Flt1 and AA4 (B-C, stages 37/34), Flt1 and Flt4 (D, stage 26) in Eto2-MO and Eto2-MO2 morphants. Red arrowhead, DA; black arrow, PCV; yellow arrow, trunk vasculature; green arrowhead, DLP. (C) Uncleared embryos, bracket indicates intersomitic vessels (ISVs) sprouting from the PCV in wild-type embryos; the graphs show the number of ISVs observed in wild-type, Eto2-MO and Eto2-MO2 embryos. Numbers at the bottom of the panels indicate the number of embryos with the given phenotype out of the total number examined. Whole mounts are shown with anterior to the left, dorsal to the top.

Figure S3. Related to Figure 4. Eto2 is not expressed in the DA - Expression pattern of Eto2-related transcripts during development

(A) Laser Capture Microdissection (LCM) was used to isolate specific tissues from stage 39 embryonic sections; the DA and surrounding mesenchyme (DA mes), the neural tube (NT) and gut. NT tissues were intended as a positive control for amplification of the Eto2 transcripts and the gut as a negative control (as expected from WMISH and ISHS results, Figure 4). Gene expression analysis was carried out for Eto2 and the known HSC markers Scl and Runx1 by Reverse Transcription Real Time PCR. Results were normalised to ODC. Errors bars represent the standard deviation from two independent experiments. Unlike the HSC markers, Eto2 expression in the DA mesenchyme is not significantly different from that observed in gut when compared to the high levels observed in the NT.

(B, C) Expression of Eto, Mtgr1, Mtgr1-like1 and Mtgr1-like2 was examined during Xenopus development (B) Expression of Eto is detected in the heart fields (stage 22, yellow arrowhead), in the heart (stage 27, yellow arrowhead), in neural cells (stage 22; neural tube at stages 27, 36, 39, white arrowheads) and in the PCV (stages 36/39, blue arrowhead). There was no expression in the DA at stage 39 (red arrowhead). (C) Expression of Mtgr1, Mtgr1-like1 and Mtgr1-like2 is observed in neural cells (white arrowheads stage 22; neural tube at stages 26 and 35), and in the PCV (initiates anteriorly at stage 26, is established by stage 35; blue arrowheads). Mtgr1-like2 is expressed at low levels in the somites (stage 26, orange arrowhead). ISHS at stage 43 shows no expression of Mtgr1 and Mtgr1-like1 in the DA (red arrowhead) and a faint staining for Mtgr-like2 in the region of the DA and surrounding mesenchyme. However, given the lack of homology in the sequences targeted by the Eto2 MOs, it is highly unlikely that expression of Mtgr-like2 was affected in the knock-down experiments.

Whole mounts are shown with anterior to the left and dorsal to the top. Sections are in transverse orientation with dorsal to the top.
Figure S4, related to Figures 1-4. Zebrafish ETO2 is required for HSC emergence

(A-C) As in Xenopus, Eto2 is expressed in the trunk somites (A) but not in the dorsal aorta (DA), as seen in transverse sections along the trunk (B, C), at 24hpf (hours post-fertilisation); s, somites; ICM, intermediate cell mass. (D) To test the function of ETO2 in hematopoietic development, we knocked-down its expression using a morpholino that targets the zebrafish Eto2 (Meier et al. 2006). A dose-dependent loss of the HSC markers runx1 and cmyb (another HSC marker (Murayama et al. 2006)) in the DA (black arrowheads) was observed with increasing amounts of Eto2 MO, at 28hpf. In contrast, expression of the arterial marker notch1b was unaffected. (E) At 28hpf, none of the arterial markers analysed (dll4, dlC, notch3/5; DA, black and white arrowheads (Lawson and Weinstein 2002; Nicoli et al. 2008; Rowlinson and Gering 2010)) was affected in Eto2 morphants, whereas runx1 expression was severely downregulated (red arrowheads). Flk1 expression is grossly normal in Eto2 morphants confirming normal endothelialisation. Therefore, down-regulation of Eto2 in zebrafish embryos leads to non cell-autonomous phenotypic defects that are very similar to those observed in Xenopus Eto2 morphant embryos.

Figure S5, related to Figure 5. Expression of a panel of markers implicated in the development of the DA/HSC progenitors in stage 27 WT and Eto2 morphant embryos.

(A) Schematic diagram detailing the signalling events that are proposed to be involved in the specification of the DA and the HSC program; adapted from (Diez et al. 2007).

(B) Expression of selected markers was examined in stage 27 WT and Eto2 morphant embryo sections by ISHS. There was no discernible alteration in the expression level or the pattern of these markers in the Eto2 morphants.

Figure S6, related to Figure 6. Vegfa hypomorph embryos and Vegfa-MOi6e7 morphants phenocopy the Eto2 morphants

(A) Expression of endothelial (Ami, AA4, Flt4), arterial (Cx37) and hemangioblast (Scl and Flk1) genes was examined in Vegfa hypomorph embryos, by WMISH at the stages indicated. Note that AA4 expression was examined on uncleared embryos.

(B) Vegfa medium/long isoform morphants (Vegfa-MOi6e7) recapitulate the Eto2 morphant phenotype in the DA. WMISH analysis of hematopoietic (Gfi1, Gata2) endothelial (Flk1, CD31), arterial (Cx37 and Notch4) and hemangioblast (Scl, Flk1) markers reveals that endothelialisation, arterialisation and
hematopoietic specification do take place in the morphant DA (red arrowheads). Note, however, the absence of ISVs (AA4, uncleared embryos, brackets), the weak staining of the PCVs (black arrows) and the limited development of the trunk vasculature (yellow arrows) in the *Vegfa*-MOi6e7 morphants. Numbers at the bottom of the panels indicate the number of embryos with the given phenotype out of the total number examined. Whole mounts are shown with anterior to the left and dorsal to the top.

Red arrowheads, DA; black arrows, PCV; brackets, ISVs; pink arrowheads, DLP; yellow arrowheads, VBI; dark blue arrowheads, *Scl* expression in the neural tube; green arrowheads, *Flk1* expression in the trunk endothelium; light blue arrowheads, notochord; yellow arrows, trunk vasculature. Numbers at the bottom of the panels indicate the number of embryos with the given phenotype out of the total number examined. Whole mounts are shown with anterior to the left and dorsal to the top.
Supplemental Experimental Procedures

Laser Capture Microdissection (LCM)

*Xenopus* embryos were washed in 30% sucrose:OCT and 100% OCT. Individual embryos were then transferred and orientated in a cryostat mould filled with OCT and snap-frozen. 20 μm sections (20-30 sections from the DA region; ~ 10 sections from gut and NT regions) were cut from the blocks in a cryostat and transferred to Membraneslides (Leica). The slides were stained with Toluidine Blue (0.1%) before microdissection. RNA was extracted from pooled micro-dissected samples using a QIAGEN RNeasy micro kit.

Real-Time quantitative PCR

Primers for both TAQMAN and SYBRGREEN PCR were designed using the Primer Express Software Version 3.0 program (Applied Biosystems). Details of primers used are below. PCR reactions were performed using TaqMan® Universal PCR Master Mix or SYBR® GREEN PCR Master Mix (Applied Biosystems). All results were normalised to levels of ornithine decarboxylase (ODC).

### Real-Time and standard PCR primers

#### SYBRGreen Primers

| Primer Name | Sequence | Function |
|-------------|----------|----------|
| XLVEGFA122_F | CAACATCACCATGCAGATAATGAA | Detection of Xenopus VEGFA<sub>122</sub> |
| XLVEGFA122_R | CGTGGCTTTTTCACATTTTTTC | |
| XLVEGFA170ALL_F | AATCATTTGTAGCCTTGCAACAG | Detection of Xenopus VEGFA<sub>170</sub> |
| XLVEGFA170ALL_R | GGCTTTTCACATCGCAAGTCC | |
| XLVEGFA190_F | CGAGGGAAGGCGCTTAACAG | Detection of Xenopus VEGFA<sub>190</sub> |
| XLVEGFA170ALL_R | GGCTTTTCACATCGCAAGTCC | |

#### TaqMan Primers

| Primer Name | Sequence | Function |
|-------------|----------|----------|
| XLETO2-F | CAGAAAGCCTGTGGTCGGAAGCAG | Detection of Xenopus XLETO2 |
| XLETO2-R | TGAATGTCTGTCGTCACCAAAAGT | |
| XLETO2-Probe | FAM-AGCATCCTCATACTTTTGGAACGCACCAAG-TAMRA | |
| XSCL-F | CCATGCTCTATGGCCTCAATC | Detection of Xenopus XSCL |
| XSCL-R | AAGGTGTCTGGTCACCAAAAGT | Walmsley et al, Blood, 2007 |
| XSCL-Probe | FAM-CCCCCTGGCGTCAGATAACAGTGCG-TAMRA | |
| Xrunx1-F | GGATCCTACACCGTCTCTAT | Detection of Xenopus Xrunx1 |
| Xrunx1-R | CCCGTGAAGCTTTGTG | Walmsley et al, Blood, 2007 |
| Xrunx1-Probe | FAM-ATCTCCGCTCGATCTTCTCCA-TAMRA | |
| XODC-F | CCTGCGCGCTCAGTGAAAXOCF | Detection of Xenopus XODC |
| XODC-R | GCAAGGCCTCGCAACATG | Walmsley et al, Blood, 2007 |
| XODC-Probe | FAM-ACCCTTTAAAAACACGGCGCTTCTGGA-TAMRA | |

VegfA FL Fwd: ccccttaacggaatatcat (at ATG)
VegfA FL Rev: tgtcccttttggatagc (at 3'UTR) 

designed using BC169428, 793bp PCR fragment
Fish maintenance and morpholino injections
Fish were bred, maintained and staged as described (Westerfield 2007). A morpholino oligonucleotide (MO) was used to target zebrafish *Eto2* (Meier et al. 2006).

In situ hybridization, sections and image acquisition (zebrafish)
Whole mount hybridization was carried out as described (Jowett and Yan 1996). An EST containing the zebrafish *Eto2* cDNA (Accession number: CK693846, Imagenes, Germany) was used as a template to generate an *Eto2* probe. DIG-labelled antisense RNA probes were transcribed from linearized templates using T3, T7 or Sp6 RNA polymerases (Roche, Burgess Hill, United Kingdom). Embryos were bleached and prepared for whole mount imaging as described (Monteiro et al. 2011); alternatively, embryos were embedded in JB-4 resin (Electron Microscopy Sciences) and sectioned according to the manufacturer’s protocol. Sections were counterstained with neutral red and mounted in Pertex (Leica).

Whole mount photography was done on a Nikon DXM 1200 digital camera and Nikon ACT-1 software (version 2.12) mounted on a Nikon SMZ 1500 zoom stereomicroscope (Nikon, Melville, NY); sections were imaged on a Nikon DMX1200C camera and Nikon Elements software mounted on a Eclipse E600 microscope.

Probes used for *in situ* hybridisation

| Gene Name | EST ID     | Accession Number | Restriction Enzyme | RNA Polymerase | Reference                          |
|-----------|------------|------------------|--------------------|----------------|-----------------------------------|
| *Runx1*   |            |                  | SalI              | T7             | Tracey et al., 1998               |
| *Gata2*   |            |                  | XbaI              | SP6            | Walmsley et al., 1994             |
| *VegfA*   |            |                  | BamHI             | T7             | Cleaver et al., 1997              |
| *Scl*     |            |                  | XhoI              | SP6            | Ciau-Uitz et al., 2010            |
| *Flk1*    | NIBB XL087o23 BJ092634 | NotI or SacI or SmaI | T7             | Ciau-Uitz et al., 2010 |
| *αT4-globin* |            |                  | EcoRI             | SP6            | Walmsley et al., 1994             |
| *Eto2*    | NIBB XL185m19 BJ635188 | NotI or BamHI    | T7             | This report                  |
| *Eto2*    | IMAGE 5130002 BX852558 | Sall or SmaI     | T7             | This report                  |
| *Eto*     |            |                  | EcoRI             | T7             | Koyano-Nakagawa & Kintner, 2005   |
| *MTGR1*   | IMAGE 5571316 BC044006 |                | EcoRI             | T7             | This report                  |
| *MTGR1-like1* | IMAGE 4680246 BC057713 | Sall or EcoRI or EcoRV | T7             | This report                  |
| *MTGR1-like2* | IMAGE 5156021 CA790039 | Sall or EcoRI or EcoRV | T7             | This report                  |
| *SpiB*    | IMAGE 5537169 AAH46671 | Sall or EcoRI    | T7             | Ciau-Uitz et al., 2010         |
| *Lmo2*    | IMAGE 4174203 AAh97502 | Sall or SmaI     | T7             | Ciau-Uitz et al., 2010         |
| *Gfi1*    | IMAGE 8547327 EB645267 | EcoRI or ClaI    | T7             | Ciau-Uitz et al., 2010         |
| *Notch4*  | IMAGE 4684242 BQ735158 | Sall or SmaI     | T7             | Ciau-Uitz et al., 2010         |
| *Dll4*    | IMAGE 7876232 DT435811 | EcoRI or ClaI or BamHI | T7             | Ciau-Uitz et al., 2010         |
| *EphrinB2a* | IMAGE 4724740 BC057724 | Sall or SmaI     | T7             | Ciau-Uitz et al., 2010         |
| Gene       | Accession | Restriction Enzymes | Vector | Source                          |
|------------|-----------|---------------------|-------|--------------------------------|
| Notch1     | IMAGE 7020309 | NotI or SacI       | T7    | This report                    |
| Hesr1      | IMAGE 7204180 | SmaI or EcoRI      | T7    | This report                    |
| Ami        | IMAGE 5512615 | EcoRV or EcoRI or SmaI | T7    | This report                    |
| Tie2       | NIBB XL064i22 | NotI or SacI or SmaI | T7    | Ciau-Uitz et al., 2010        |
| Cx37       | IMAGE 7638101 | Sall or SmaI or EcoRI | T7    | This report                    |
| Vecad      | IMAGE 5515354 | Sall or Clal       | T7    | This report                    |
| AA4        | IMAGE 4959298 | Sall or SmaI       | T7    | Ciau-Uitz et al., 2010        |
| Flt4       | IMAGE 4970772 | Sall               | T7    | Ciau-Uitz et al., 2010        |
| Etv6       | NIBB XL153n17 | NotI or SacI       | T7    | Ciau-Uitz et al., 2010        |
| Ptc1       | IMAGE 6631163 | Sall or SmaI or EcoRI | T7    | This report                    |
| HIF1a      | IMAGE 4930371 | SmaI or EcoRI      | T7    | This report                    |
| Arnt/HIF1β | NIBB XL032m15 | NotI               | T7    | This report                    |
| Shh        | NIBB XL096o20 | NotI or SacI or SmaI | T7    | This report                    |
| Nrp1       | IMAGE 4969039 | Sall or EcoRI or EcoRV | T7    | This report                    |
| Apelin     | IMAGE 6631163 | Sall or SmaI or EcoRI | T7    | This report                    |

**Zebrafish probes**

| Gene  | Accession | Restriction Enzymes | Vector | Source                          |
|-------|-----------|---------------------|-------|--------------------------------|
| Eto2  | CK693846  |                     |       | See Extended Experimental Procedures |
| Runx1 | HindIII   | T7                  | Kalev-Zylinska et al., 2002 |
| cMyb  | EcoRI     | T7                  | Thompson et al 1998 |
| Notch1b| IMAGE 3725324 | HindIII  | T7    | This report                    |
| Dll4  | SpeI      | T7                  | Rowlinson and Gering, 2010 |
| DeltaC| Xbal      | T7                  | Rowlinson and Gering, 2010 |
| Notch3| PstI      | T7                  | Gering and Patient, 2005  |
| Flk1  | EcoRI     | T7                  | Fouquet et al, 1997       |
Supplemental References

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