The role of *meis1* in primitive and definitive hematopoiesis during zebrafish development

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Supplementary Design and Methods

Meis1 antisense oligomers and mRNA injections

Morpholino (MO) targeting zebrafish *meis1* atg (5'-GTATATCTTCGTACCTCTGCGCCAT-3'), *meis1* splice (5'-TCGTACTGCACACACACGAATGGCA-3'), *pbx1* atg (5'-CTGGTCATCCATCCTCGCCGCTGTT-3') and standard control MO (5'-CCTCTTACCTCAGTTACAATTTATA-3') were obtained from GeneTools LLC (Philomath, OR, USA). The oligomers were resuspended in sterile water and approximately 1 nL was injected into zebrafish embryos, at the one- to two-cell stage. For *meis1* atg a concentration of 3 μg/μL was used and for splice MO and standard control MO concentrations of 6 μg/μL were used. The *pbx1* MO was injected at 1.5 μg/μL.

Capped zebrafish *meis1* sense RNA was synthesized, using the mMESSAGE mMACHINE kit (Ambion, Warrington, UK) according to the manufacturer's protocol, from linearized pTNTEGFP plasmids containing the complete coding region of zebrafish *meis1* cDNA. Meis1-EGFP sense mRNA (200 ng/μL) was injected immediately after the injection of *meis1* atg MO into one-cell embryos.

Reverse transcription polymerase chain reaction

The efficiency of the splice-site MO-mediated gene knockdown was determined by reverse transcription (RT) of RNA followed by polymerase chain reaction (PCR) amplification of template. Samples of total RNA were isolated from control and *meis1* splice MO-injected embryos using the RNeasy mini kit (Qiagen, Crawley, UK) and subjected to cDNA synthesis by RT using meis1F79 (5'-GATTGATTGACAGCCGGAGT-3') and meis1R (5'-CATGTAGTGCCACTGTCCCTC-3') primers and the One-step RT-PCR kit (Qiagen, Crawley, UK). The following program of cycling was used for the PCR: 50°C for 30 min, 94°C for 15 min, 94°C for 30 sec, 58°C for 1 min, 72°C for 1.5 min (35 cycles), and 72°C for 5 min.

Online Supplementary Movie 1. Differential interference contrast time-lapse movie of circulatory blood flow in the 2 dpf control MO-treated embryos. Images were recorded at 1-second intervals over a period of 1 min. Dorsal side down; anterior to the left. SEE MOVIE

Online Supplementary Movie 2. Differential interference contrast time-lapse movie of circulatory blood flow in the 2 dpf *meis1* MO-treated embryos. Images were recorded at 1-second intervals over a period of 1 min. Dorsal side down; anterior to the left. SEE MOVIE

Online Supplementary Movie 3. Differential interference contrast movie of the heart in the 2 dpf control MO-treated embryos shows a fully developed zebrafish heart with a heart beat of 90 beats per minute. SEE MOVIE

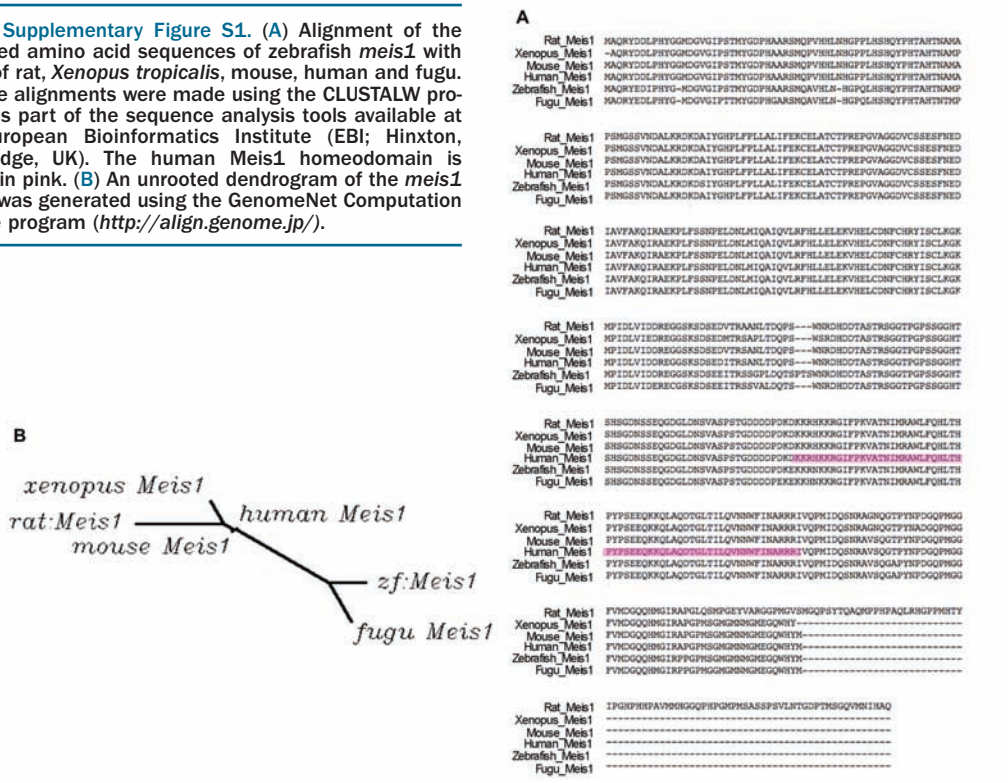
Online Supplementary Movie 4. Differential interference contrast movie of the heart in the 2 dpf *meis1* MO-treated embryos shows an under-developed heart with a heart beat of 42 beats per minute. SEE MOVIE

Online Supplementary Table S1. Percent identity between human, mouse, rat, fugu, and *Xenopus tropicalis* to zebrafish translated amino sequence of Meis1.

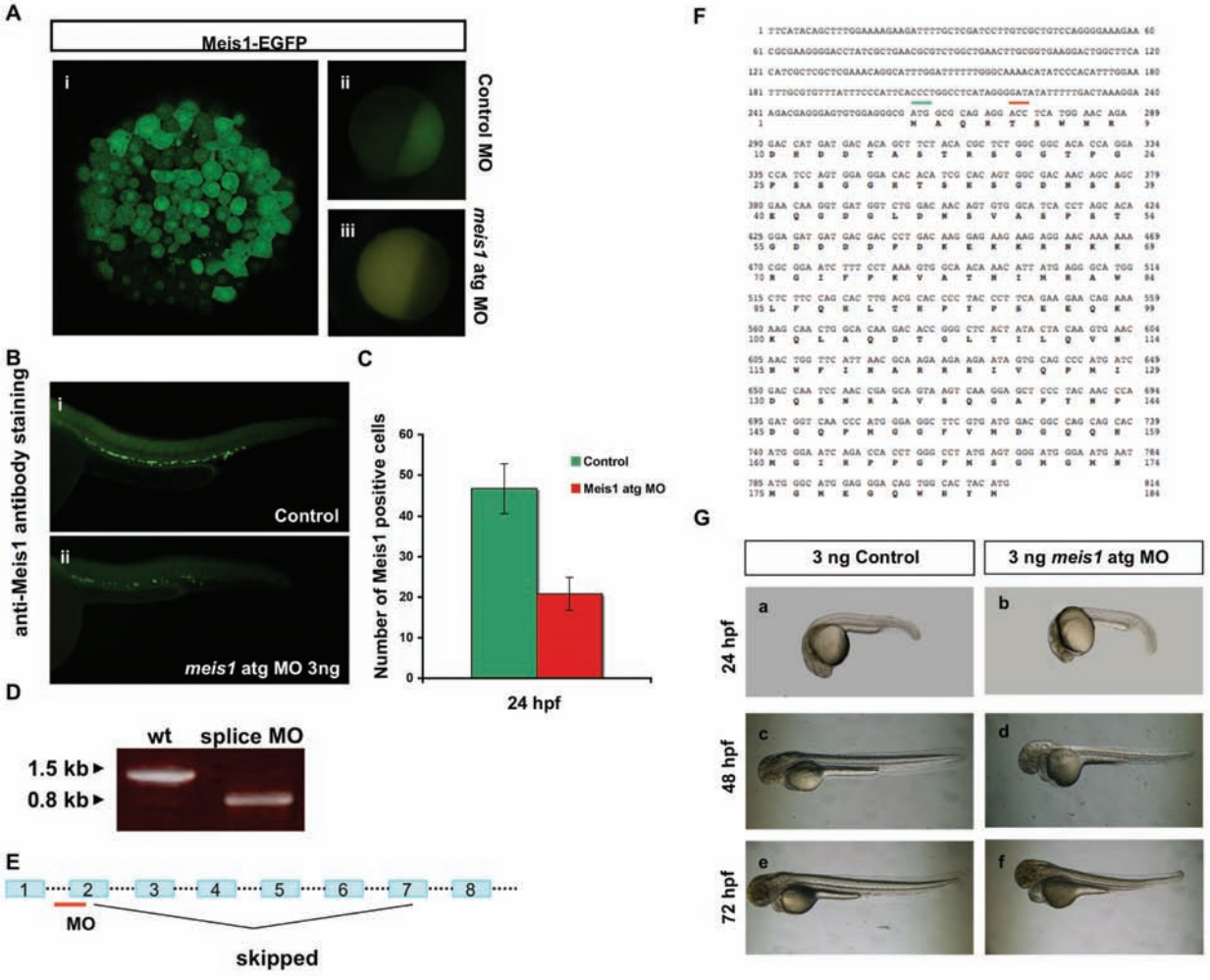
Species	Ensembl Gene ID	% of identity *
Human (*Homo sapiens*)	ENSG00000143995	94
Mouse (*Mus musculus*)	ENSMUSG00000020160	94
Rat (*Rattus norvegicus*)	ENSRNOG00000004606	93
Fugu (*Takifugu rubripes*)	ENSTRUG00000011993	96
Xenopus tropicalis	ENSXETG00000011473	93

*% identity of zebrafish to other vertebrates translated amino acid sequence was calculated using BLAST

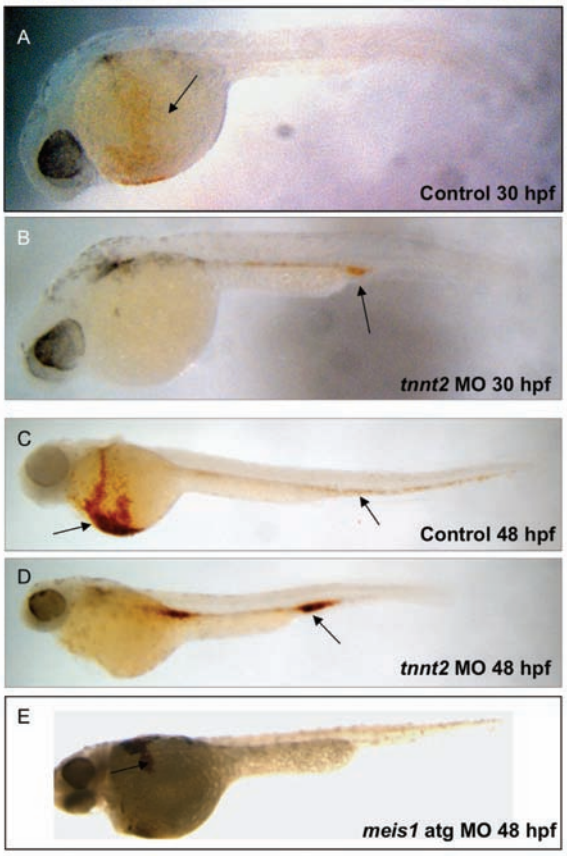
Online Supplementary Figure S1. (A) Alignment of the predicted amino acid sequences of zebrafish *meis1* with those of rat, *Xenopus tropicalis*, mouse, human and fugu. Multiple alignments were made using the CLUSTALW program as part of the sequence analysis tools available at the European Bioinformatics Institute (EBI; Hinxton, Cambridge, UK). The human Meis1 homeodomain is shown in pink. (B) An unrooted dendrogram of the *meis1* family was generated using the GenomeNet Computation Service program (*http://align.genome.jp/*).



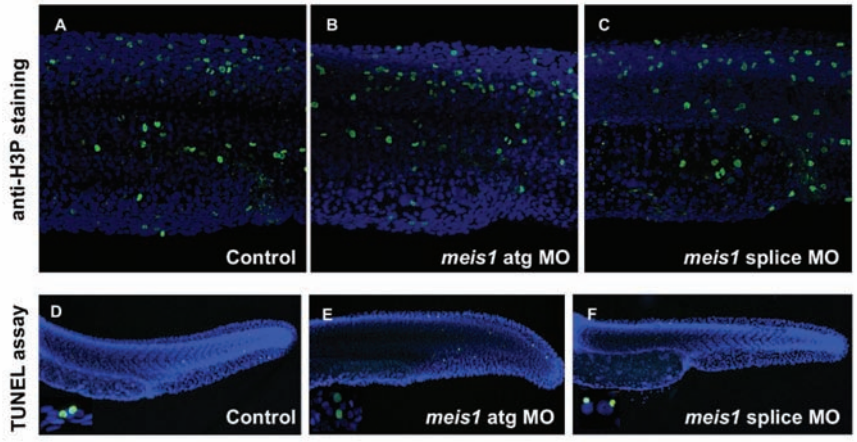
Online Supplementary Figure S2. (A) An illustration of the expression of the *meis1-EGFP* reporter construct injected on its own (i), or together with either control MO (ii) or *meis1* atg MO (iii) at the one-cell stage. (B) Whole-mount immunohistochemistry was used to determine Meis1 protein levels and to verify the effectiveness of the *meis1* atg MO in the control (i) *versus* the MO-injected (ii) embryos. (C) Graph to illustrate the decrease in Meis1 protein expression in *meis1* atg MO-injected embryos in comparison with the control; the number of individual Meis1-positive cells was reduced by 55%. Error bars are the standard error of the mean (SEM). (D) The splice modification caused by *meis1* splice MO was assayed by RT-PCR, using primers meis1F79 and meis1R, and is seen as a band shift after gel electrophoresis of the RT-PCR products. (E) Schematic diagram illustrating the first eight exons/introns of the *meis1* gene. Eight blue boxes (1-8) and dashed lines indicate the exons and introns, respectively. The splice-site target is shown as the solid, red line, marked MO. RT-PCR and cDNA sequencing results showed that exons 2 to 7 were removed by splicing in *meis1* splice MO-injected embryos, as indicated by the solid, black lines on the diagram. The loss of these six exons created a transcript encoding a truncated protein lacking most of the amino terminal domain of *meis1*. (F) Alignment of the nucleotide and deduced amino acid sequence of Meis1 in *meis1* splice MO-injected embryos. Alignment of the protein sequence is from the first methionine (marked by a solid, green line) and is given in the single letter amino acid code. The alternative transcript generated by the *meis1* splice MO has an open reading frame and the 5' end of exon 8 is marked with a solid, red line. Numbers at the end of the sequence elements aligned refer to the number of nucleotides (upper number) and number of amino acids (lower number). (G) One-cell stage embryos were injected with the standard control MO (left column: a, c, e) and *meis1* atg MO (right column: b, d, f). The optimal dose for microinjection of MO was determined as that which resulted in specific defects but did not cause gross lethality or global defects. The morphology of the embryos was observed at 24, 48 and 72 hpf.



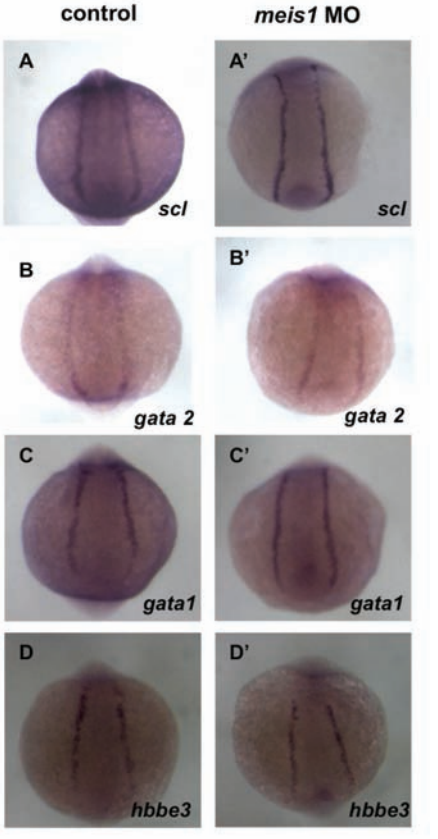
Online Supplementary Figure S3. (A-E) O-dianisidine staining was used to detect the distribution of hemoglobin-positive cells in control, *tnnt2* MO (*sih*) and *meis1* MO-injected embryos at 30 and 48 hpf. (A-B) In control embryos (A) hemoglobin-positive cells were found on the yolk sac soon after circulation started at 30 hpf; however, *tnnt2* MO-injected embryos (B) had erythrocytes accumulated in the trunk due to the lack of circulation. (C-E) At 48 hpf the number of o-dianisidine-positive cells was severely reduced in *meis1* atg MO-injected embryos (E) in comparison with the control (C) and *tnnt2* MO-injected embryos. Although lack of blood circulation in *sih* morphants led to accumulation of erythrocytes in the trunk there were no changes in their number in comparison to in the wild-type embryos.



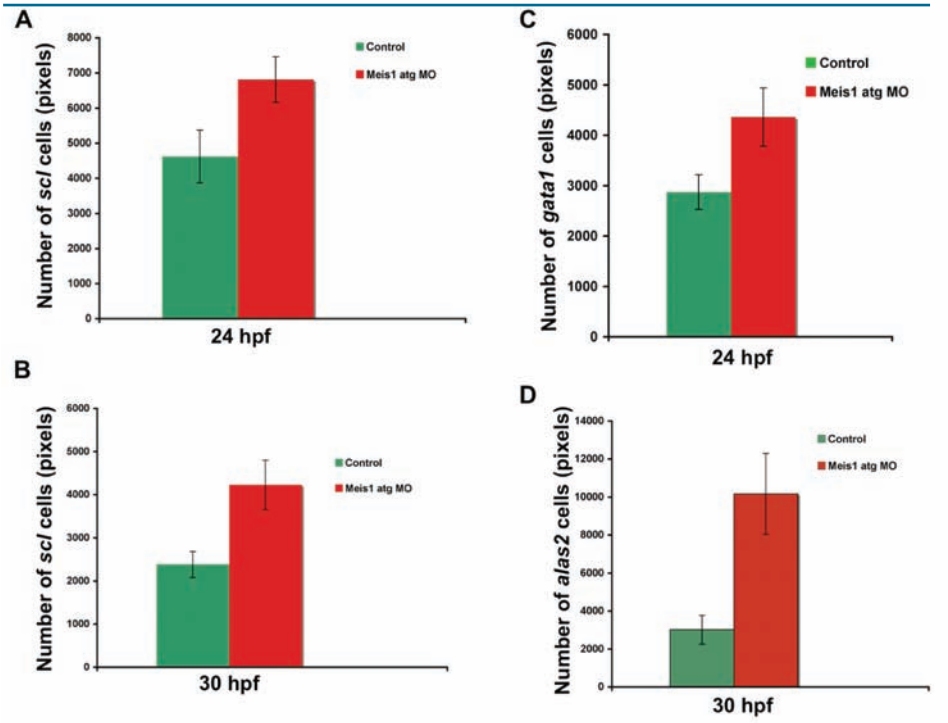
Online Supplementary Figure S4. Cell proliferation and cell death appear to be normal in *meis1*-depleted embryos. (A-B) Proliferating cells were examined at 24 hpf in the ICM of embryos by immunohistochemistry using an anti-H3P antibody. The rate of H3P-positive cells was not affected by injection of the *meis1* atg MO (B) or by that of the splice MO (C), in comparison with the control (A). (D-F) Apoptotic cells were examined in the ICM of control (D), *meis1* atg MO-injected (E) and *meis1* splice MO-injected (F) embryos by a TUNEL assay at 24 hpf. No increased cell death was observed in *meis1* MO-injected embryos.

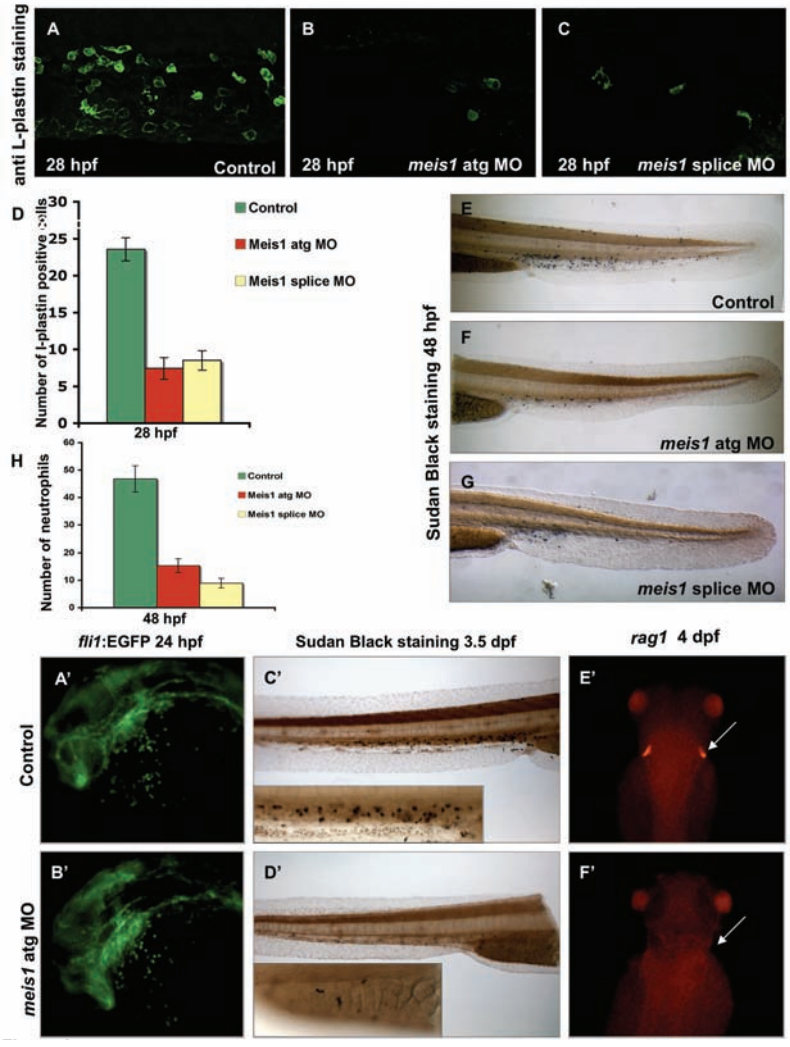


Online Supplementary Figure S5. *In situ* hybridization of the hematopoietic markers *scl*, *gata2*, *gata1* and *hbbe3* shows normal expression in *meis1* MO-injected embryos at the 10-somite stage (A'-D') when compared to control embryos (A-D).



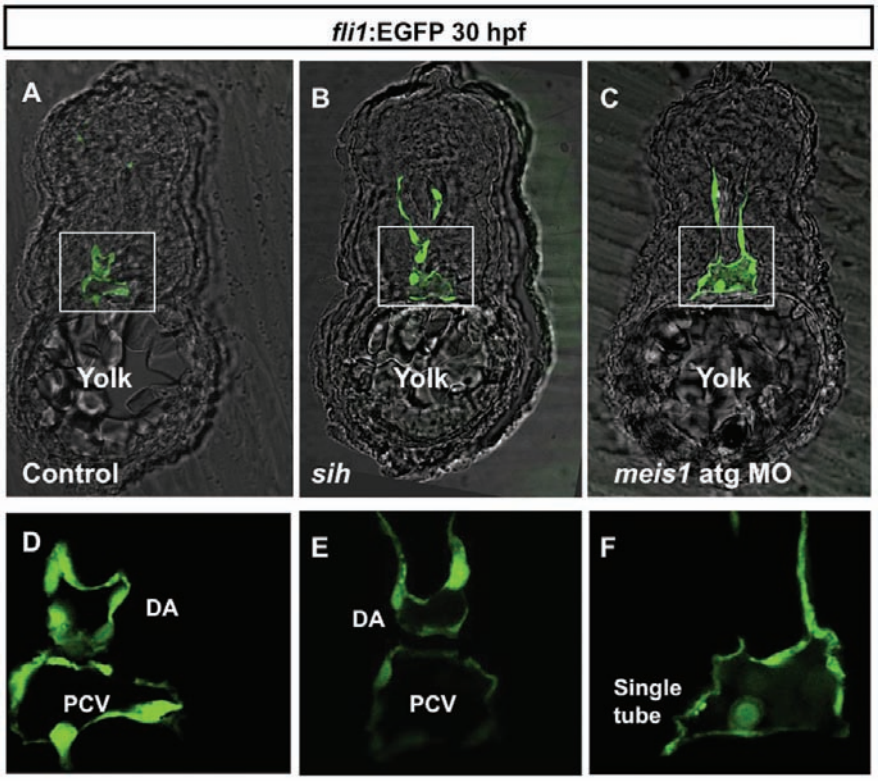
Online Supplementary Figure S6. (A-E) Graphs to illustrate the difference in number of *scl* (A) and *gata1* (C) positive cells at 24 hpf and number of *scl* (B) and *alas2*-positive cells (D) at 30 hpf. Error bars are the standard error of the mean (SEM).



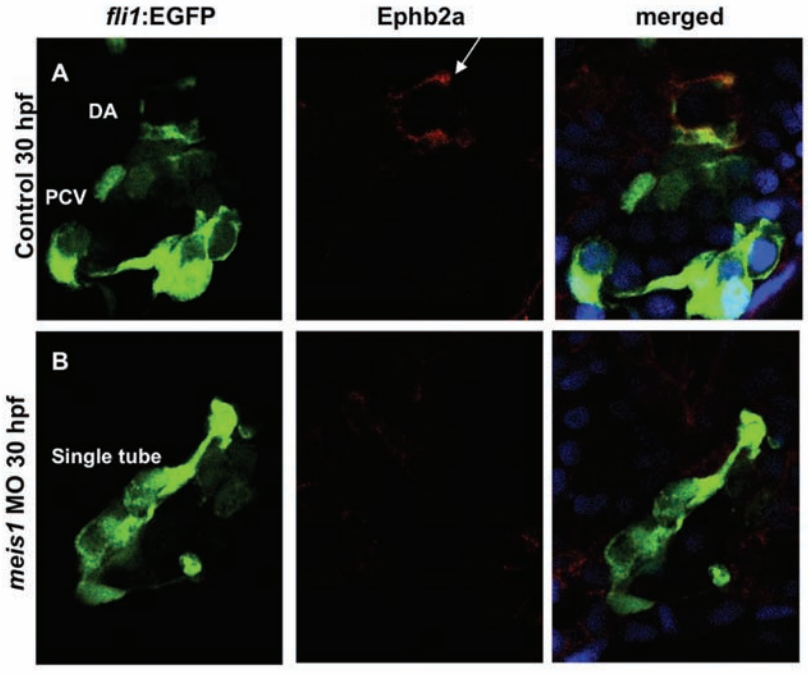


Online Supplementary Figure S7. The number and distribution of neutrophils and macrophages are affected in *meis1* MO-injected embryos. (A-C) At 28 hpf in *meis1* atg MO-injected embryos (B) a mean of 7.1±1.5 (mean±SEM) *l-plastin*-positive cells was observed at the posterior blood island by comparison to 23.6±1.6 (mean±SEM) cells in control MO-injected embryos (A). A similar phenotype was observed in *meis1* splice MO-injected embryos (8.5±1.3, mean±SEM) (C). (D) Graph to illustrate the difference in the mean number of *l-plastin*-positive cells in control MO, *meis1* atg MO and splice MO-injected embryos. (E-G) At 48 hpf a 67% reduction in the total number of Sudan black-positive cells in *meis1* atg (F) and splice (G) MO-injected embryos was observed in comparison with the control (E). (H) Graph to illustrate the difference in mean number of Sudan black-positive cells in control MO, *meis1* atg MO and splice MO-injected embryos. Error bars are the standard error of the mean (SEM). (A'-B') Distribution of the primitive macrophages at 24 hpf in *Tg(fli1:EGFP)* embryos. Lateral view of the head and yolk sac in control (A') and *meis1* atg MO-injected embryos (B'). Higher magnification view of the Sudan black staining in the ventral vein region in a control (C') and *meis1* MO-injected embryos (D') at 3.5 dpf (E'-F'). Whole-mount *in situ* hybridization for *rag-1* transcripts in control (E') and morphant embryos (F') at 4 dpf. Bilateral *rag-1* expression in the thymus is indicated by white arrows.

Online Supplementary Figure S8. *Meis1* depletion leads to impaired vascular development. (A-F) Transverse view of the vessels in the trunk region of *Tg(fli1:EGFP)* embryos at 30 hpf. Two distinct lumenized vessels (dorsal aorta, DA; posterior caudal vein, PCV) can be observed in control (A,D) and *sih* (B,E) embryos. However, vascular tube formation is perturbed in *meis1* MO-injected embryos. At most locations in *meis1* MO-injected embryos, the DA is apparently fused with the PCV (C,F).



Online Supplementary Figure S9. Transverse agarose sections (250 µm thick) of a *Tg(fli1:EGFP)* embryo at 30 hpf visualized for EGFP (green) and Ephb2 (red) in control (A) and *meis1* MO-injected (B) embryos. The arrow marks Ephb2 expression within the dorsal aorta. *Meis1* MO-injected embryos show a severe decrease in Ephb2 (B). Dorsal side up; DA, dorsal aorta; PCV, posterior caudal vein.



Online Supplementary Figure S10. Lateral view of a *Tg(fli1:EGFP)* embryo at 30 hpf. Dual immunohistological detection of EGFP (green) and *in situ* hybridization detection of *flt4* (red) in control (A) and *meis1* MO-injected (B) embryos. At most locations in *meis1* MO-injected embryos, the dorsal aorta (DA) is apparently fused with the posterior caudal vein (PCV) and it expresses the venous marker flt4 (B). Anterior to the left, dorsal side up in all images.