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Supplemental Information

Accumulation of the Vitamin D Precursor Cholecalciferol Antagonizes Hedgehog Signaling to Impair Hemogenic Endothelium Formation

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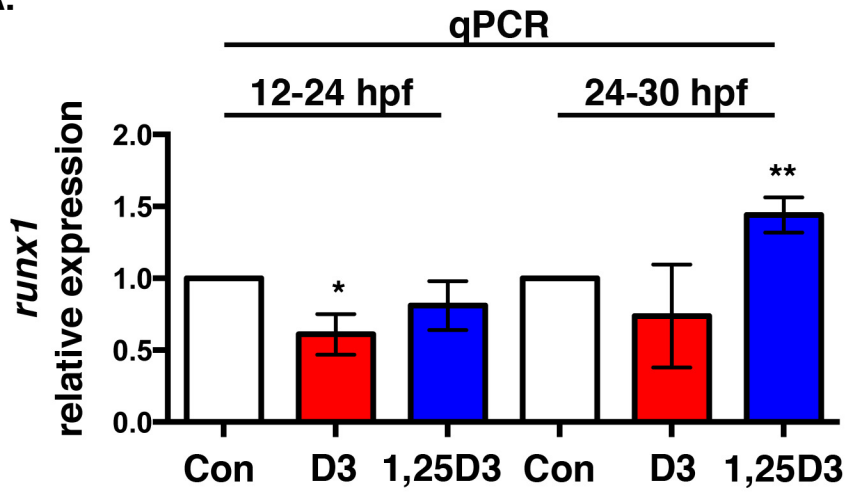
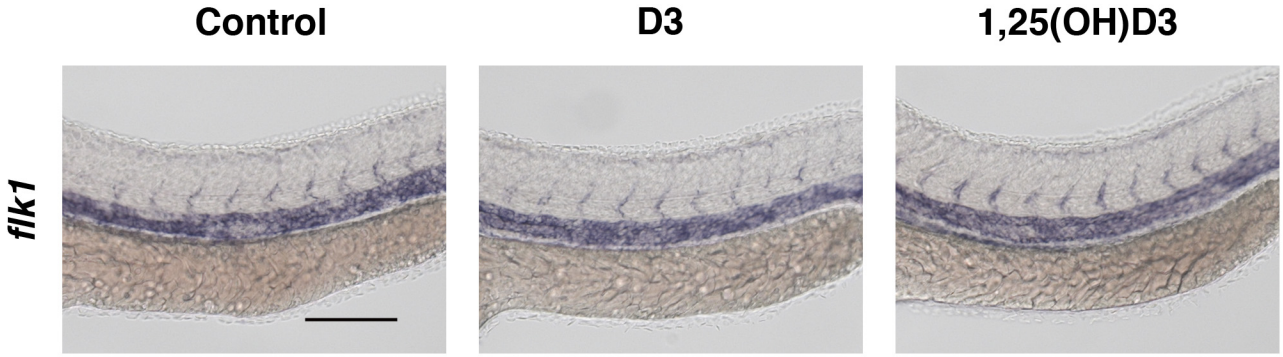
Supplemental Figure 1.

A.

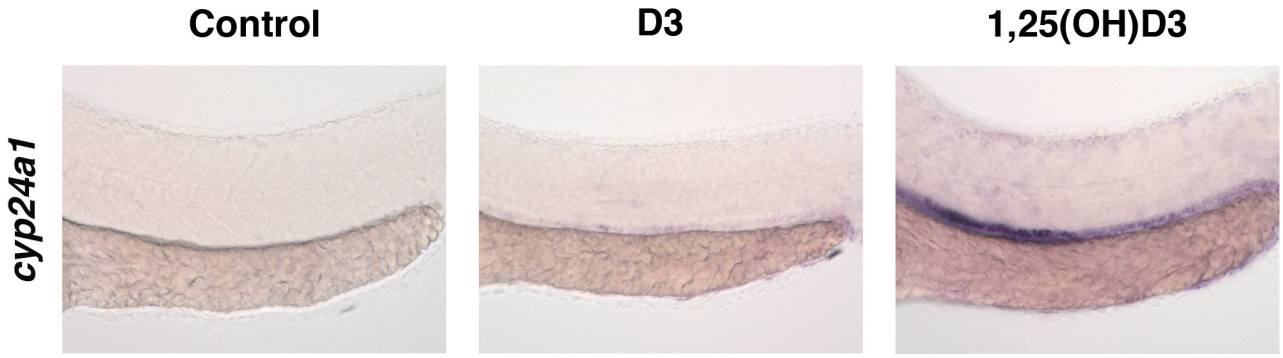


B.



12-36 hpf

C.



12-36 hpf

Figure S1. Vitamin D3 intermediates differentially affect HSPC expression and VDR activation (*related to Figure 1*)

A. qPCR analysis confirmed D3 significantly decreased *runx1* expression only when treatment occurred during hemogenic niche formation (12-24hpf) (*p=0.014); in contrast, 1,25(OH)D3 increased *runx1* expression (24-30hpf: **p=0.003) (40 pooled embryos/condition x 3 replicates).

B. WISH for *flk1* indicated normal vasculature in embryos treated with either D3 or 1,25(OH)D3 from 12-36hpf (n>25 embryos/condition); scale bar =100μm.

C. Embryos treated with 1,25(OH)D3 showed differential regulation of the known VDR transcriptional target *cyp24a1* compared with those exposed to D3 from 12-36hpf (n>20 embryos/condition); scale as S1B.

Supplemental Figure 2.

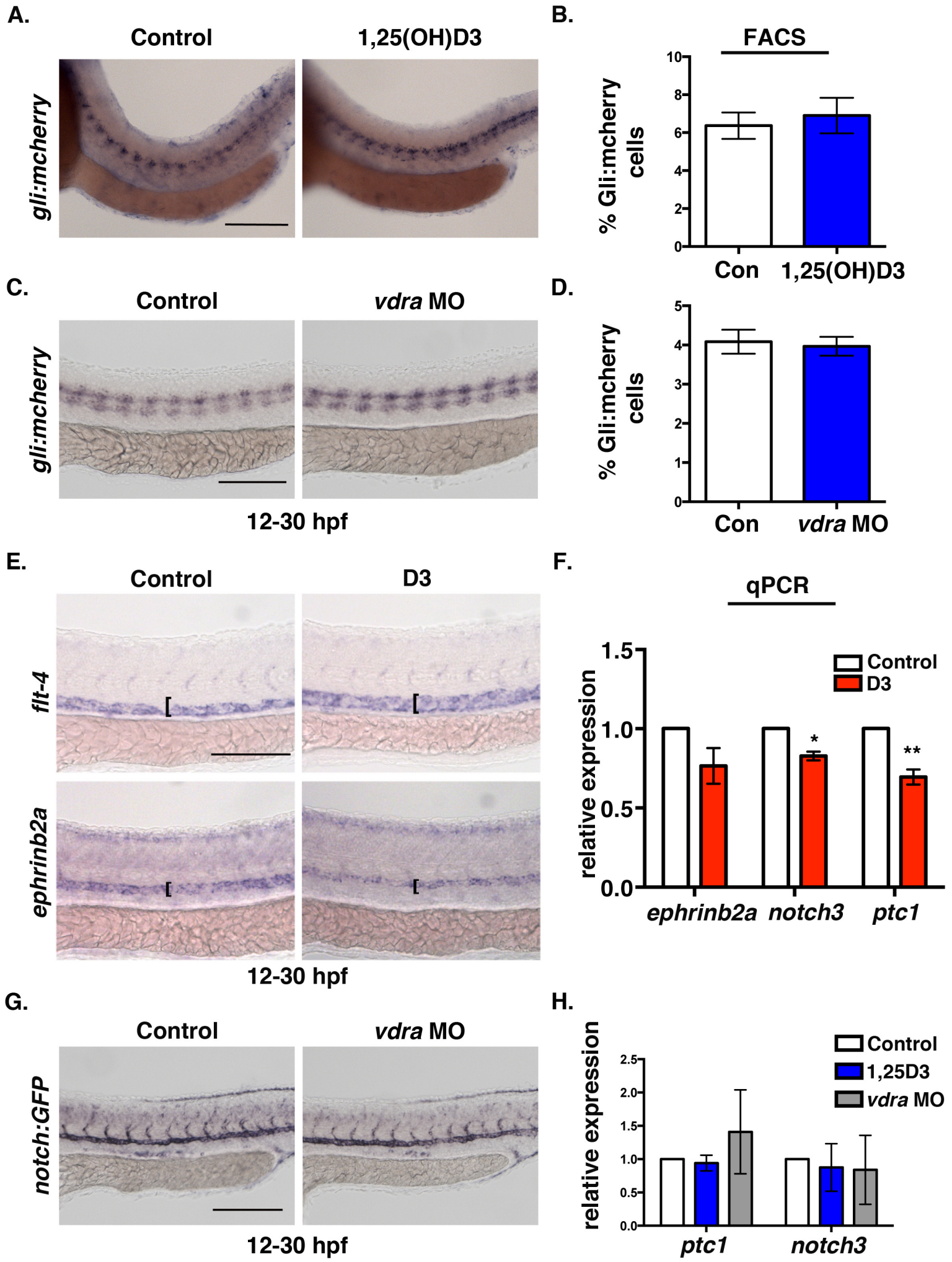


Figure S2. D3, but not 1,25(OH)D3, negatively impacts Hedgehog activity and vascular specification (*related to Figure 2*)

A. WISH analysis of Gli:mCherry embryos showed no significant decrease in Hh activity with 1,25(OH)D3 treatment (n>20 embryos/condition); scale bar =200μm.

B. FACS quantification confirmed the Gli:mCherry expression was unaltered by 1,25(OH)D3 (p=0.396) (5 embryos per sample x 4 replicates/condition).

C. WISH analysis of Gli:mCherry embryos showed no significant impact on Hh activity with *vdra* knockdown (n>20 embryos/condition); scale bar =100μm.

D. FACS quantification confirmed that Gli:mCherry expression was unaltered in *vdra* morphants (p=0.53) (n-value as in S2B).

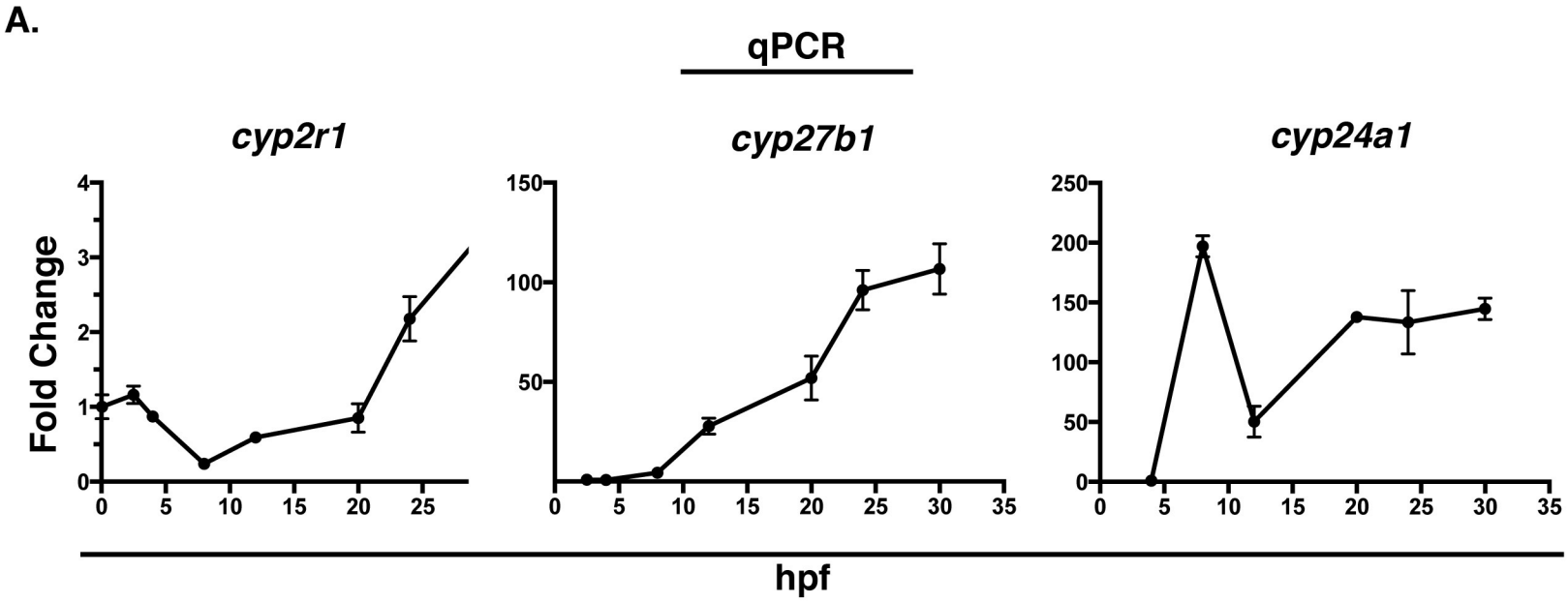
E. Embryos treated with D3 (12-30hpf) showed expansion (black brackets) of the venous marker *flt-4 (upper panels)* and reduced/restricted expression of the arterial marker *ephrinb2a (lower panels)* (n>20 embryos/condition); scale bar =100μm.

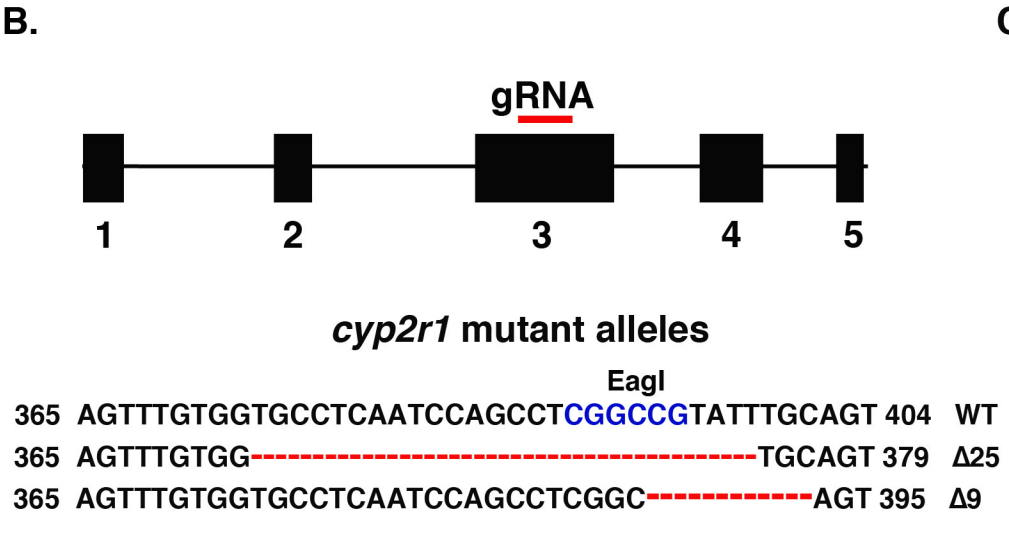
F. qPCR analysis of D3-treated embryos confirmed reduced expression of arterial markers *ephrinb2a* and *notch3* (one-tailed t-test, *p=0.012), along with the hedgehog marker *ptc1* (one-tailed t-test, **p=0.013) (40 pooled embryos/condition x 3 replicates).

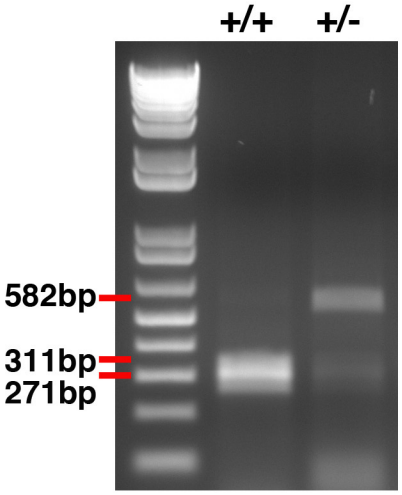
G. Notch-reporter embryos showed no impact on Notch activity by *vdra* knockdown as determined by WISH for *GFP* expression (n>20 embryos/condition); scale bar =100μm.

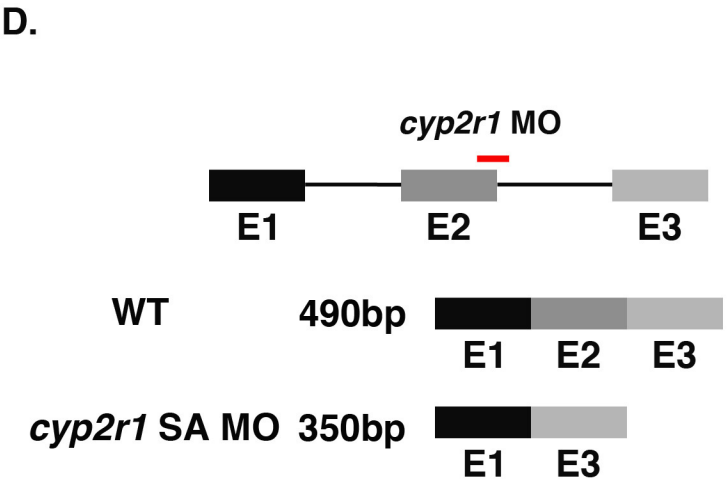
H. qPCR analysis of 1,25(OH)D3-treated or *vrda* morphant embryos confirmed that neither *ptc1* (one-tailed t-test, p=0.32) nor *notch3* (one-tailed t-test, p=0.62) were impacted by VDR modulation (n-value as in S2F).

Supplemental Figure 3.









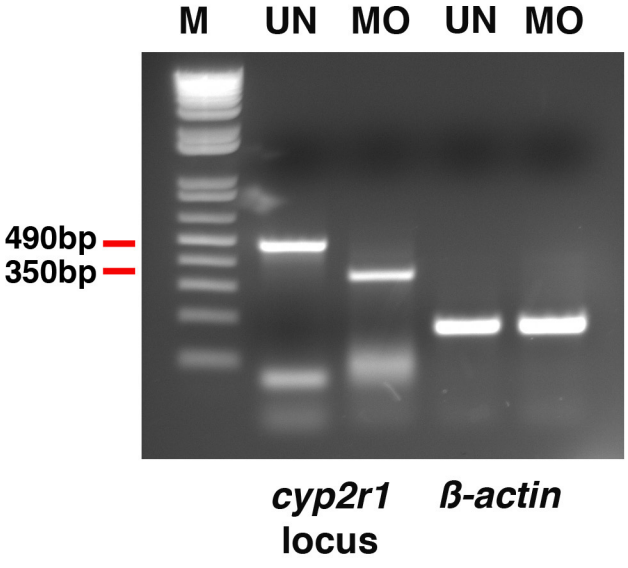


Figure S3. Targeting D3 hydroxylation via *cyp2r1* mutation and knockdown (*related to Figure 4*)

A. qPCR analysis indicated expression of *cyp2r1*, *cyp27b1*, and *cyp24a1* was upregulated by 24hpf, at the onset of circulation and definitive hematopoiesis (40 pooled embryos/ condition x 3 replicates).

B. Schematic representation of the CRISPR/cas9-mediated targeted deletions in *cyp2r1*.

C. Gel electrophoresis of depicting loss of EagI restriction site in *cyp2r1* heterozygote mutant compared to WT control sibling.

D. Schematic representation *cyp2r1*-MO targeting site and predicted spliced product.

E. Confirmation of MO-mediated knockdown of *cyp2r1* by efficient splicing of exon-3, as determined by PCR amplification of *cyp2r1* loci in control and MO-injected embryos.

Supplemental Figure 4.

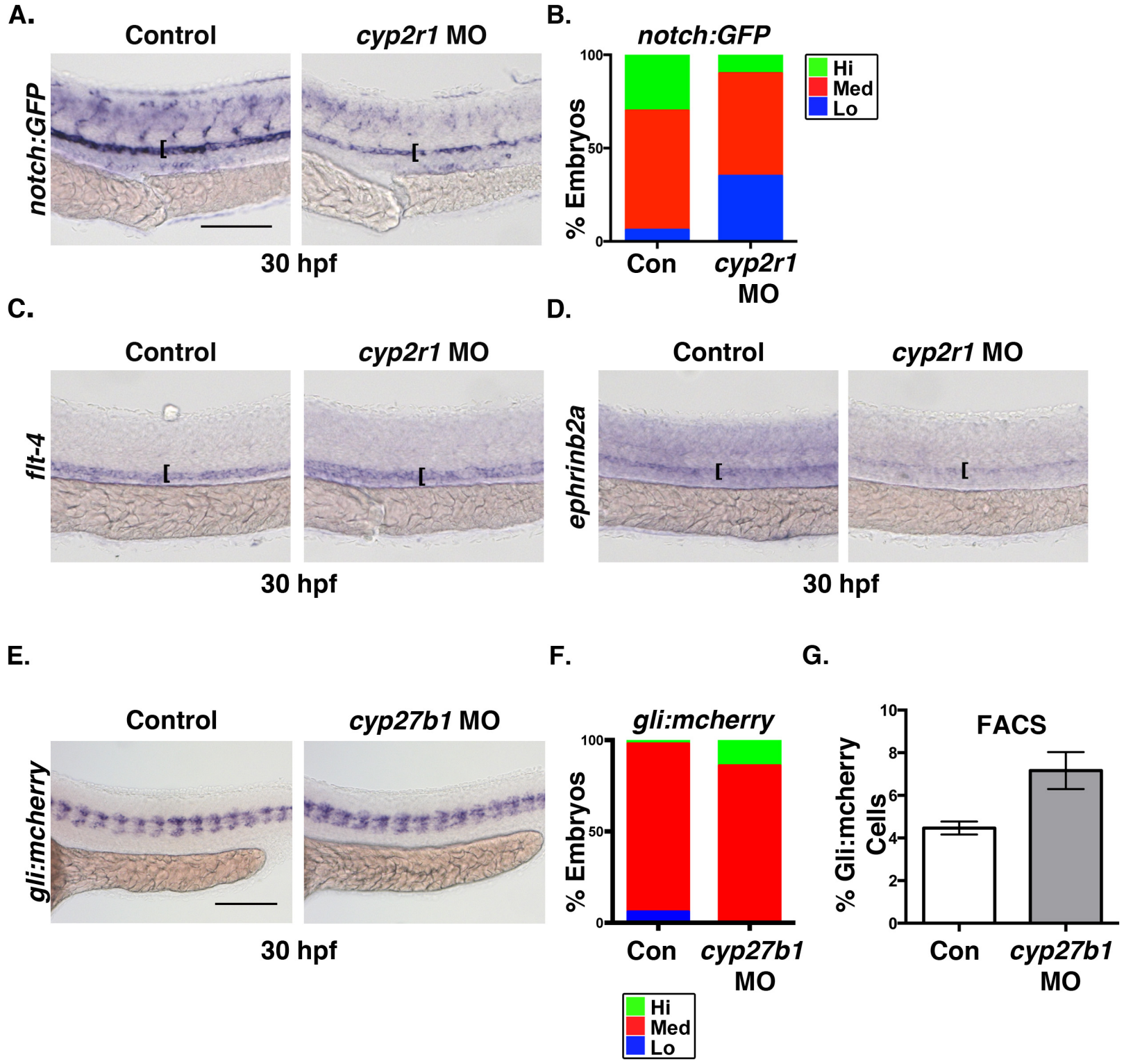


Figure S4. Reduction in *cyp2r1* function impairs Hh-mediated specification of the vascular niche (*related to Figure 4*)

A. *cyp2r1* morphants had reduced Notch-reporter activity in the AGM as determined by WISH; scale bar =100μm.

B. Qualitative phenotypic distribution of embryos from panel S4A scored for high, medium, and low Notch activity (n>35 embryos/condition).

C. *cyp2r1* morphants exhibited expanded *flt-4* (black brackets) expression at 30hpf (n>20embryos/condition); scale as S4A.

D *ephrinb2a* expression was reduced in *cyp2r1* morphants (n>20 embryos/condition); scale as S4A.

E. Morpholino knockdown of *cyp27b1* showed no significant impact on Gli-reporter activity by WISH; scale bar =200μm.

F. Qualitative phenotypic distribution of embryos from panel S4E scored for high, medium, and low Gli-reporter expression (n>35 embryos/condition).

G. Gli-reporter FACS analysis in *cyp27b1* morphants shows no alteration in Hh activity (5 embryos per sample x 4 replicates/condition).

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Table S1. Quantitative RT-PCR Primers

Gene	Forward	Reverse
18s	TCGCTAGTTGGCATCGTTTAT	CGGAGGTTCGAAGACGATCA
runx1	CGTCTTCACAAACCCTCCTCAA	GCTTTACTGCTTCATCCGGCT
ephrinB2	CAAGGACAGCAAATCGAATG	TGAGCCAATGACTGATGAGG
notch3	GCATTGACCGACCTAATGGA	TGCTCTCACACAGTCTTCCTTC
ptch2	TGACTATGCCCGTTCTCAAAG	CATCAAAAGTGGCTTGCAGAC
ptch1	TTATTTGTCTGCCTGGGTGAG	TGCATATTCGATGGGCTCAG