Supplemental Information

Supplemental methods

Measurement of Ca^{++} flux

VMSC were incubated in DMEM containing 2% FBS and 5 µM Fura-2-AM for 30 min. The cells were washed in medium warmed for 15 to 20 min to remove extracellular dye. They were re-cultured in HBSS with Ca^{++} (1.26 mM) and placed under a Zeiss microscope with environmental controls (37°C and 5% CO_2). The cells were stimulated with PE (20 µM) and were imaged for 5 min at a rate of 6 pictures per min. Excitation wavelengths were switched between 340 nm and 380 nm with illumination time of 180 ms, and emission wavelength was 510 nm. Signals from more than 15 randomly-selected cells were recorded and the results expressed as ratios of fluorescent intensity at 510 nm excited by 340 nm versus 380 nm.

Quantitative immunofluorescemce microscopy for Efnb1, Efnb2, Enb3, type 1a α adrenoreceptor and angiotensin II receptor Ia

VSMC were cultured in 24-well plates with cover glass placed at the bottom of the wells. After four to five days, the cells were washed twice with PBS and fixed with paraformaldehyde (4%) for 15 minutes. For cell surface Ag staining, cells were blocked with 10% goat IgG in PBS for 20 minutes and then incubated with various first Abs (2 µg/ml): goat anti-mouse EFNB1 Ab (R&D System, Minneapolis, MN, USA); goat anti-mouse EFNB2 Ab (R&D Systems); goat anti-mouse EFNB3 Ab (R&D Systems); rabbit anti-mouse type 1a α adrenoreceptor (α_{1a} -AR) Ab, (Abcam Inc., Cambridge, MA, USA); and rabbit anti-mouse AngII receptor Ia (ATR_{1a}) Ab (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4 °C.

Cells were then reacted with corresponding second Abs (i.e., rhodamine-conjugated donkey anti-goat Ab, 0.15 µg/ml, (Jackson ImmunoResearch Laboratories, West Grove, PA, USA); and FITC-conjugated sheep anti-rabbit IgG, 0.2 µg/ml, (Chemicon International, Billerica, MA, USA) for overnight at 4 °C. The stained cells were examined under a Zeiss microscope. The total fluorescent intensity of a cell and cell size were measured using software AxionVision (Zeiss) and the results are presented as arbitrary fluorescent intensity per unit cell area.

In situ hybridization (ISH)

A 1,700-bp fragment of EphB6 cDNA (positions 859 to 2,559) in the pGEM-3Z vector was used to generate sense and antisense riboprobes with SP6 and T7 RNA polymerase for both ^{35}S-UTP and ^{35}S-CTP incorporation. Tissues were frozen in -35°C isopentane and kept at -80°C until cut. ISH was performed on 10-µm cryostat sections. ISH microscopy was undertaken by photographic emulsion followed by 8-day exposure.

Supplemental tables

Supplemental Table I. Sequences of primers for RT-qPCR.
Supplemental Table II. siRNA sequences of Dishevelled, PDZ-RGS3, Grip1 and controls.

Legends to supplemental figures

Supplemental Figure 1. Plasmid construct used to generate β-actin promoter-driven Ephb6Δ (Ephb6 without its intracellular domain) Tg mice.

Ephb6Δ Tg mice were generated by linearized ClaI/ClaI fragment from plasmid pAC-Ephb6Δ. The fragment contained the human β-actin promoter followed by the Ephb6 coding sequence with the intracellular domain truncated, and the poly A sequence of β-actin.

Supplemental Figure 2. BP and HR of male and female Ephb6 KO mice.
BP and HR were measured for 3 days by radiotelemetry, starting at least 7 days after transmitter implantation. Mouse numbers per group are shown. Values are expressed as mean 24-h BP and HR for each day ± SE. SP: systolic pressure; DP: diastolic pressure; MAP: mean arterial pressure; HR: heart rate. Data were analyzed by repeated ANOVA, and the *p* values are reported.
A: males; B: females.

Supplemental Figure 3. Plasma and serum hormone levels in Ephb6 KO mice.
Plasma AngII (A) and serum aldosterone (B) of male, female and castrated EphB6 KO and WT mice were measured by ELISA. For castrated mice, samples were collected at more than 4 weeks after castration. Mouse numbers per group (n) are indicated. Means + SD of hormone concentrations are shown. No statistically significant differences were found (Student's *t* test).

Supplemental Figure 4. Castrated Ephb6Δ/KO mice present no BP increase.
BP and HR of male (A) or castrated (B) male Ephb6Δ/KO mice were measured by radiotelemetry as described in Fig. 3. Data were analyzed by repeated ANOVA, and the *p* values are reported.

Supplemental Figure 5. Ephb6 expression in the adrenal gland medulla according to ISH.
Med: medulla; Cx: cortex. Scale: 1 mm.

A. Adult adrenal gland labeled with anti-sense probe.
B. Adult adrenal gland labeled with sense probe.

Supplemental Figure 6. Expression of EFNBs, Ephb6, AR, AT1, and Grip1 in VSMC and endothelial cells.
A. Normal expression of Efnb1, Efnb2, Efnb3, type 1a α-adrenoreceptor *(AR) and AngII receptor (AT1) in Ephb6 KO VSMC according to immunofluorescence..*
VSMC from male, female and castrated male Ephb6 KO or WT mice were stained with Abs against Efnb1, Efnb2, Efnb3, AR or AT1 as indicated. VSMC were identified with anti-α-actin Ab staining. Normal goat IgG served as isotypic control. For each staining, more than 10 α-actin-positive cells were randomly selected and their total immunofluorescence intensity and cell size were recorded by Zeiss AxioVision software. The experiment was repeated at least twice. The means + SD of fluorescence intensity per arbitrary unit cell area of all cells examined (more than 10 per experiment) in a representative experiment are shown.

B and C. Efnbs, Ephb6 and Grip1 expression in endothelial cells according to q-PCR.
Efnb1, Efnb2, Efnb3 Ephb6 and Grip1 mRNA levels in freshly isolated mesenteric arteries with endothelium stripped (B) or endothelial cells from mesenteric arteries (C) of males, females and castrated males of WT or KO mice were determined by RT-qPCR in triplicate. The data are expressed as means $\pm$ SD of the ratios of the target gene signal versus the β-actin signal. No

significant difference between KO samples and their WT counterparts was found (Student's *t* tests), except Ephb6, which was deleted in KO mice.

D. Efnbs, Ephb6 and Grip1 expression according to q-PCR in VSMC after sex hormone treatment in vitro.

VSMC from female WT mice were cultured in the presence of testosterone (10 μg/ml) for 4 days. Conversely, VSMC from male WT mice were cultured in the presence of estrogen (10 μg/ml) for 4 days. The cells were harvested and their mRNA levels of Efnb1, Efnb2, Efnb2, Ephb6 and Grip1 were determined by RT-qPCR. The data are expressed as means $\pm$ SD of the ratios of the target gene signal versus the β-actin signal. No significant difference between KO samples and their WT counterparts was found (Student's *t* tests), except Ephb6, which was deleted in KO mice.

Supplemental Figure 7. Ca^{++} flux in PE-stimulated VSMC.

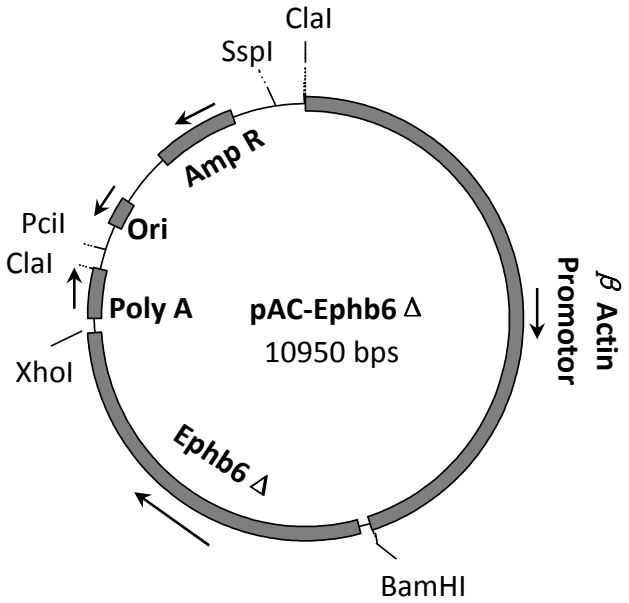
The experiments in this figure were repeated more than twice, and a representative dataset is shown.

A. Ca^{++} flux in PE-stimulated VSMC from WT and Ephb6 KO mice.

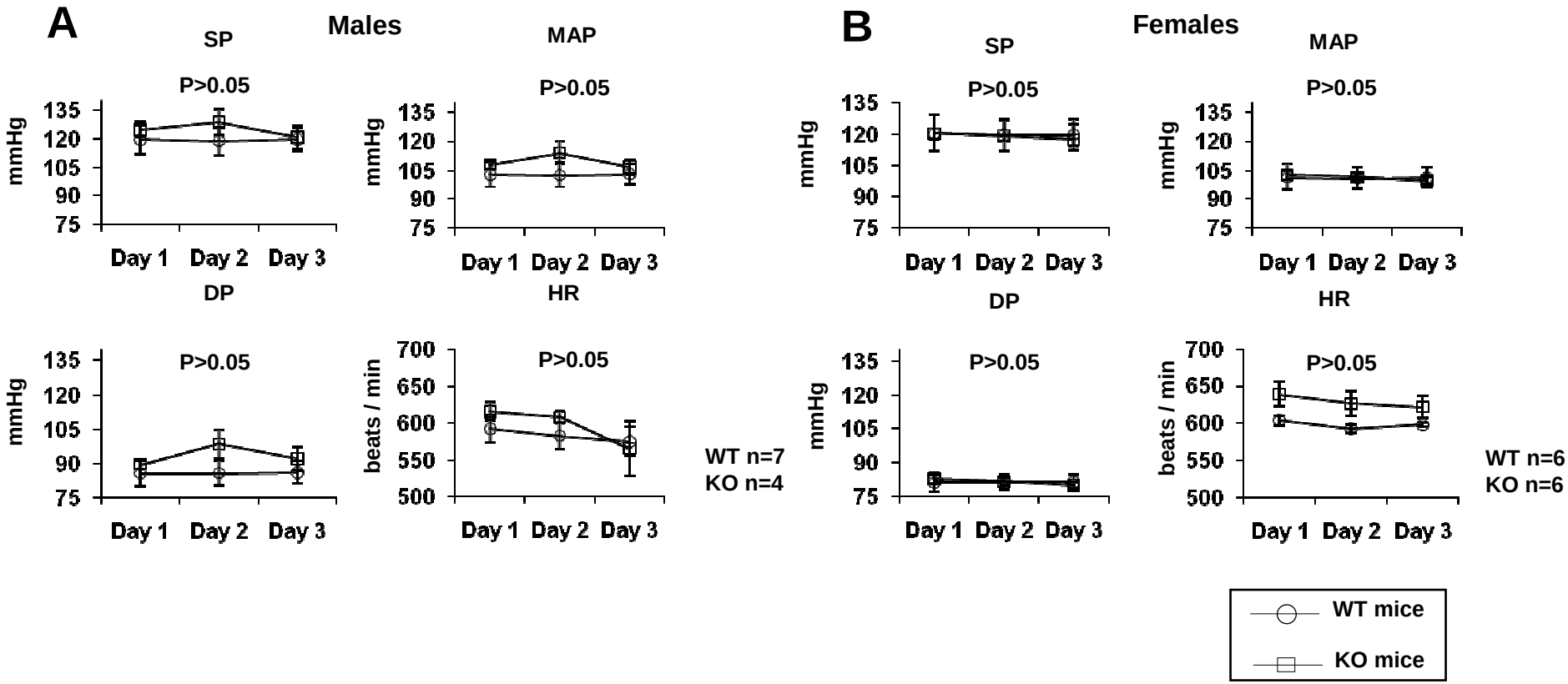
VSMC from male (left panel), female (middle panel) or castrated male (right panel) WT or Ephb6 KO mice were cultured for 4 days and then loaded with Fura2 (5 μM). They were then placed in HBSS containing 1.26 mM Ca^{++} at 37°C and stimulated with PE (20 μM). The ratio of emission at 510 nm triggered by 340 nm versus 380 nm excitation in each cell was registered every 10 s for 5 min, and the means + SD of the ratio of more than 15 randomly-selected VSMC are shown. Arrows indicate the time point when PE was added.

B. Ca^{++} flux in PE-stimulated WT VSMC cultured in wells coated with Ephb6-Fc.

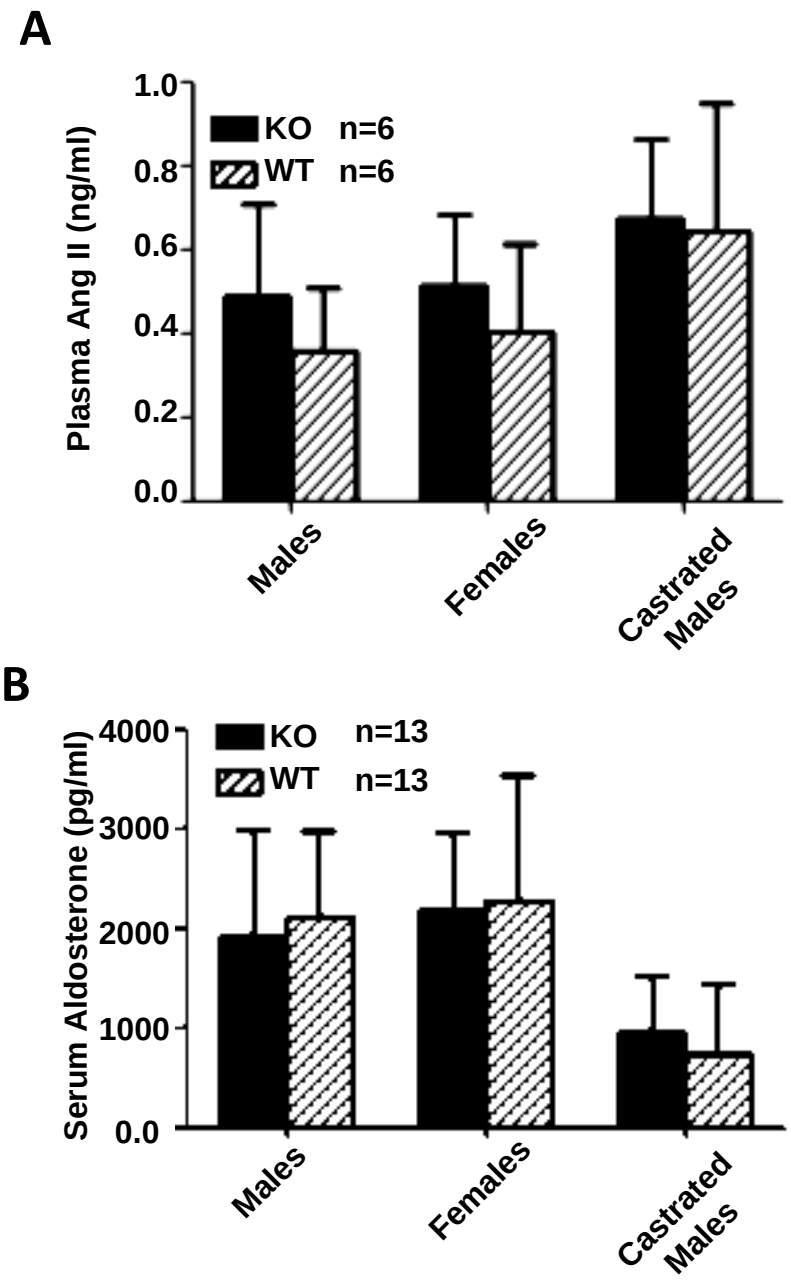
VSMC from WT male, female or castrated male mice were cultured for 4 days in wells coated with Ephb6-Fc or NHIgG (2 μg/ml during coating). Ca^{++} flux after PE stimulation (20 μM) was measured as described above.



Supplemental Figure 1



Supplemental Figure 2



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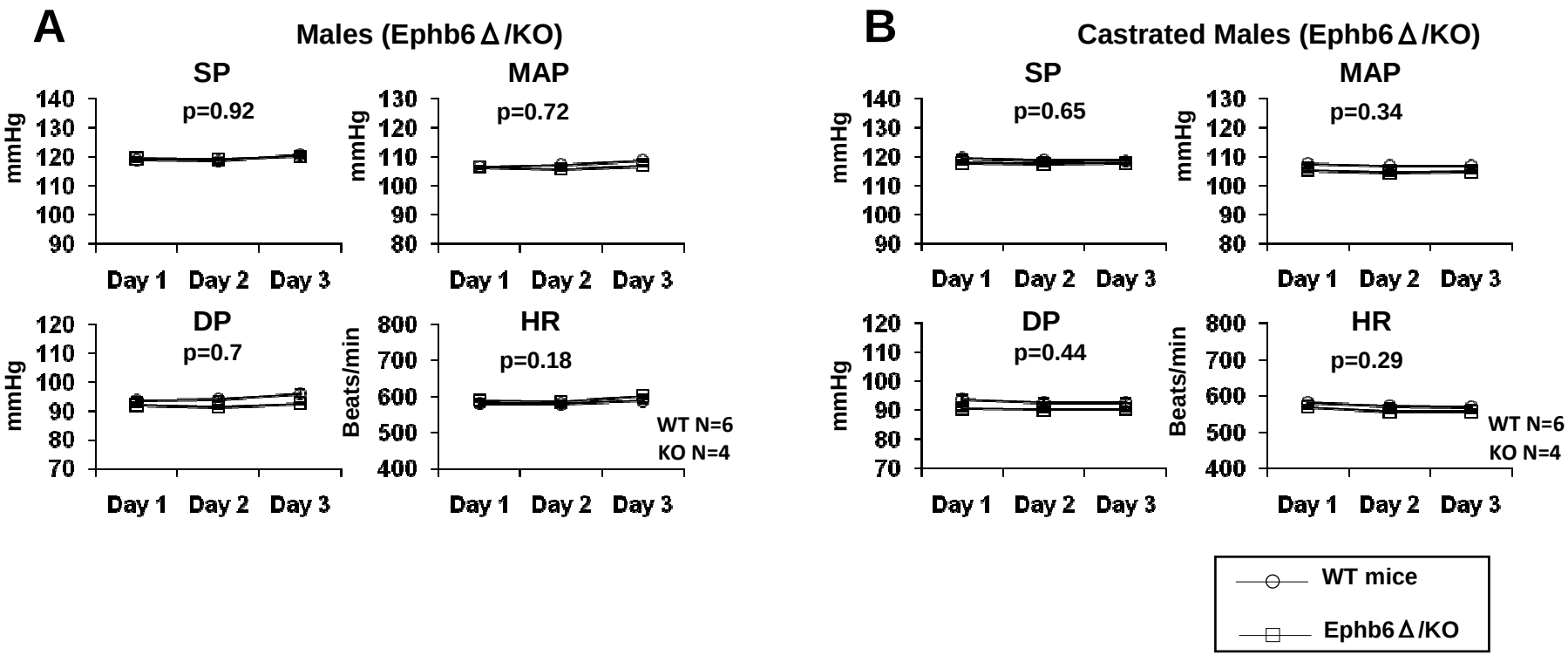

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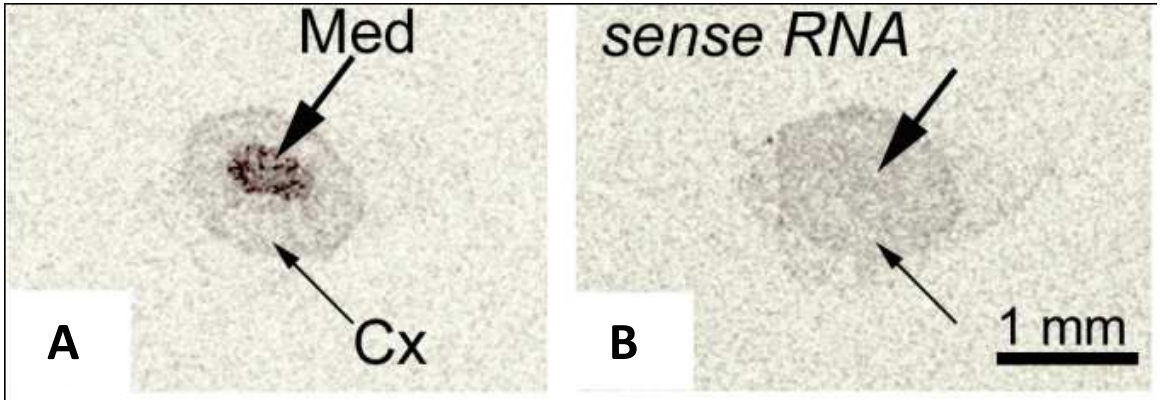

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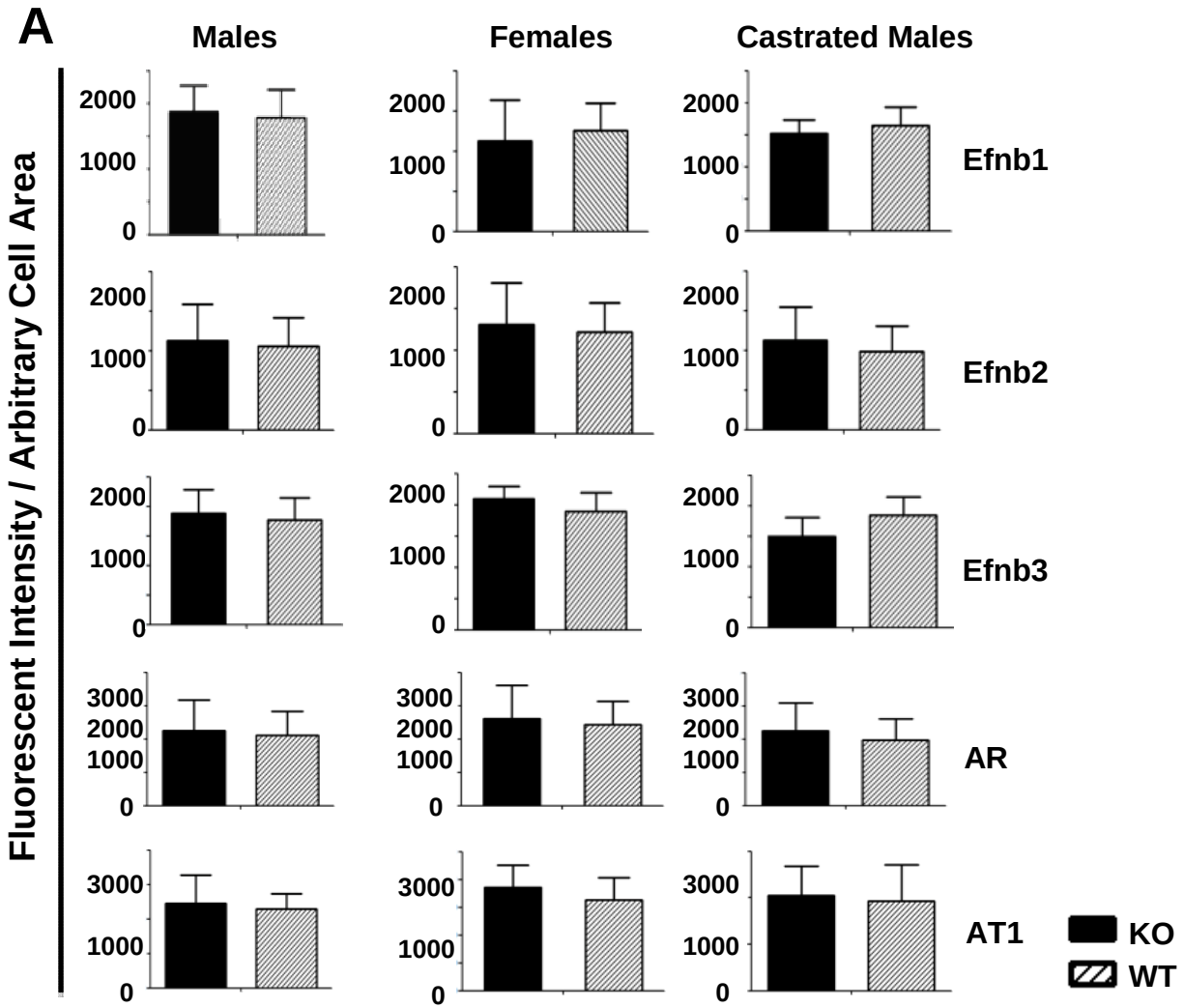
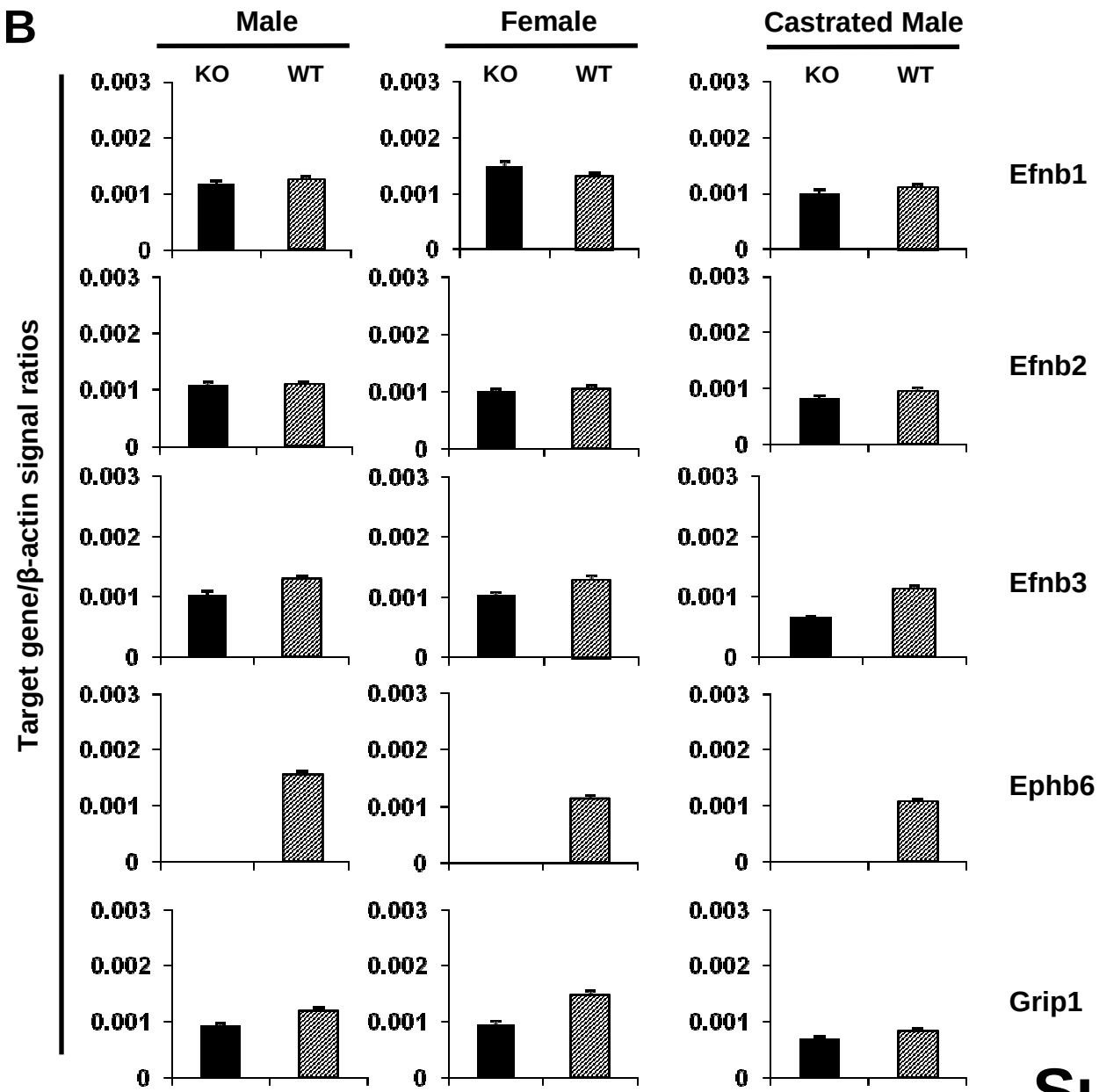


Supplemental Figure 6



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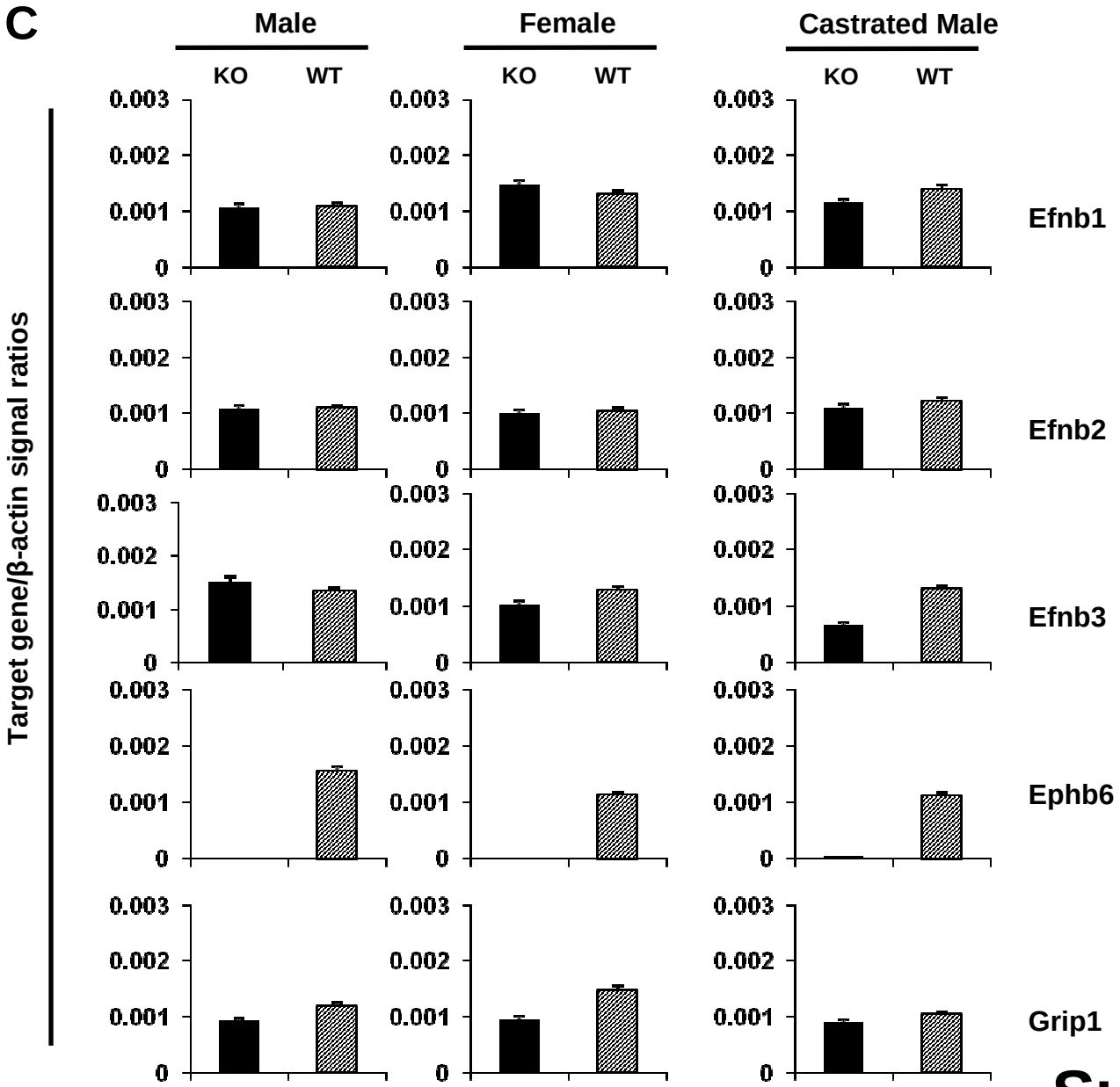

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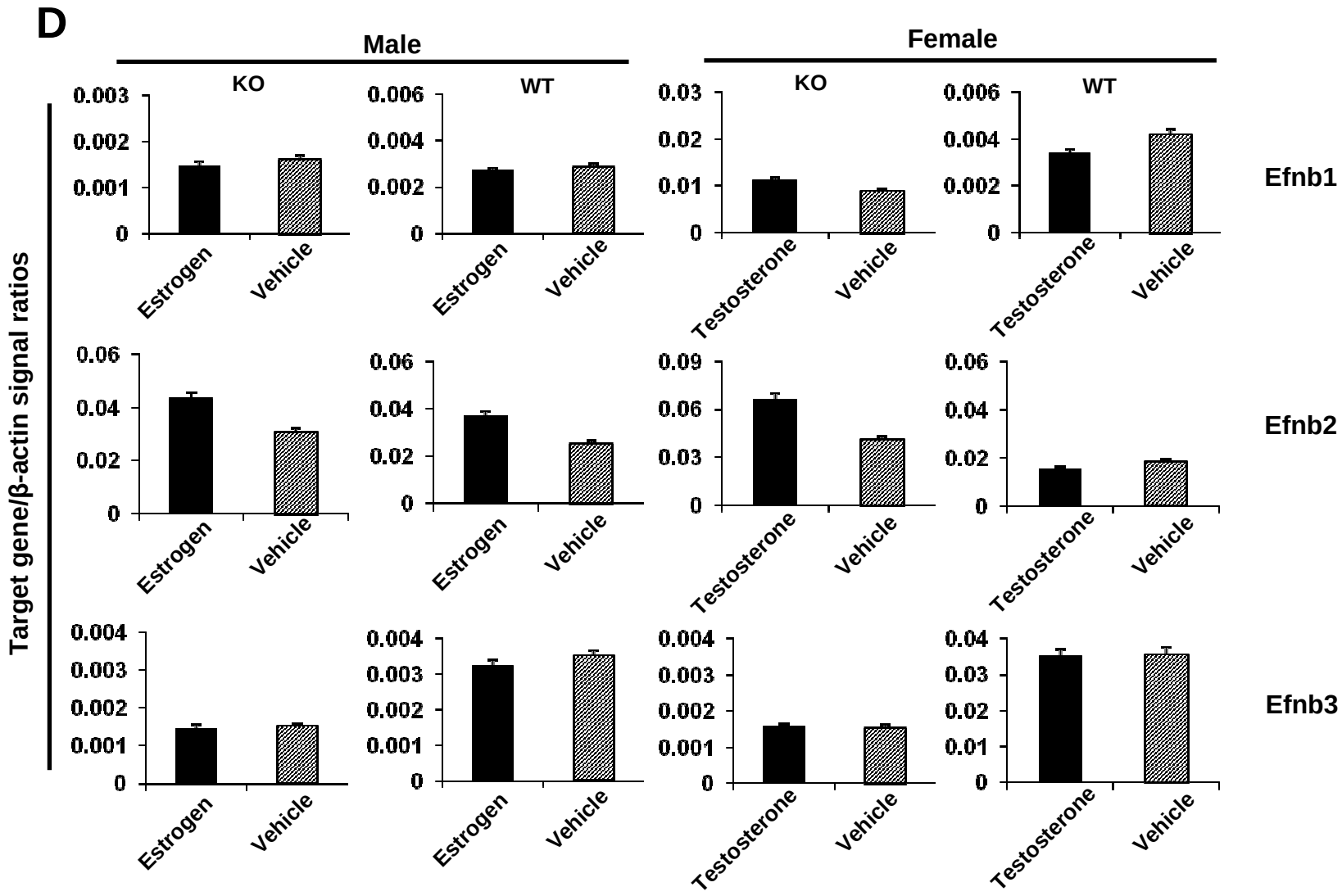

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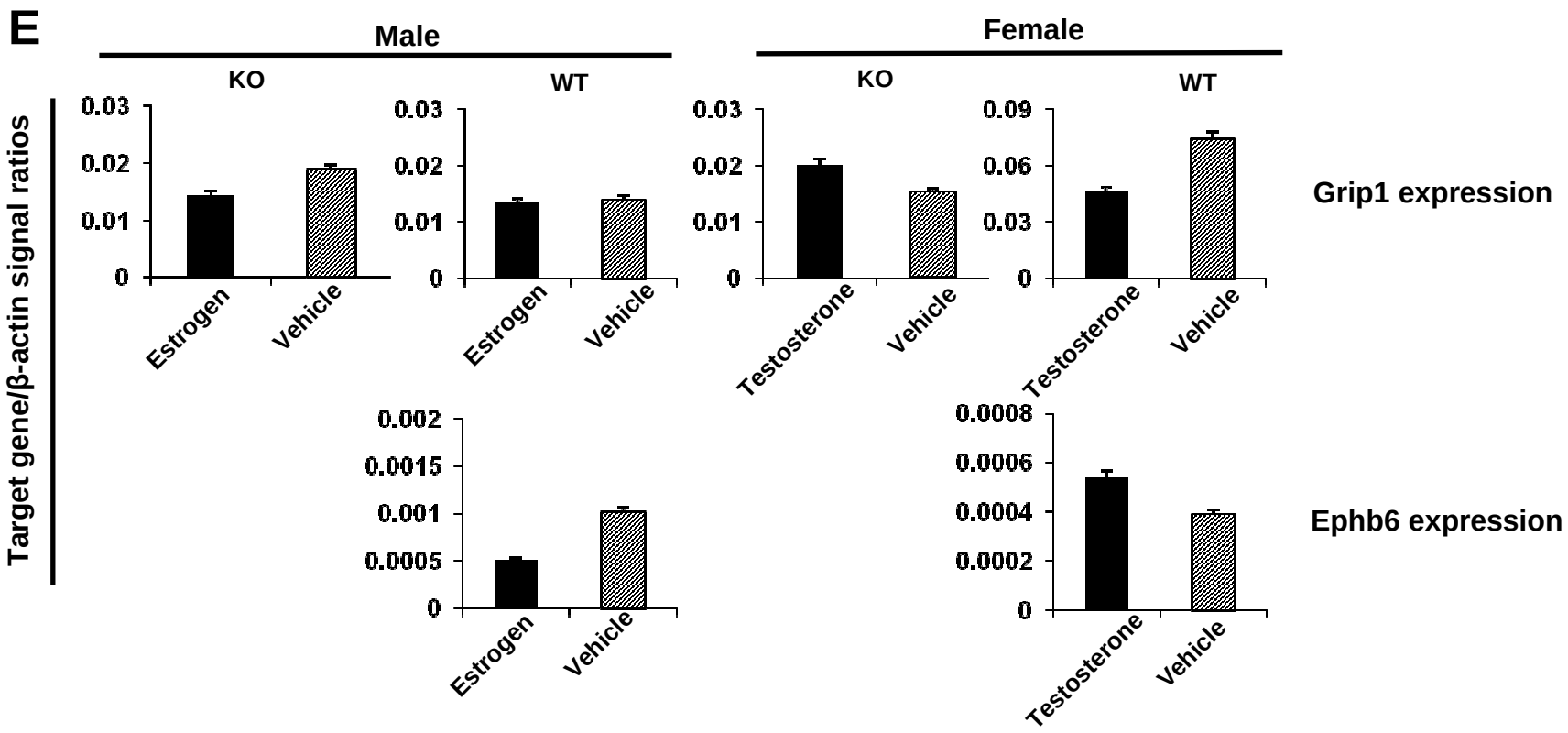

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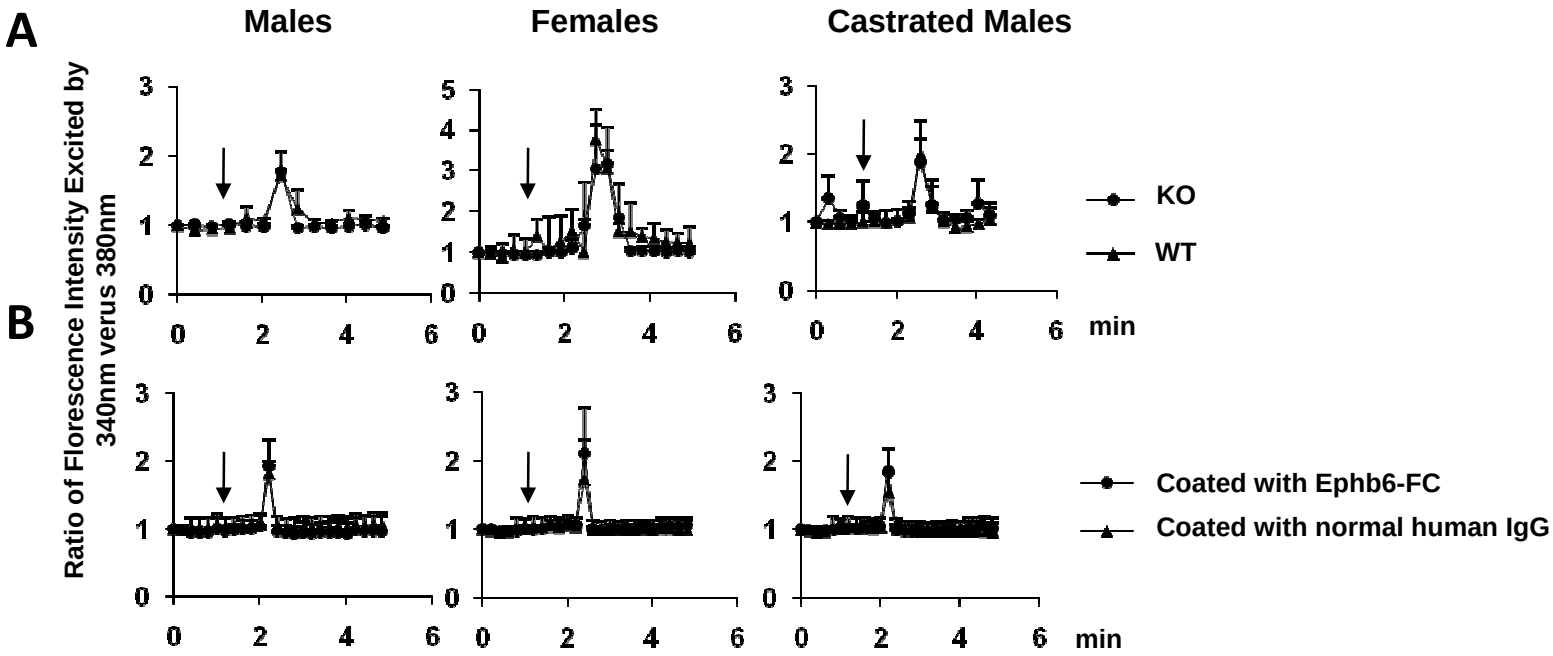

Supplemental Figure 7

Supplemental Table I. qPCR Primer Sequences

Gene	qPCR primer sequences		length of PCR products
	sense sequences	antisense sequences	
β-actin	5’-TCGTACCACAGGCATTGTGATGGA-3’	5’-TGATGTCACGCACGATTTCCCTCT-3’	200bp
Ephb6	5’-CTTTGCCTTTGTTCACCGAGCACT-3’	5’-AGCAAGGAACTTGAACCCTGAGGA-3’	111bp
Efnb1	5’-ACCAGGAAATCCGCTTCACCATCA-3’	5’-ACAGCATTTGGATCTTGCCCAACC-3’	199bp
Efnb2	5’-TTCTGCTGGATCAGCCAGGAATCA -3’	5’-TCCTGATGCGATCCCTGCGAATAA-3’	196bp
Efnb3	5’-AGTTCCGATCCCACCACGATTACT-3’	5’-AGAAGCACCTTCATGCCTCTGGTT -3’	112bp
Dishevelled 1	5'-TCTCGGCTAGTTCGGAAGCACAAA-3'	5'-TGATGTTCAGGGACATGGTGGAGT-3‘	112bp
PDZ-RGS3	5'-TGGCAACAGGAGGAACAGTGGTAT-3'	5'-ATGTCTTCCAGCAGGAATGGGTCA-3‘	170bp
Grip1	5'-ACAAGTCCCGTCCGGTTGTGATAA-3'	5'-TCTATCAGCAGCGTGGCTTCTTGT-3‘	181bp

Supplemental Table II. siRNA Sequences

Gene	sets of siRNA sequences	
	sense sequences	antisense sequences
Disheveled 1	5'-rGrCrU rArGrU rUrCrG rGrArA rGrCrA rCrArA rArUrG rCrCG T-3'	5'-rArCrG rGrCrA rUrUrU rGrUrG rCrUrU rCrCrG rArArC rUrArG rCrCrG-3'
	5'-rArCrA rGrCrU rCrArA rGrUrA rUrCrU rArUrA rGrArG rUrCT T-3'	5'-rArArG rArCrU rCrUrA rUrArG rArUrA rCrUrU rGrArG rCrUrG rUrArC-3'
	5'-rCrGrC rCrUrA rCrArA rArUrU rCrUrU rCrUrU rCrArA rGrUC C-3'	5'-rGrGrA rCrUrU rGrArA rGrArA rGrArA rUrUrU rGrUrA rGrGrC rGrUrG-3'
PDZ-RGS3	5'-rGrGrG rArGrA rGrArA rCrArC rCrArA rArUrA rArArU rCrAA C-3'	5'-rGrUrU rGrArU rUrUrA rUrUrU rGrGrU rGrUrU rCrUrC rUrCrC rCrUrG-3'
	5'-rGrCrC rArCrA rUrArA rArUrC rArCrG rCrUrA rArGrA rArGC A-3'	5'-rUrGrC rUrUrC rUrUrA rGrCrG rUrGrA rUrUrU rArUrG rUrGrG rCrArG-3'
	5'-rCrCrU rArCrA rGrArG rArGrG rArArG rArArA rCrUrU rCrAC C-3'	5'-rGrGrU rGrArA rGrUrU rUrCrU rUrCrC rUrCrU rCrUrG rUrArG rGrUrG-3'
Grip1	5'-rArGrA rUrArA rCrUrC rArGrA rCrGrA rGrCrA rArGrA rGrAG T-3'	5'-rArCrU rCrUrC rUrUrG rCrUrC rGrUrC rUrGrA rGrUrU rArUrC rUrUrC-3'
	5'-rArGrC rGrUrG rGrArA rCrUrU rGrGrA rArUrA rArCrC rArUC A-3'	5'-rUrGrA rUrGrG rUrUrA rUrUrC rCrArA rGrUrU rCrCrA rCrGrC rUrGrU-3'
	5'-rArCrA rCrUrA rGrArA rArUrC rGrArG rUrUrU rGrArU rGrUT G-3'	5'-rCrArA rCrArU rCrArA rArCrU rCrGrA rUrUrU rCrUrA rGrUrG rUrGrA-3'
Control	5’-rCrUrU rCrCrU rCrUrC rUrUrU rCrUrC rUrCrC rCrUrU rGrUG A-3’	5’-rUrCrA rCrArA rGrGrG rArGrA rGrArA rArGrA rGrArG rGrArA rGrGrA-3’

Supplemental Tables