

# **SUPPLEMENTAL MATERIAL**

## Data S1.

### Supplemental Methods

***Reagents:*** For immunohistochemistry, antibodies used were purified rat anti-mouse CD31 Clone MEC 13.3 from BD Pharmingen (550274), anti-SM22 alpha antibody from abcam (ab14106), purified rat anti-mouse Flk-1 Clone Avas 12alpha1 from BD Pharmingen (550549), anti-activated Notch1 antibody from Abcam (ab8925), Cleaved Caspase-3 (Asp175) antibody from Cell Signaling Technology (9661) and alexa fluor secondary antibodies (Life Technologies). Custom antibodies to RGS6<sup>1,2</sup> and ox-CaMKII<sup>3</sup> were prepared in our labs. Fluorescent mounting medium (ProLong® Diamond Antifade Mountant with DAPI) was purchased from Invitrogen (P36962). For western blotting, RGS6, ox-CaMKII, mouse anti-NICD (Developmental Studies Hybridoma Bank, University of Iowa) and GAPDH (Cell signaling Technology; 2118) antibodies were used.

***Mouse and embryo genotyping:*** Postnatal and embryonic mice were genotyped using the primers shown in supplementary **Table S1** and representative gel pictures are shown in **Figure S1**.

***Immunohistochemistry:*** All pregnant female mice were euthanized with CO<sub>2</sub> or isoflurane for isolation of embryos according to approved animal procedures. For immunostaining, embryos and placenta sections were deparaffinized using an automated staining platform (Leica Biosystem) followed by heat induced antigen retrieval at pH 9.0. Then sections were permeabilized in PBST (PBS plus 0.05% Triton X-100) and incubated in blocking buffer (5% goat serum in PBST) containing antibodies to FLK1, PECAM1, Caspase 3, Sm22, and NICD at a concentration of 1:100. Following washing four times in PBS (5 min each), sections were incubated for 1 h at room temperature with Alexa Fluor secondary antibodies<sup>4</sup>.

Whole-mount immunostaining by anti-PECAM1 antibodies (at 1:50 dilution) was performed with a modified protocol described previously<sup>4</sup>. In brief, embryos were fixed with 4% paraformaldehyde at 4°C overnight. Then embryos were washed in PBS twice with gentle shaking (each for 5 min) and dehydrated with 25% to 100% methanol in PBT (0.2% BSA, 0.1% Triton X-100 in PBS). Embryos were bleached (5% hydrogen peroxide in methanol) for 4-5 h, washed in PBST with gentle shaking and blocked with PBSMT (3% instant skim milk, 0.1% Triton X-100 in PBS) at room temperature for 2 h. Then embryos were incubated with PECAM1 antibody (dilution, 1:100) in PBSMT at 4°C for 2-3 days with gentle shaking, followed by incubation with Alexa fluor secondary antibodies (dilution 1:100) in PBSMT at 4°C for 1 day. Finally, embryos were transferred onto glass slides and mounted with anti-fade medium. Images were acquired using a BX61 microscope (Olympus) equipped with an HV-C20 TV camera (Hitachi) and were analyzed using ImageJ software. Dihydroethidium staining was performed in embryos section derived from RGS6<sup>+/+</sup>/CaMKII<sup>MM</sup>, RGS6<sup>-/-</sup>/CaMKII<sup>MM</sup> and RGS6<sup>-/-</sup>/CaMKII<sup>VV5</sup>.

qPCR: Embryos at E10.5 were dissected in PBS. Yolk sacs and hearts were removed, flash frozen in liquid nitrogen, and stored at -80°C until the genotype was determined. Total RNA was extracted from tissues including embryos, yolk sacs and hearts (4 hearts of the same genotype were pooled together) using Trizol (Invitrogen)<sup>6</sup>. One µg of DNase-treated RNAs was reverse transcribed using SuperScript III kit (Invitrogen). The resulting cDNA was used directly for qPCR using iQ SYBR Green Supermix (Bio-Rad) according to the manufacturer's protocol<sup>7</sup>. GAPDH and beta actin were used as an internal controls to normalize RNA levels. Primers used in qPCR analysis of all genes examined are given in Table S1<sup>8-10</sup>.

Western blot analysis: Western blotting was performed as described previously<sup>7</sup>. In brief, embryos were lysed in RIPA lysis buffer (150 mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl pH 8.0) containing protease and phosphatase inhibitors cocktail (Sigma P8340 and P5726). Lysates were centrifuged for 10 min at 12,000 x g

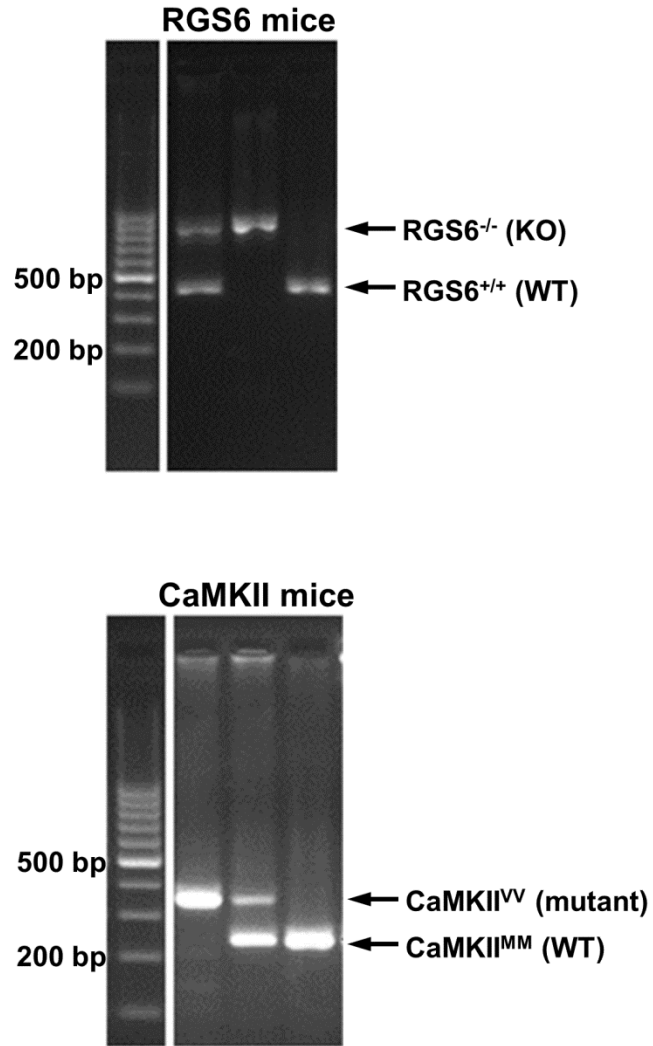
at 4°C. Protein concentrations were determined using DC™ protein assay reagents (Bio-Rad). Antibodies used were RGS6, ox-CaMKII, GAPDH, and N1ICD, described in the supplemental Reagents section. Western blot signals were recorded and measured using the Odyssey infrared imaging system (LI-COR Biosciences).

*Hematopoietic Colony-Forming Assay:* A hematopoietic colony-forming assay was performed as described by Wu et al<sup>4</sup>. Briefly, yolk sacs from RGS6<sup>-/-</sup>/CaMKII<sup>MM</sup> and RGS6<sup>-/-</sup>/CaMKII<sup>VV</sup> embryos were incubated at 37°C for 1 h in Hank's PBS solution containing 0.1% collagenase D, 10% FCS, and 1x Pen/Strep. The cells were then harvested and counted. Cells (1.5×10<sup>4</sup>) were seeded into 1 ml of methocellulose media supplemented with interleukin 3, interleukin 6 and stem cell factor (M3434, Stem Cell Technologies). Clones (>20 cells) were scored as Burst forming unit-erythroid (BFU-E) at day 7 of culture.

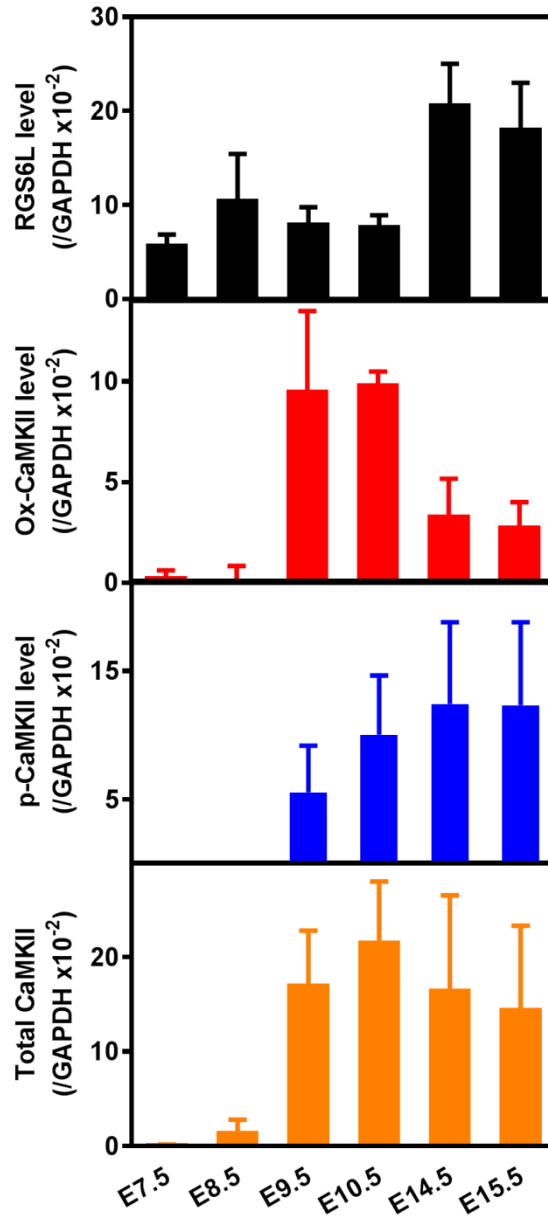
**Table S1. Primers used for qPCR and genotyping**

<b>Primers</b>	<b>Sequence (5' - 3')</b>
<b>Gja4</b>	Forward Primer: AGTGCCTCAGACCCTTACC
	Reverse Primer: GAGTGACATTAGCCCCAGAT
<b>Gja5</b>	Forward Primer: AGAGCCTGAAGAAGCCAACT
	Reverse Primer: GGCGTGGACACAAAGATGA
<b>VE-cadherin</b>	Forward Primer: AGGACAGCAACTTCACCCTCA
	Reverse Primer: AACTGCCCATACTTGACCGTG
<b>FLT1</b>	Forward Primer: GGGCAGACTCTTGTCCTCAACT
	Reverse Primer: CAGCTCATTGACACCCTCGT
<b>FLK1</b>	Forward Primer: ACAGACCCGGCCAAACAA
	Reverse Primer: TTCCCCCCTGGAAATCCTC
<b>Ephrinb2</b>	Forward Primer: GCGGGATCCAGGAGATCCCCACTTGGACT
	Reverse Primer: GTGCGCAACCTTCTCCTAAG
<b>EphB4</b>	Forward Primer: GCGGGATTCCAGCGCTCTGGACAAGATGAT
	Reverse Primer: CATCTCAAAGGAGCCGAATC
<b>Notch1</b>	Forward Primer: CTGACGCCCTTGCTCTGCCTAA
	Reverse Primer: AGTGCCACCATGGTCCACAACG
<b>Notch 3</b>	Forward Primer: CACTGAAGGCTCGTTCCA
	Reverse Primer: GGTTGCTCTCGCATTCA
<b>Jag1</b>	Forward Primer: GCCGAGGTCCTACACTTTGCT
	Reverse Primer: GTGGGCAATCCCTGTGTTTT
<b>Jag2</b>	Forward Primer: GATTGGCGGCTATTACTGTG
	Reverse Primer: AGGCAGTCGTCAATGTTCTC
<b>DLL4</b>	Forward Primer: TGACCACTTCGGCCACTATG
	Reverse Primer: AGTTGGAGCCGGTGAAGTTG
<b>Hey1</b>	Forward Primer: TGAGCTGAGAAGGCTGGTAC
	Reverse Primer: ACCCAAACCTCCGATAGTCC
<b>Hey2</b>	Forward Primer: TGAGAAGACTAGTGCCAACAGC
	Reverse Primer: TGGGCATCAAAGTAGCCTTTA
<b>Hey1L</b>	Forward Primer: GTGGCACAGGGTTCTTTGAT
	Reverse Primer: GCTGAGATAGGGTAAGGGGG
<b>c-Kit</b>	Forward Primer: GATCTGCTCTGCGTCCTGTT
	Reverse Primer: CTTGCAGATGGCTGAGACG
<b>Lmo2</b>	Forward Primer: CGAAAGGAAGAGCCTGGAC
	Reverse Primer: AGCGGTCCCCTATGTTCTG
<b>SCL</b>	Forward Primer: CCCGCAACTAGAGGGACA
	Reverse Primer: GCGCTCCTAGCTCGATGA
<b>GATA1</b>	Forward Primer: GAGCTGACTTTCCAGTCCTT
	Reverse Primer: CACACACTCTCTGGCCTCAC
<b>RUNX1</b>	Forward Primer: CTCCGTGCTACCCACTCACT
	Reverse Primer: ATGACGGTGACCAGAGTGC
<b>Beta globin</b>	Forward Primer: TTTCTACTCATTTTGCCAAGGAA
	Reverse Primer: CATTGGCCACTCCAATCAC

<b>Beta major</b>	Forward Primer: GTGACAAGCTGCATGTGGAT
	Reverse Primer: GTGAAATCCTTGCCCAGGT
<b>Tbx2</b>	Forward Primer: ACAGGGGAACAGTGGATGG
	Reverse Primer: CTTGTGCATGGAGTTCAGGA
<b>BMP2</b>	Forward Primer: AGATCTGTACCGCAGGCACT
	Reverse Primer: GTTCCTCCACGGCTTCTTC
<b>RGS6WT</b>	Forward Primer GAGGGAGTCATCATCGGTGCCATCGC
	Reverse Primer AACATGGTCTGAGATTGGGAAGATGTAGCC
<b>RGS6KO</b>	Reverse Primer GACTCTTCCACAACCTATCCAACCTCACAACGT
<b>CaMKII</b>	Forward Primer AAGGCCATCAGGTGATGCATGTCCTTAGTC
	Reverse Primer AAGTCTATCTGTCTGTCTGTCTCTCCCCTC

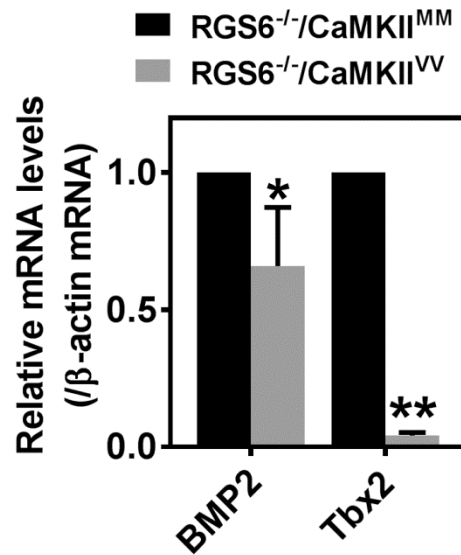


**Figure S1. RGS6 and CaMKII genotyping of mice and embryos.** Representative gel images used within the analysis of mouse RGS6 and CaMKII. Key bands used in the determination of RGS6 and CaMKII genotype are indicated to the right of each respective gel. A 100bp DNA ladder was used in this analysis. We have identified the 200bp and 500bp bands within the ladder at the left of each gel image. Primers required for this analysis can be found in Table S1.

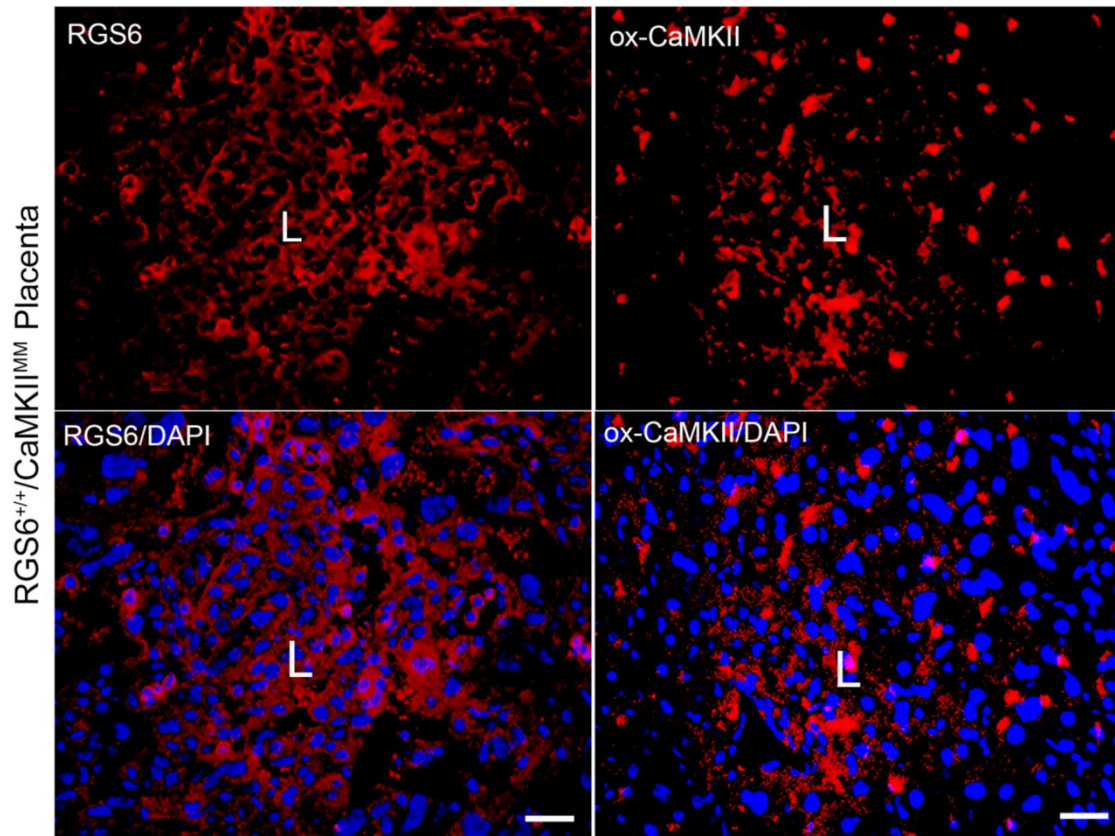


**Figure S2. Summary data of expression of RGS6, ox-CaMKII, p-CaMKII and total CaMKII in mouse embryos at different gestation periods.** Western blot analysis of RGS6, ox-CaMKII, p-CaMKII and total CaMKII expression levels in mouse embryos at different gestation stages was performed and quantified using ImageQuant 5.2 program (N = 3). Representative blot can be seen in **Figure 1A**.

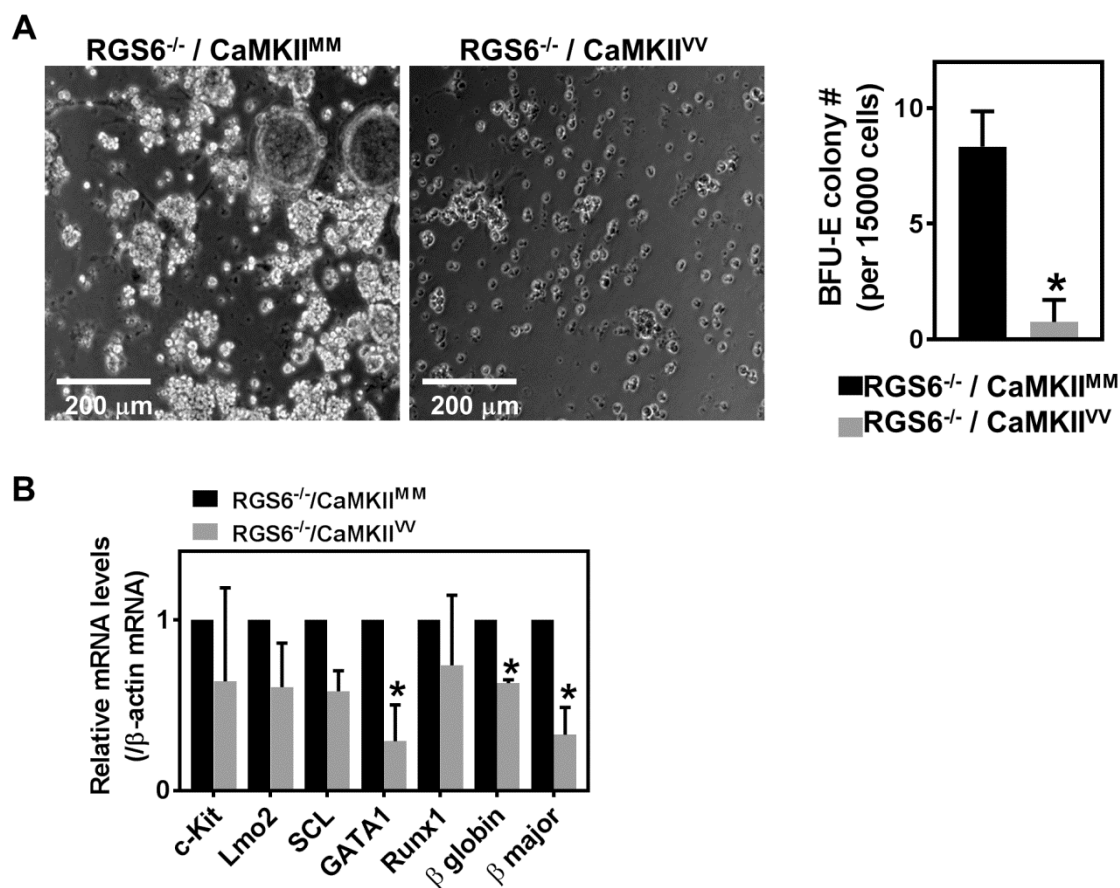




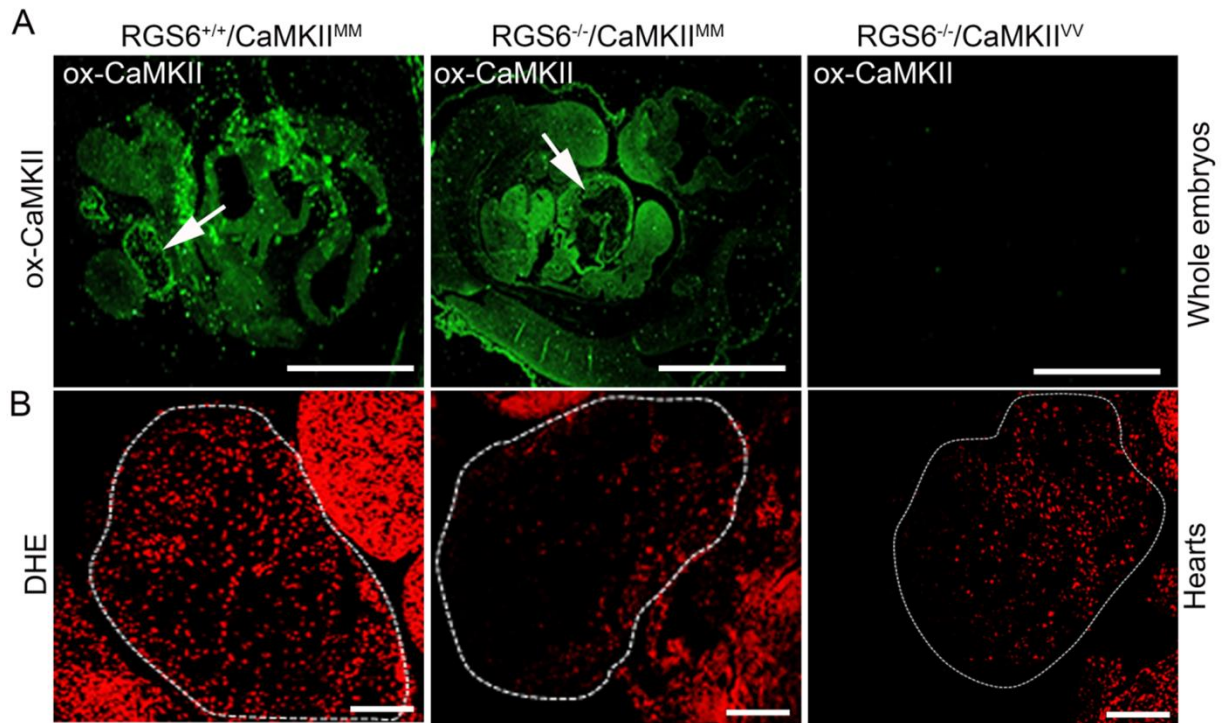
**Figure S3. Expression of BMP2 and Tbx2 in E10.5 hearts.** Analysis of BMP2 and Tbx2 expression, quantified via qPCR, revealed that Tbx2, but not BMP2, is significantly down-regulated in RGS6<sup>-/-</sup>/CaMKII<sup>VV</sup> hearts relative to RGS6<sup>-/-</sup>/CaMKII<sup>MM</sup> hearts. \* p < 0.05, \*\*p < 0.01.



**Figure S4. RGS6 and ox-CaMKII expression at E10.5 in  $RGS6^{+/+}/CaMKII^{MM}$  placentas.**  
RGS6 and ox-CaMKII (red) were expressed in the labyrinthine region of placentas as detected by immunohistochemistry. Scale bars = 20 $\mu$ m.

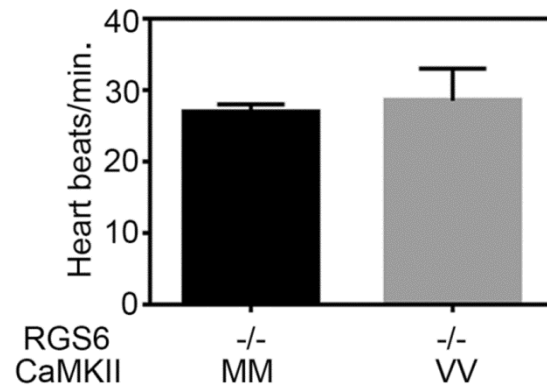


**Figure S5. Hematopoietic defects in RGS6<sup>-/-</sup>/CaMKII<sup>VV</sup> yolk sacs.** **(A)** *In vitro* differentiation analysis of yolk sac hematopoietic cells derived from RGS6<sup>-/-</sup>/CaMKII<sup>MM</sup> and RGS6<sup>-/-</sup>/CaMKII<sup>VV</sup> yolk sacs at E10.5 using methylcellulose colony assay. The number of BFU-E colonies (> 20 cells) produced from RGS6<sup>-/-</sup>/CaMKII<sup>MM</sup> and RGS6<sup>-/-</sup>/CaMKII<sup>VV</sup> yolk sacs were quantified after 7 days in culture, as shown in the representative images. This analysis revealed that the combined loss of RGS6 and ox-CaMKII reduces the ability of hematopoietic cells to form colonies. Scale bars = 50μm. N = 3. \*p < 0.01. **(B)** qPCR analysis of hematopoietic gene expression in E10.5 yolk sac revealed a significant reduction in the expression of the GATA1, β globin, and β-major genes in RGS6<sup>-/-</sup>/CaMKII<sup>VV</sup> yolk sacs relative to RGS6<sup>-/-</sup>/CaMKII<sup>MM</sup> yolk sacs. N = 3. \*p < 0.01.



**Figure S6. ox-CaMKII and ROS expression in hearts of embryos of different genotypes.**

**(A)** Detection of ox-CaMKII (green) levels in WT (RGS6<sup>+/+</sup>/CaMKII<sup>MM</sup>), RGS6<sup>-/-</sup> (RGS6<sup>-/-</sup>/CaMKII<sup>MM</sup>) and RGS6<sup>-/-</sup>/CaMKII<sup>VV</sup> double mutant embryo hearts (arrows) by immunostaining reveals that RGS6 loss does not alter ox-CaMKII expression. Note: embryos were not in same sectioning planes. Scale bars = 200μm. **(B)** Effects of combined loss of RGS6 and ox-CaMKII on superoxide (red) generation in hearts of embryos as shown by DHE staining. Scale bars = 50μm.



**Figure S7. Heart rates in  $RGS6^{-/-}/CaMKII^{MM}$  and  $RGS6^{-/-}/CaMKII^{VV}$  live embryos.** The heart rate of live E10.5 embryos was manually counted by viewing the embryo through a dissection microscope. This analysis revealed no significant difference in the heart rate of  $RGS6^{-/-}/CaMKII^{VV}$  double mutant embryos relative to  $RGS6^{-/-}/CaMKII^{MM}$  embryos. N = 3.

## Supplemental References:

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