

Crim1-GCE Allele Characterization

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Version Final

Findings: **VALIDATED**

Our analysis confirms the expression of Crim1 driven eGFP CreER^{T2} in a subset of cells in renal vasculature at 15.5 dpc. Native GFP expression was not detectable in our samples and could not be visualized by immunohistochemistry. Cre dependent R26R-LacZ expression was observed in a subset of PDGFR- β positive endothelial cells following Tamoxifen treatment.

Crosses

The Crim1-GCE strain was generated by homologous recombination in ES cells. The expectation is that integration of the Crim1-GCE DNA into the Cysteine rich transmembrane BMP regulator 1 (chordin-like) domain disrupts the allele however we did not intercross heterozygotes to confirm the null phenotype. We crossed two Crim1^{GCE/+} males with Rosa26R^{lacZ/+} (R26R) female mice and injected Tamoxifen in order to test correct expression of eGFP CreER^{T2} in Crim1^{GCE/+}; R26R^{lacZ/+} embryos at 15.5dpc.

Genotyping

Tail samples of the embryos were collected and incubated in tail digestion buffer overnight at 55°C. PCR was performed as per the protocol below and the PCR products were run on a 1.5% agarose gel (Fig 1).

Oligonucleotides: for Wt allele Size: 742bp

DNA sequence (forward): 5'-GTCGTCTTCCCCGGCAATC-3'

DNA sequence (reverse 1) 5'-CTTCGCAGACGCCACTTC-3'

Oligonucleotides: for targeted/transgenic allele Size: 471bp

DNA sequence (forward): 5'-GTCGTCTTCCCCGGCAATC-3'

DNA sequence (reverse 2) 5'-GTCCAGCTCGACCAGGATGG-3'

Amplifies 5' arm into GFP sequence within GFP-Cre region.

Rxn Buffer and Conditions: (25μl reaction)

10X GSB	2.5ul			
25mM dNTP	1ul	94°C	3min	1 cycle
10uM primer F	1ul	94°C	30sec	
10uM primer R1	1ul	56°C	30sec	35cycles
10uM primer R2	1ul	72°C	45sec	
DMSO	2.5ul	72°C	10min	1 cycle
2-mercaptoethanol	0.125ul			
Amplify Taq	0.3ul (5u/ml)			
5x cresol red dye	5ul			
Genomic DNA	1ul			

10X Gitschier Buffer (GSB):
670 mMTris, pH 8.8
166 mM Ammonium Sulfate
65 mM MgCl₂
0.1% gelatin

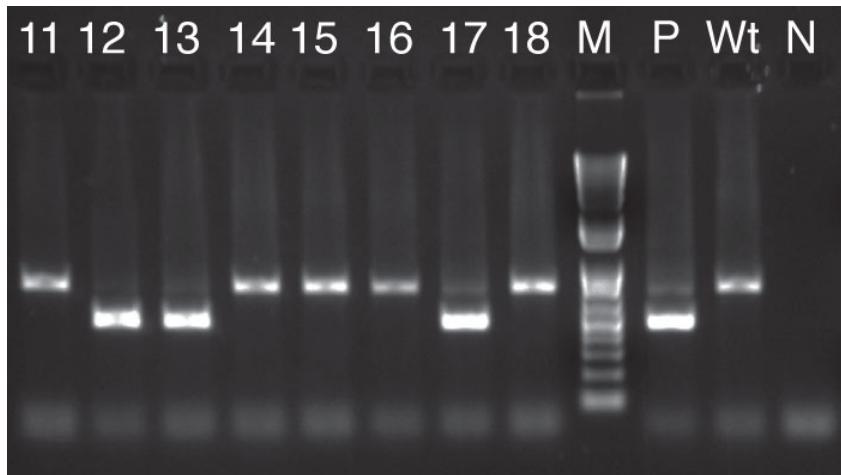


Fig1: Numbers 12, 13 and 17: $\text{Crim1}^{\text{GCE}/+}$, numbers 11, 14,15,16 and 18 Wildtype
P: $\text{Crim1}^{\text{GCE}/+}$ positive control; **W:** Wildtype control; **N:** Negative control.

Data:

Native Fluorescence

Whole embryos as well as dissected UGS samples were examined with a fluorescent microscope to view GFP expression. However, GFP was not detectable under these conditions.

Cre-recombinase Activity

$\text{Crim1}^{\text{GCE}/+}$ males were mated to $\text{Rosa26R}^{\text{lacZ}/+}$ (R26R) female mice to generate $\text{Crim1}^{\text{GCE}/+}; \text{Rosa26R}^{\text{lacZ}/+}$ embryos. In order to activate β -galactosidase (β -gal) reporter expression from the $\text{R26R}^{\text{lacZ}/+}$ allele, an intraperitoneal injection of Tamoxifen in corn oil was either: injected into pregnant female mice at 13.5dpc (2mg to 40g body weight) collecting the UGS at 15.5dpc or injected into pregnant female mice at 10.5 and 12.5dpc (2mg to 40g body) with collections at 15.5dpc. A control group was injected with the same volume of corn oil. Dissected UGS samples were stained with X-gal to assay for β -gal activity. Tamoxifen dependent Cre activity was detected in $\text{Crim1}^{\text{GCE}/+}; \text{R26R}^{\text{lacZ}/+}$ samples in the adrenal glands and renal vasculature (Fig. 2 and 3).

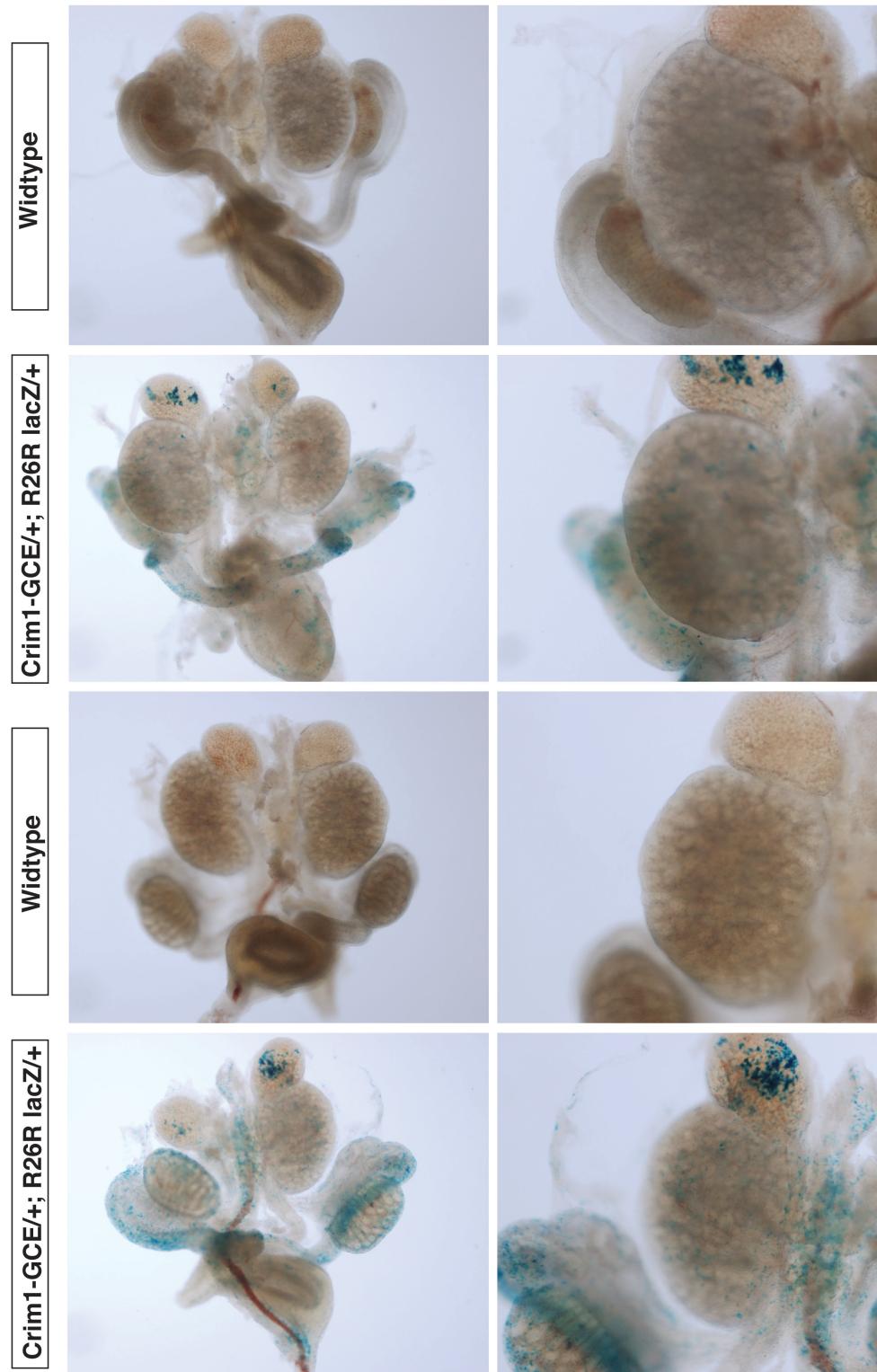


Fig 2. Cre-dependent β -gal activity in $\text{Crim1}^{\text{GCE}+/}; \text{R26R}^{\text{lacZ}+}$ UGS samples. Two doses of Tamoxifen (lower four panels: 2mg/40g) results in a small number of X-gal positive cells in the adrenal glands, kidney and the male and female reproductive systems in $\text{Crim1}^{\text{GCE}+/}; \text{R26R}^{\text{lacZ}+}$ embryos at 15.5dpc.

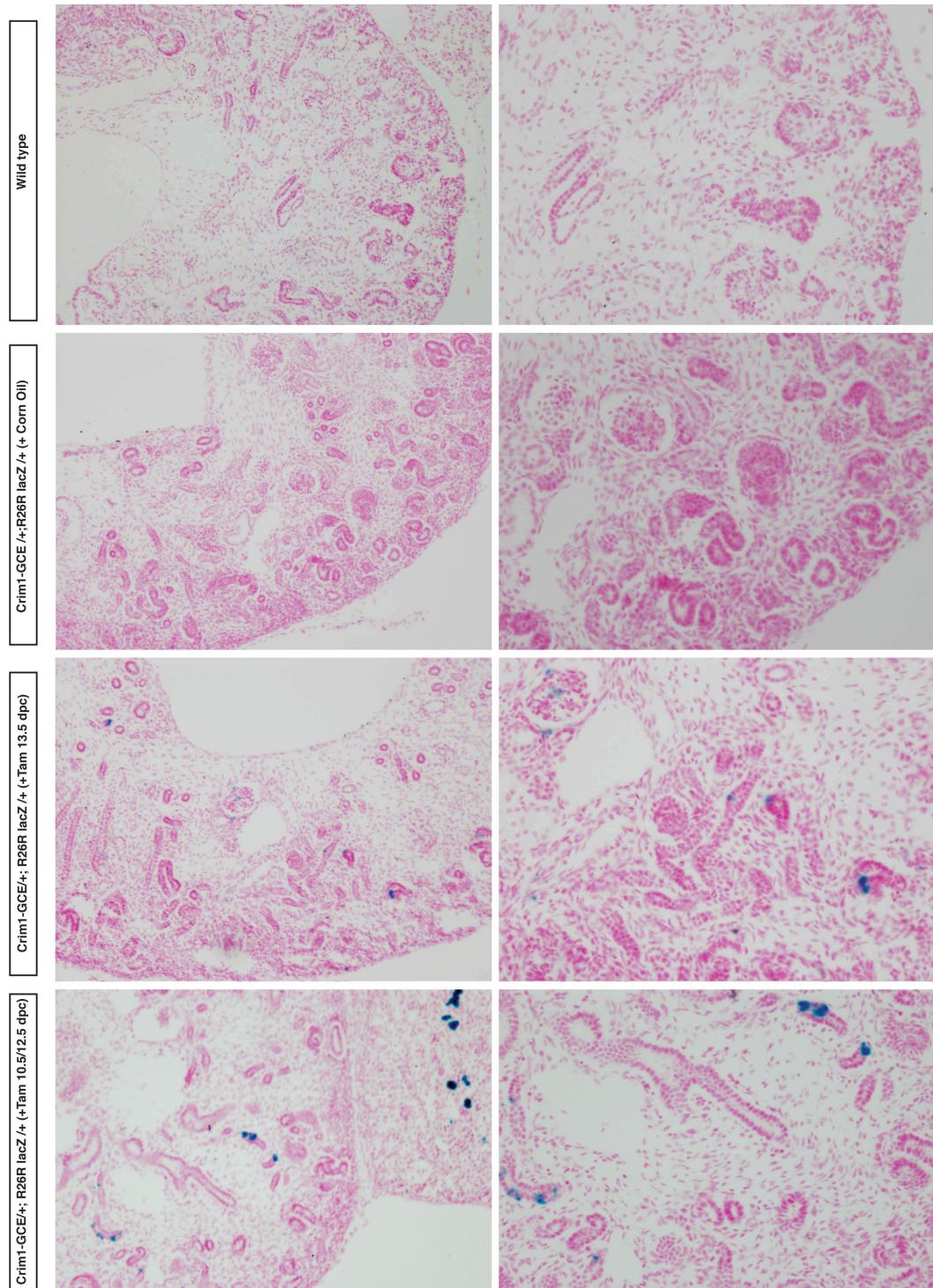


Fig 3. Cre-dependent β -gal activity in Crim1^{GCE/+}; R26R^{lacZ/+} 15.5dpc UGS samples.
A single dose of Tamoxifen at 13.5dpc results in a small number of X-gal positive cells in the microvasculature of Crim1^{GCE/+}; R26R^{lacZ/+} embryos at 15.5dpc. With two doses of Tamoxifen, given at 10.5 and 12.5dpc, the number of positive cells increased in the Adrenal gland and renal blood vessels.

Immunohistochemistry

Immunohistochemistry was performed to examine if the eGFP CreER^{T2} allele was expressed in the expected Crim1 domain. To test for Cre function, 15.5 dpc Crim1^{GCE/+}; R26R^{lacZ/+} and R26R^{lacZ/+} embryos from Tamoxifen and corn oil injected mice were assayed.

Whole UGS samples were fixed in 4% paraformaldehyde at 4°C 2 hours, washed 3 times in PBS, equilibrated in 30% sucrose overnight, embedded in OCT and flash frozen on dry ice. The samples were sectioned at 16 μ m and probed with chicken-anti-GFP/ rat-anti-Pecam CD31/ rabbit-anti- β -Gal and rabbit-anti- β -gal/chicken-anti-GFP/ Mouse anti-Smooth muscle Actin (Table 1).

Construct	Primary Antibody	Company	Catalog #	Dilution	Secondary	Company	Dilution
Crim1-GCE	Chicken-anti-GFP	Aves Labs, Inc	GFP-1020	1/500	Goat-anti-chicken-A488	Invitrogen	1/500
	Rabbit-anti- β -gal	MP Biomedicals, LLC	55976	1/20,000	Donkey-anti-rabbit-A555	Invitrogen	1/500
	Rat anti-PDGFR-beta-CD140b	eBiosciences	14-1402	1/100	Goat-anti-rat-A633	Invitrogen	1/250
	Rat-anti-Pecam CD31	BD Pharmingen	553370	1/1000	Goat-anti-rat-A633	Invitrogen	1/250
	Mouse anti-Actin, a-Smooth muscle IgG2a	Sigma	A5228	1/500	Goat-anti-mouse IgG2a-A633	Invitrogen	1/500

Table 1. Summary of antibodies used to screen Crim1^{GCE/+}; R26R^{lacZ/+} and R26R^{lacZ/+} 15.5 dpc embryo sections.

eGFP was not detected above background levels by immunohistochemistry in Crim1^{GCE/+}; R26R^{lacZ/+} embryos. Co-localization of a small number of β -gal positive cells were detected upon Tamoxifen induction in PDGFr-b and Smooth muscle actin positive cells in the microvasculature of the kidney (Fig 4 & 5).

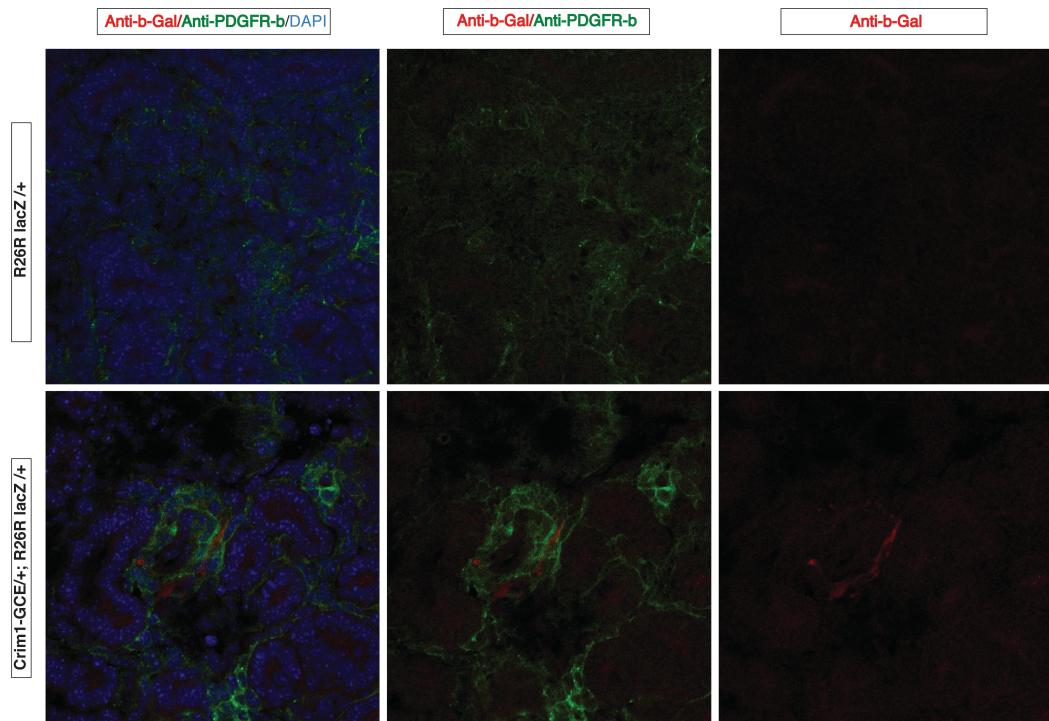


Fig 4. β -gal positive cells are detected in a subset of PDGFR- β positive endothelial cells lining microvasculature in Crim 1^{GCE/+}; R26R^{lacZ/+} embryos at 15.5dpc. Antibodies: rat-anti-PDGFR- β / rabbit-anti- β -gal.

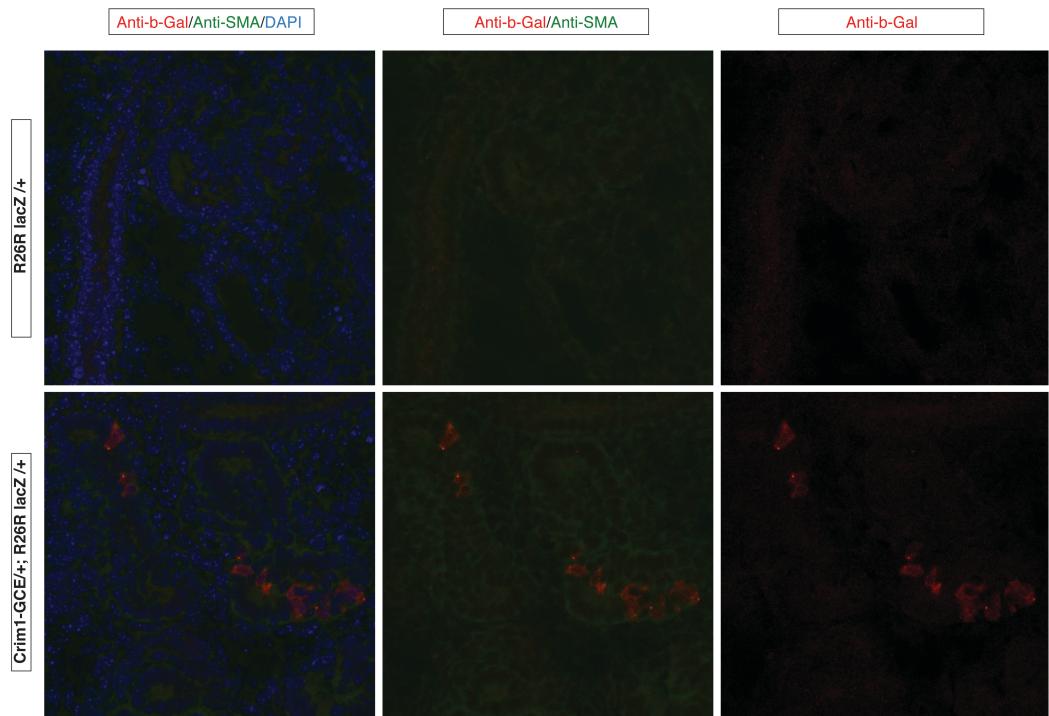


Fig 5. β -gal positive cells are detected in a percentage of Smooth muscle actin positive arterioles in Crim 1^{GCE/+}; R26R^{lacZ/+} embryos at 15.5dpc. Antibodies: mouse-anti-SMA/ rabbit-anti- β -gal