Spp2 ^{F2aeGFPT2aCE} Allele Characterization

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Findings: VALIDATED

Our analysis confirms the expression of eGFP and activity of CreErt2 under the regulation of Spp2 in proximal tubule cells of the mouse kidney and liver at 19.5 days post coitum (dpc; post natal day 0 [P0]). Low endogenous eGFP expression within the proximal tubule was confirmed by immunohistochemistry. Upon induction with tamoxifen, Cre dependent R26R LacZ and tdTomato expression was observed in Lotus tetragonolobus lectin (LTL) positive proximal tubule cells in the embryonic and adult kidney.

Data:

Crosses

The Spp2^{F2aeGFPT2aCE} (hereafter designated as Spp2^{G2aCE}) strain is a JM8A3.N1 ES cell derived knock-in of eGFP and CreErt2 into the Spp2 (secreted phosphoprotein 2) domain. Three knockout first reporter ES cell clones were obtained from the European Conditional Mouse Mutagenesis (EUCOMM) consortium. (http://www.mousephenotype.org/data/genes/MGI:1922646) The clones were screened for chromosome number and clone EPDO558_1_A04, which displayed an acceptable modal 40 chromosome karyotype (19 of 20 cells scored), was modified by dual-recombinase mediated cassette exchange (dRMCE) to generate the targeted allele (see Figure 1 below).

A two-vector system was optimized in collaboration with EUCOMMTOOLS scientists at the Sanger (www.knockoutmouse.org/about/eucommtools to give rise to a gene-targeting event in which a transcript encoding eGFP and CreERT2 is produced from the Spp2 locus. The resulting transcript is predicted to lead to the production of individual polypeptides for each of these protein products due to the failure of amino acid incorporation where the translating ribosome encounters viral target sequences upstream of eGFP (F2a) and CreERT2 (T2a).

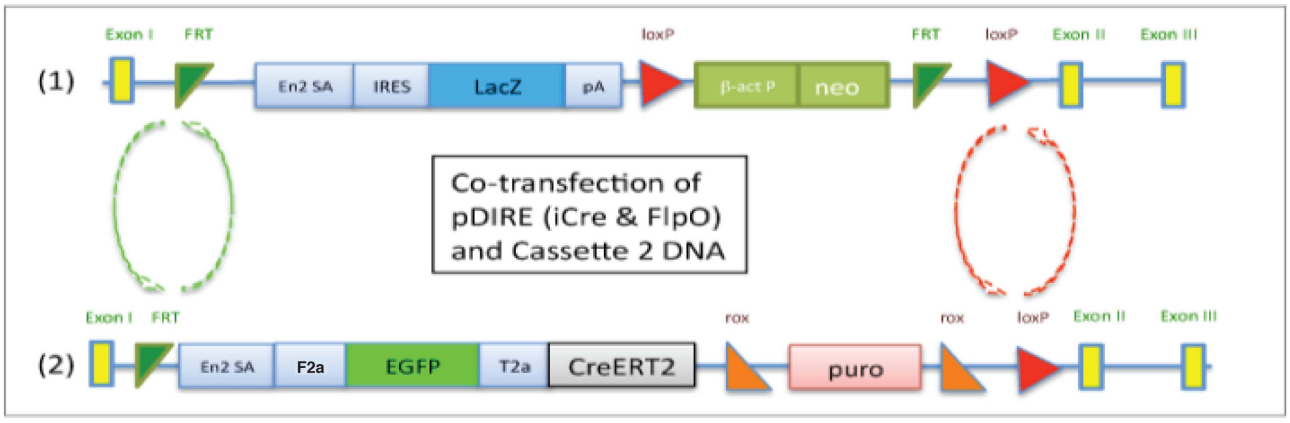


Figure 1. RCME strategy. Modified from: Osterwalder, M., et al. Dual RMCE for efficient re-engineering of mouse mutant alleles. Nat Methods. 2010 Nov;7(11):893-5.

Three correctly targeted clones were screened again by chromosome counting to increase the likelihood of germ line transmission and two clones with > 80% of cells displaying 40

chromosomes were injected into either C57BL/6J (USC) or albino B6(Cg)-Tyr<c-2J>/J (HAR) donor blastocysts. Male chimeras were mated to R26R $^{lacZ/lacZ}$ and R26R $^{tdTomato/tdTomato}$ female mice and the urogenital system (UGS) and livers were collected from 15.5-P0 embryos/pups. Of the seven chimeras tested, three (M1, M2A and M3A) transmitted the transgene and showed expression of eGFP and activity of CreERT2 in the expected cell population (Table 1).
Further analysis was carried out on the M3A line.

Line	Clone	% Chim	Embryos	# Tg	Transmission	Visible GFP	Cre Activity
Spp2-G2ACE2 M1(USC)	53	ND	17	0	No	NA	NA
Spp2-G2ACE2 M1(HAR)	53	90	40	16	Yes	Low	Yes
Spp2-G2ACE2 M2(USC)	53	ND	18	0	No	NA	NA
Spp2-G2ACE2 M3(USC)	53	ND	16	0	No	NA	NA
Spp2-G2ACE2 M1A(USC)	83	ND	22	0	No	NA	NA
Spp2-G2ACE2 M2A	83	85	33	17	Yes	Low	Yes
Spp2-G2ACE2 M3A	83	90	34	14	Yes	Low	Yes

Table 1. Transmission analysis of founders.

Genotyping

Tail samples were collected and incubated in tail digestion buffer overnight at 55ºC. PCR was performed as per the protocol below and the PCR products were run on a 1.5% agarose gel (Fig2).

Oligonucleotides: for targeted/transgenic allele Size: 362 bp
DNA sequence (Forward): 5'-GGCATTATTTAAAGTTAGGCGCG-3'
DNA sequence (Reverse) 5'-CAGATCCCTAGCTCCCAACA-3'
Amplifies 3' arm into targeted allele.
Rxn Buffer and Conditions: (25μl reaction)

10X PCR Buffer	2.5ul
1.25mM dNTP	4ul
10uM primer F	1ul
10uM primer R1	1ul
5x cresol red dye	5ul
Amplify Taq	0.2ul (5u/ul)
Genomic DNA	1ul
Total volume	**25 ul**

94ºC	3min	1 cycle
94ºC	30sec	35cycles
60ºC	30sec	
72ºC	45sec	
72ºC	10min	1 cycle

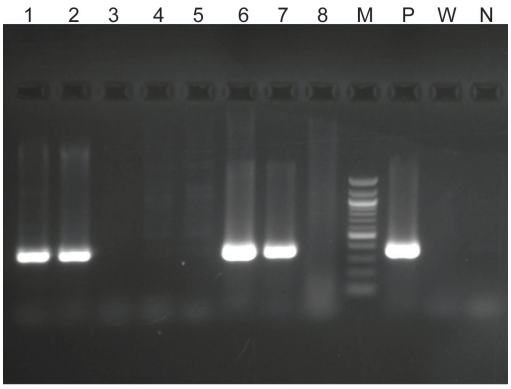
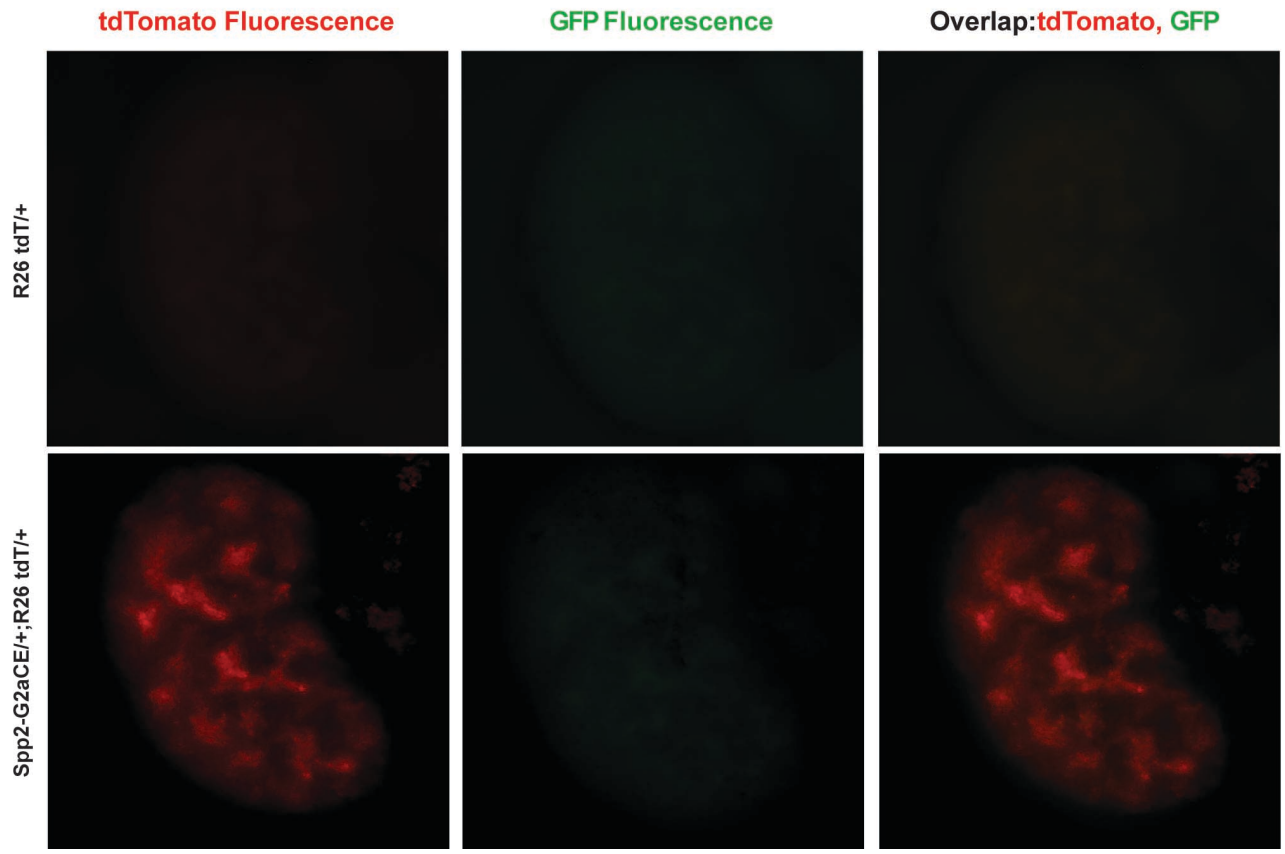


Fig 2: Number 1, 2, 6 and 7: $Spp2^{G2a\text{-}CE/+}$,$Rosa26R^{lacZ/+}$, numbers 3,4, 5 and 8: $Rosa26R^{lacZ/+}$, **P:** $Spp2^{G2a\text{-}CE/+}$ Positive control, **W**: Wildtype control, **N**: Negative control.

Native Fluorescence

Whole embryos as well as dissected UGS and liver were examined with a fluorescent microscope to view eGFP expression. Low levels of endogenous eGFP were detected in $Spp2^{G2aCE/+}$;$R26R^{tdTomato/+}$ P0 kidneys (Figure 3).

A.



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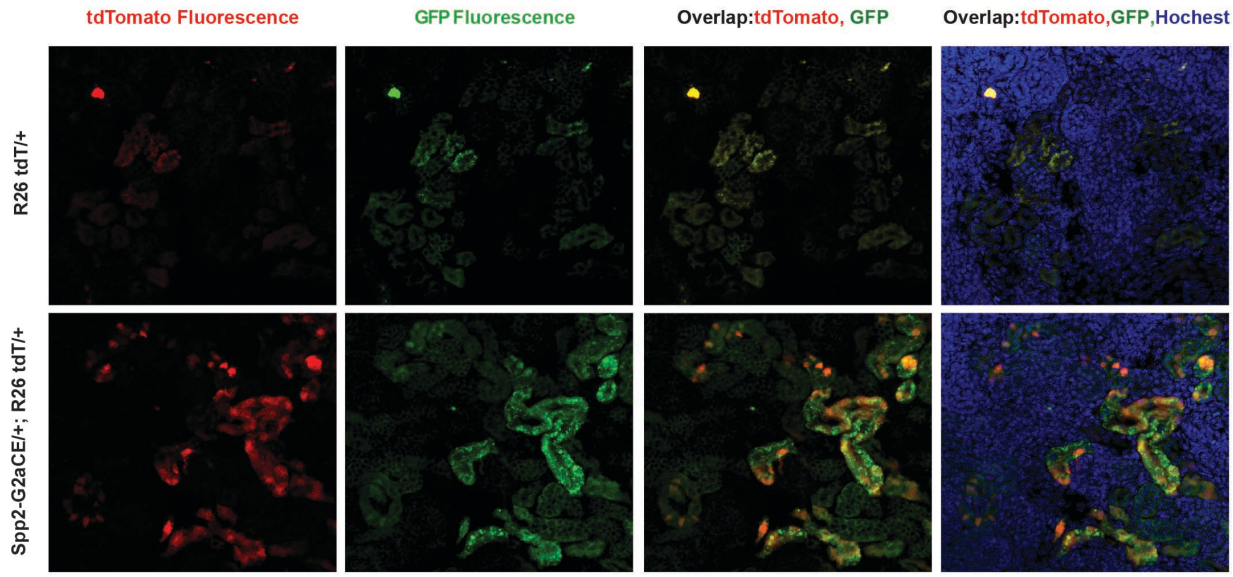

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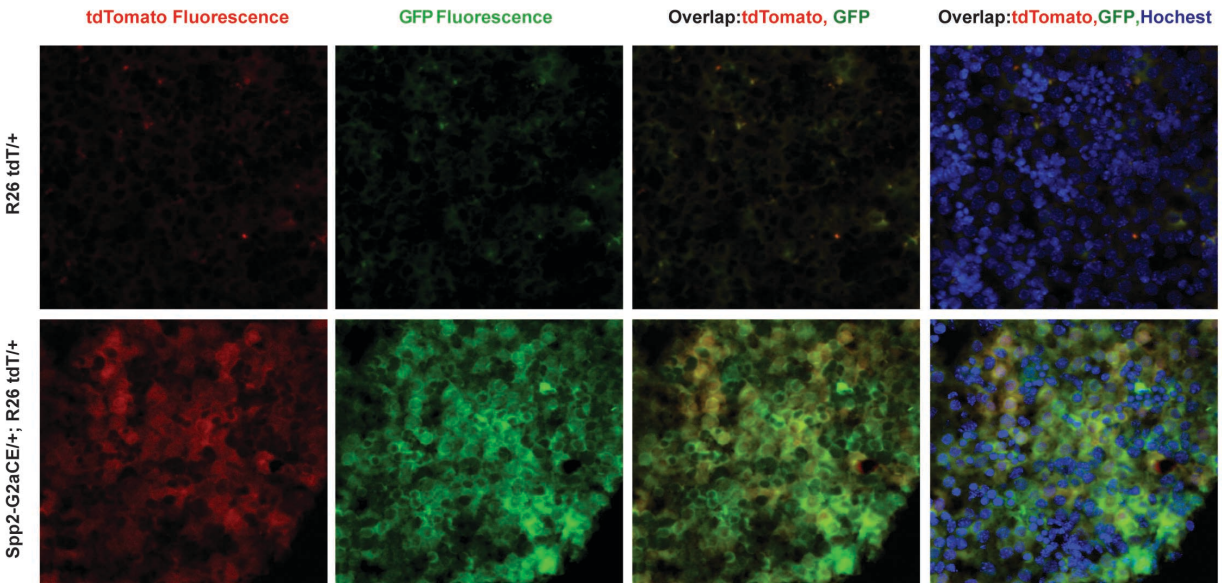
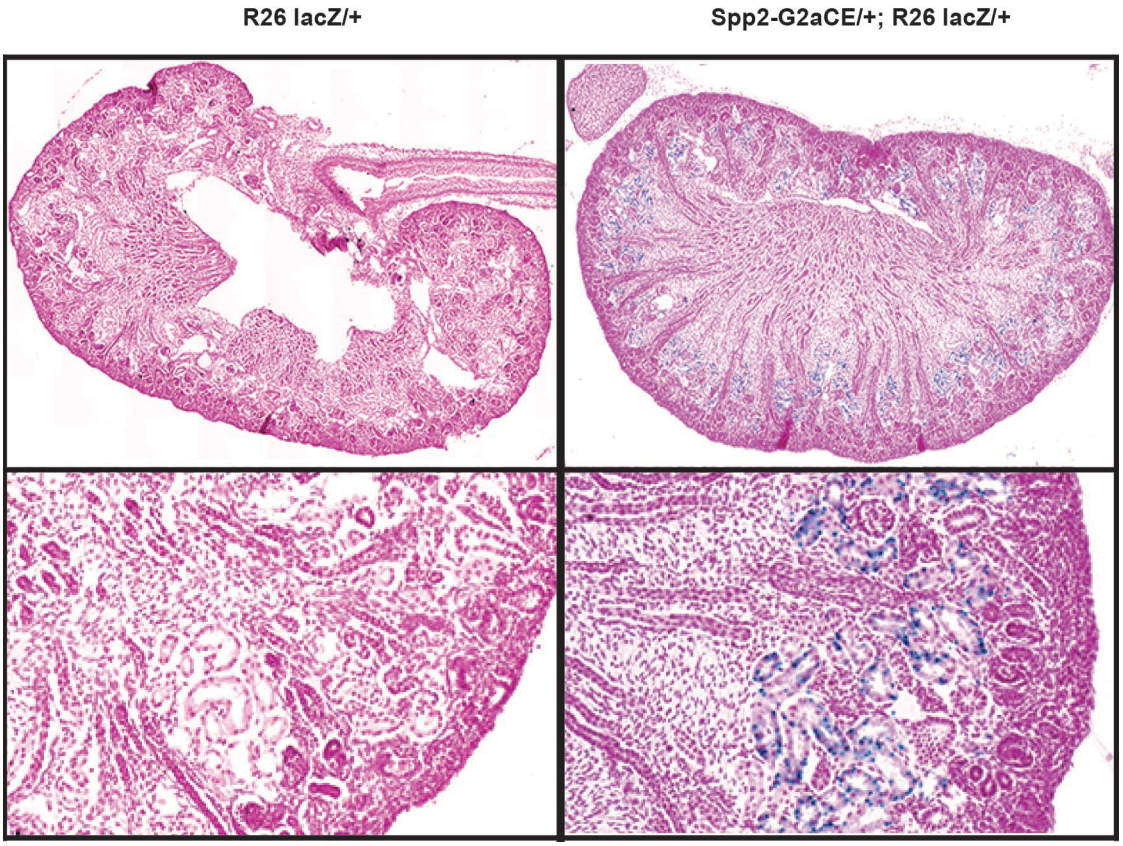


Figure 3. eGFP detection and Cre activity in P0 Spp2$^{G2aCE/+}$; R26R$^{tdTomato/+}$ kidney and liver. Low levels of endogenous eGFP activity was observed in kidneys (3A & 3B) and liver (3C) of Spp2$^{G2aCE/+}$ mice. Robust tamoxifen dependent tdTomato fluorescence was observed in proximal tubule cells and in liver cells. Tamoxifen injection at 17.5dpc, kidney and liver collection at P0.

Cre-recombinase Activity

Spp2$^{G2aCE\ /+}$ male chimeras were mated to R26R$^{lacZ/lacZ}$ and R26R $^{tdTomato/tdTomato}$ females to generate Spp2$^{G2aCE\ /+}$; R26R$^{lacZ/+}$ or R26R$^{tdTomato/+}$ embryos/pups. In order to activate ß-galactosidase (β-gal) or tdTomato reporter expression, pregnant females were injected intraperitoneally at either: 14.5,16.5 or 17.5dpc with tamoxifen in corn oil (1X 2mg to 40g body weight) and the kidneys and liver were assayed at 15.5-18.5dpc and P0. A control group was injected with the same volume of corn oil. Tamoxifen dependent Cre activity was detected in proximal tubule cells in Spp2 $^{G2aCE\ /+}$; R26R$^{lacZ/+}$ and Spp2 $^{G2aCE\ /+}$; R26R $^{tdTomato/+}$ samples (see Figures 3 and 4).

A.



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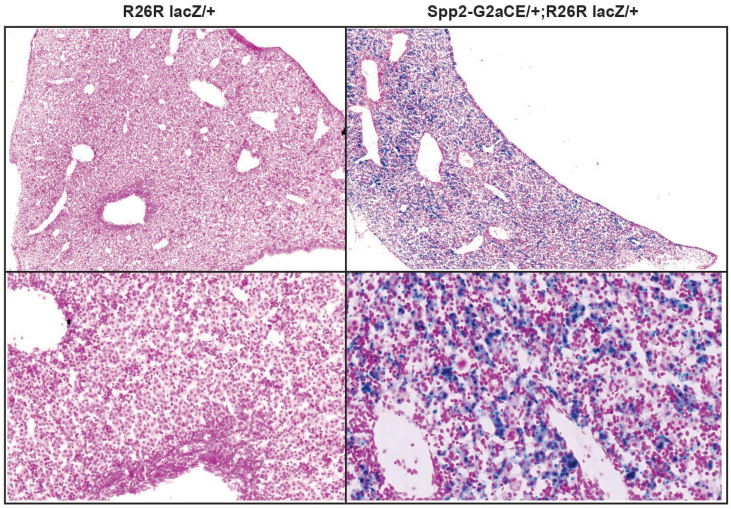

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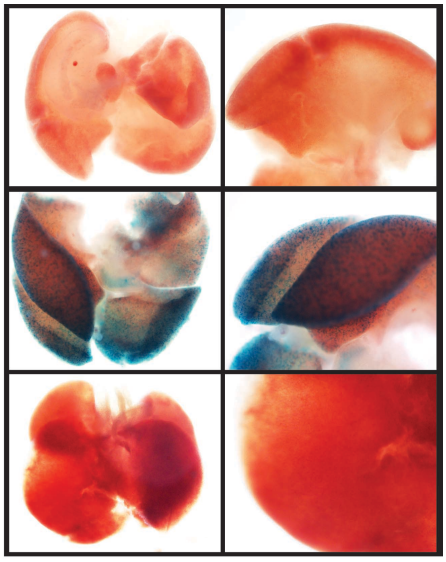


Figure 4. **Cre-dependent β-gal activity in Spp2$^{G2aCE/+}$; R26R$^{lacZ/+}$ P0 kidney and liver.** β-gal activity was detected within the proximal tubules in P0 kidneys (4A) and in the liver (4B & 4C) following tamoxifen injection at 17.5dpc.

Immunohistochemistry

Whole UGSs were fixed in 4% paraformaldehyde at 4ºC for 45 minutes, washed 3 times in PBS, equilibrated in 30% sucrose overnight then embedded in OCT and flash frozen on dry-ice. The UGSs were sectioned at 12um and probed with the antibodies listed in

(Table 2).

Primary Antibody	Company	Catalog #	Dilution	Secondary	Company	Dilution
Chicken IgY anti GFP	Aves Lab	GFP-1020	1/500	Goat anti-chicken IgG-A488	Invitrogen	1/500
LTL lectin-FITC	Vector Lab	FL-1321	1/100			

Table 2. Summary of antibodies used to screen Spp2$^{G2a\text{-}CE/+}$; R26R$^{tdTomato/+}$ 17.5 dpc UGS sections.

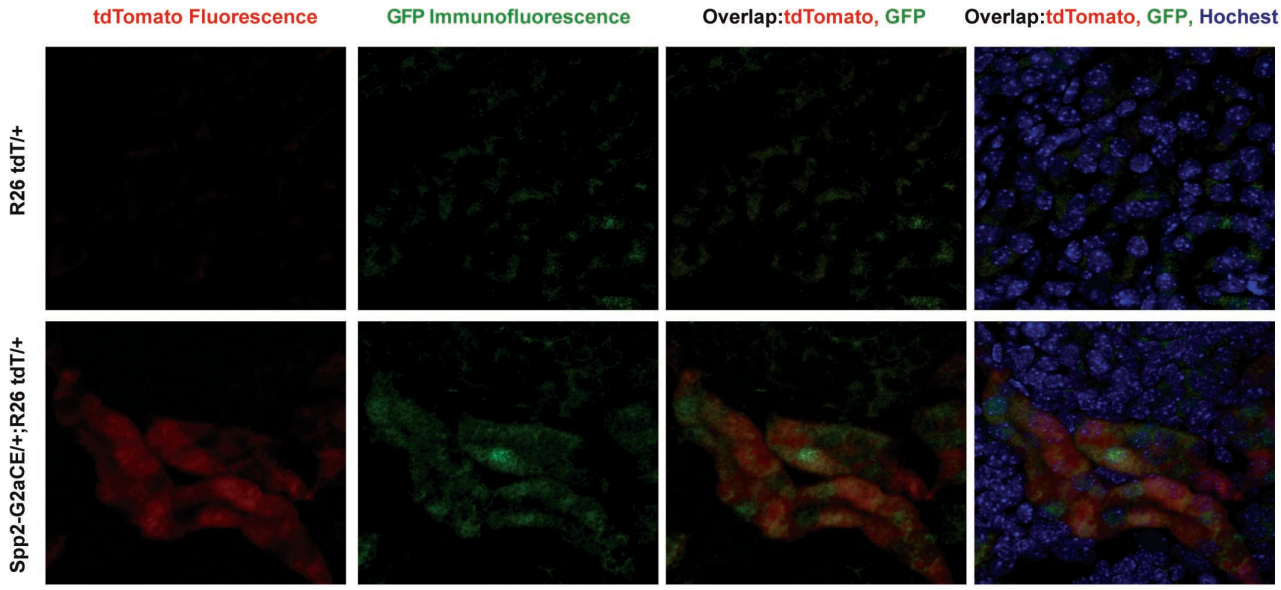
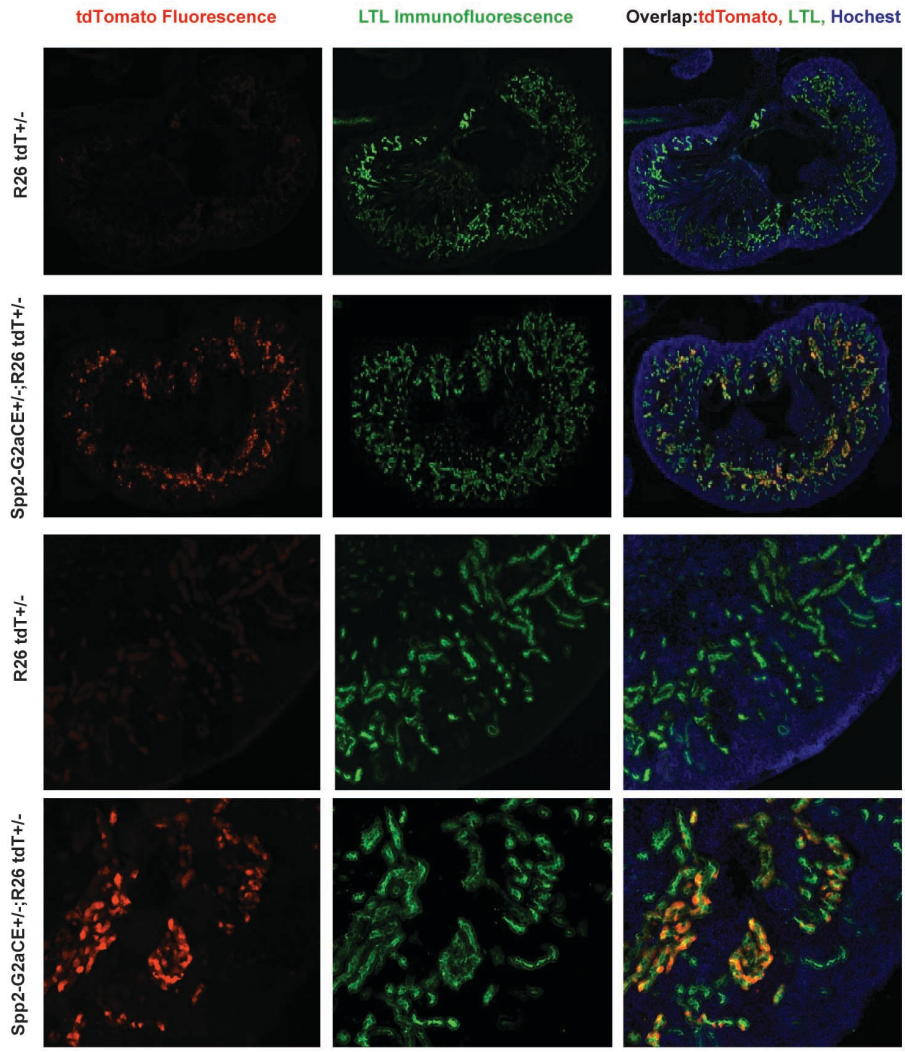


Figure 5. Tamoxifen dependent Cre activity in Spp2$^{G2aCE/+}$; R26R$^{tdTomato/+}$ P0 kidneys colocalized with eGFP expressing cells. TdTomato fluorescence was observed in a percentage of eGFP^{+}, proximal tubules within the P0 kidney following tamoxifen injection at 17.5dpc.

A.



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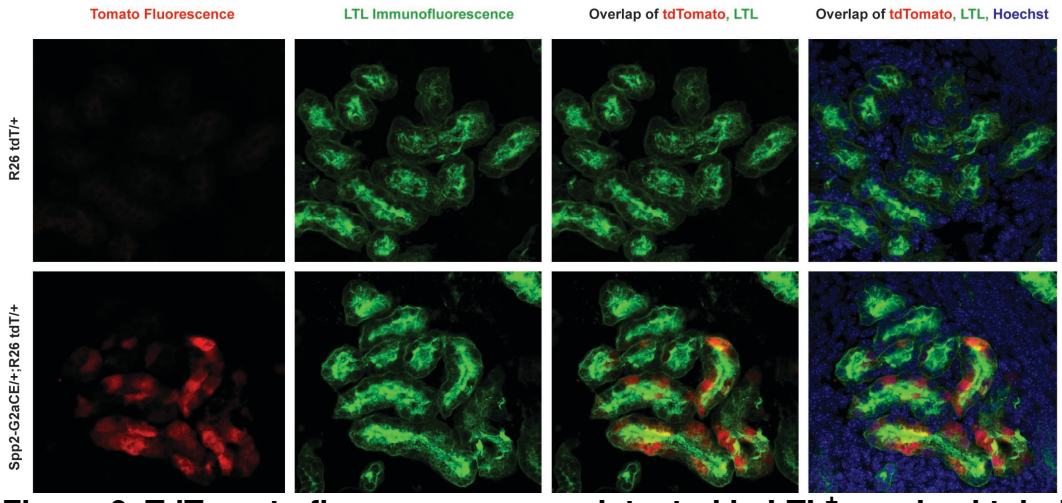

Figure 6. TdTomato fluorescence was detected in LTL^{+} proximal tubule cells in Spp2$^{G2aCE/+}$; R26R$^{tdTomato/+}$ P0 kidneys upon tamoxifen-mediated Cre recombination.

Co-localization of LTL^{+} and tdTomato^{+} cells in proximal tubules of the P0 kidney following tamoxifen injection at 17.5dpc. (6A and 6B)

Figure 7A. Cre dependent tdTomato expression was detected in proximal tubule cells following tamoxifen induction in Spp2G2aCE/+; R26R$^{tdTomato/+}$ adult kidneys. LTL^{+} and tdTomato^{+} proximal tubules were observed in the adult kidney following tamoxifen injection 3 times prior to collection: d1, d3, d5 collected at d7