

Entpd5^{F2aeGFPT2aCE} Allele Characterization

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Findings: **VALIDATED**

Our analysis confirms the expression of eGFP and activity of CreERT2 under the regulation of Entpd5 in proximal tubule cells of the mouse kidney at 19.5 days post coitum (dpc; post natal day 0 [P0]). Weak 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 Entpd5^{F2aeGFPT2aCE} (hereafter designated as Entpd5^{G2aCE}) strain is a JM8.F6 ES cell derived knock-in of eGFP and CreERT2 into the Entpd5 (ectonucleoside triphosphate diphosphohydrolase 5). One knockout first reporter ES cell clone was obtained from the Knockout Mouse Project (KOMP) consortium.

(http://www.mousephenotype.org/martsearch_ikmc_project/martsearch/ikmc_project/24177)

The clone was screened for chromosome number and clone EPDO112_5_E02, 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 Entpd5 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).

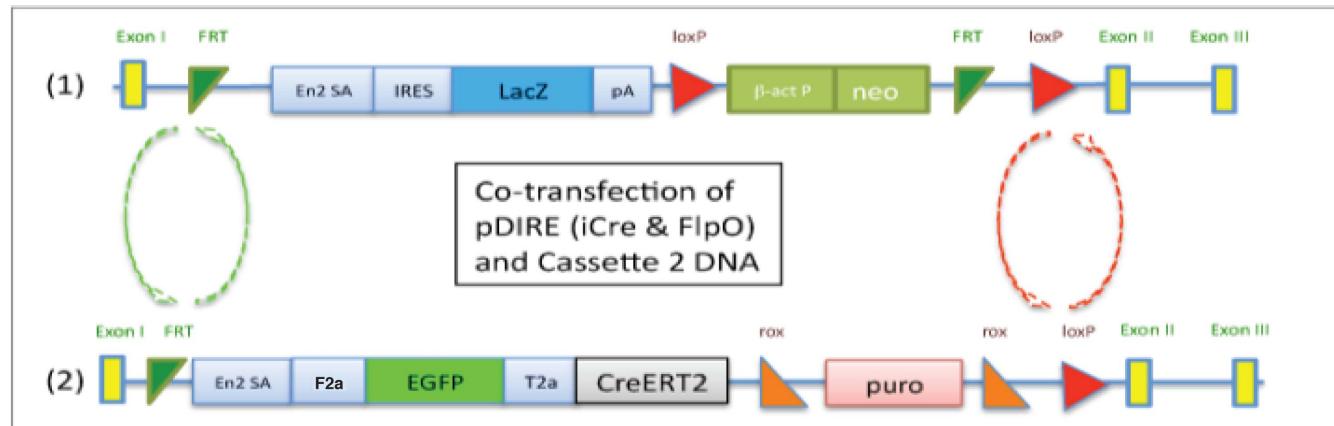


Figure 1. RCME strategy. Modified from: Osterwalder, M., et al. Dual RMCE for efficient re-engineering of mouse mutant alleles. Nat Methods. 2010 Nov;7(11):893-5.

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 albino B6(Cg)-Tyr<c-2J>/J donor blastocysts. Male chimeras

were mated to R26R ^{lacZ/lacZ} and R26R ^{tdTomato/tdTomato} female mice and the urogenital system (UGS) was collected from 15.5-P0 embryos/pups. Of the five chimeras tested, two (M4A and M5A) 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 males derived from clone 12.

Line	Clone	% Chim	Embryos	# Tg	Transmission	Visible GFP	Cre Activity
Entpd5-G2aCE M1	10	55	5	0	No	NA	NA
Entpd5-G2aCE M2	10	50	6	0	No	NA	NA
Entpd5-G2aCE M3A	12	90	24	0	No	NA	NA
Entpd5-G2aCE M4A	12	80	56	8	Yes	No	Yes
Entpd5-G2aCE M5A	12	75	78	39	Yes	No	Yes

Table 1. Transmission analysis of founders

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 (Figure 2).

Oligonucleotides: for targeted/transgenic allele Size: 446 bp

DNA sequence (Forward): 5'- GGCATTATTTAAAGTTAGGCGCG -3'

DNA sequence (Reverse) 5'- GCTAGCAAGGCCAGTAATT -3'

Amplifies 3' arm to into targeted allele.

Rxn Buffer and Conditions: (25μl reaction)

10X PCR Buffer	2.5ul			
1.25mM dNTP	4ul	94°C	3min	1 cycle
10uM primer F	1ul	94°C	30sec	
10uM primer R1	1ul	60°C	30sec	35cycles
5x cresol red dye	5ul	72°C	45sec	
Amplify Taq	0.2ul (5u/uL)	72°C	10min	1 cycle
Genomic DNA	1ul			
Total volume	25 ul			

54 55 56 57 58 59 60 61 M P W N

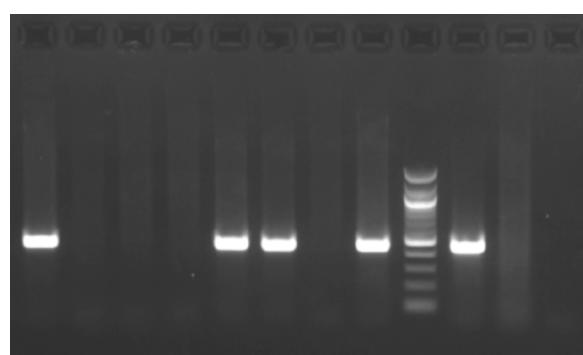
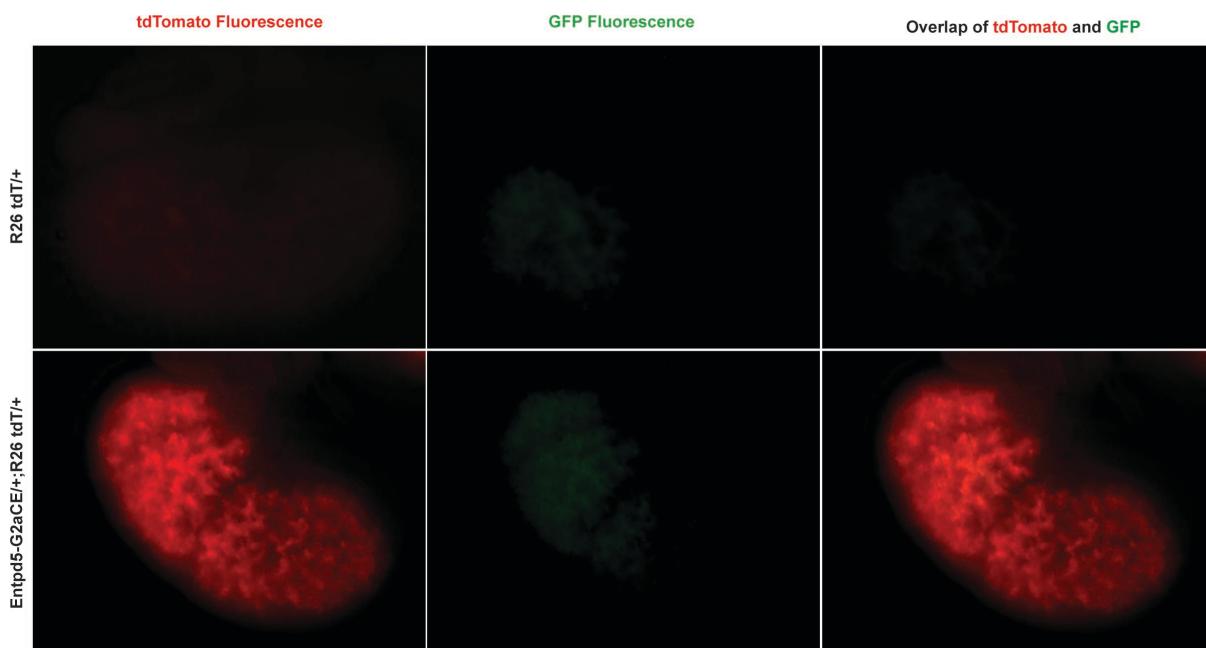


Figure 2: Number 54, 58,59 and 61: $\text{Entpd5}^{\text{G2aCE}+/+}$, numbers 53,55,56,57 and 60: Wildtype
P: $\text{Entpd5}^{\text{G2aCE}+/+}$, Positive control, **W:** Wildtype, **N:** Negative control.

Endogenous Fluorescence

Whole embryos as well as dissected UGSs were examined with a fluorescent microscope to view eGFP expression. Very low levels of endogenous eGFP were detected in $\text{Entpd5}^{\text{G2aCE}+/+}$;R26 $\text{R}^{\text{tdTomato}+/+}$ P0 kidneys (Figure 3).

A.



B.

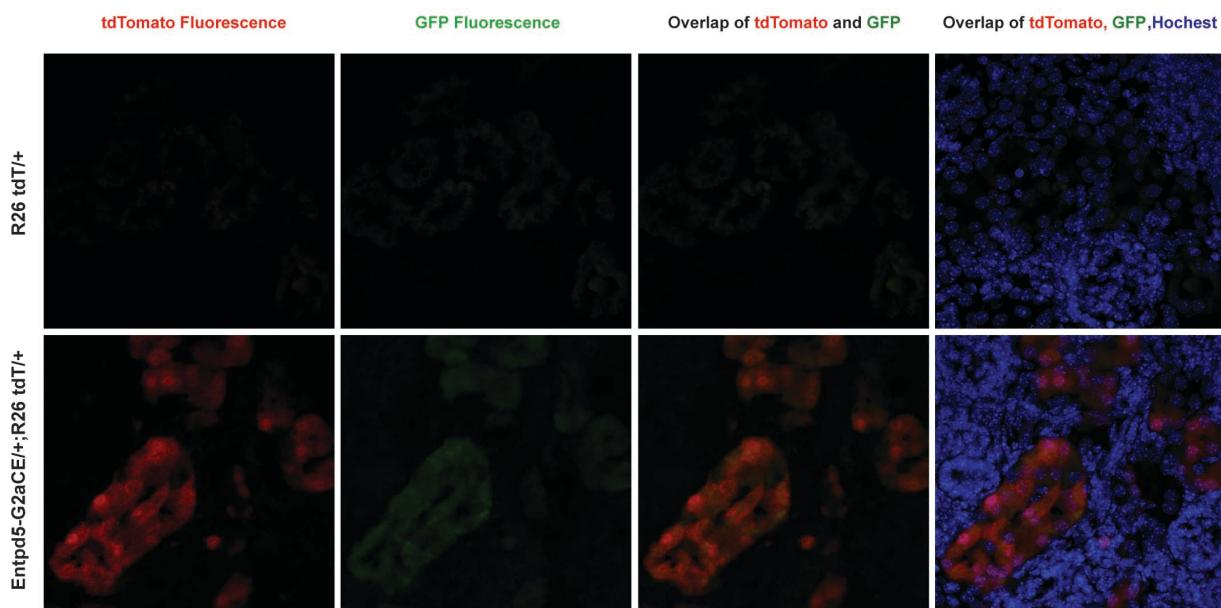


Figure 3. eGFP detection and Cre activity in P0 tdTomato $\text{Entpd5}^{\text{G2aCE}+/+}$; R26 $\text{R}^{\text{tdTomato}+/+}$ kidneys. Very low levels of endogenous eGFP activity was observed in kidneys of $\text{Entpd5}^{\text{G2aCE}+/+}$ mice. Robust tamoxifen dependent tdTomato fluorescence was observed in proximal tubule cells. Tamoxifen injection at 17.5dpc and kidney collection at P0.

Cre-recombinase Activity

Entpd5^{G2aCE/+} male chimeras were mated to R26R^{lacZ/lacZ} and R26R^{tdTomato/tdTomato} females to generate Entpd5^{G2aCE/+}; R26R^{lacZ/+} or R26R^{tdTomato/+} embryos/pups. In order to activate β-galactosidase (β-gal) or tdTomato reporter expression, pregnant females were injected intraperitoneally at 17.5dpc with tamoxifen in corn oil (1X 2mg to 40g body weight) and the kidneys assayed at P0. A control group was injected with the same volume of corn oil. Tamoxifen dependent Cre activity was detected in proximal tubule cells in Entpd5^{G2aCE/+}; R26R^{lacZ/+} and Entpd5^{G2aCE/+}; R26R^{tdTomato/+} samples (Figure 3 and 4).

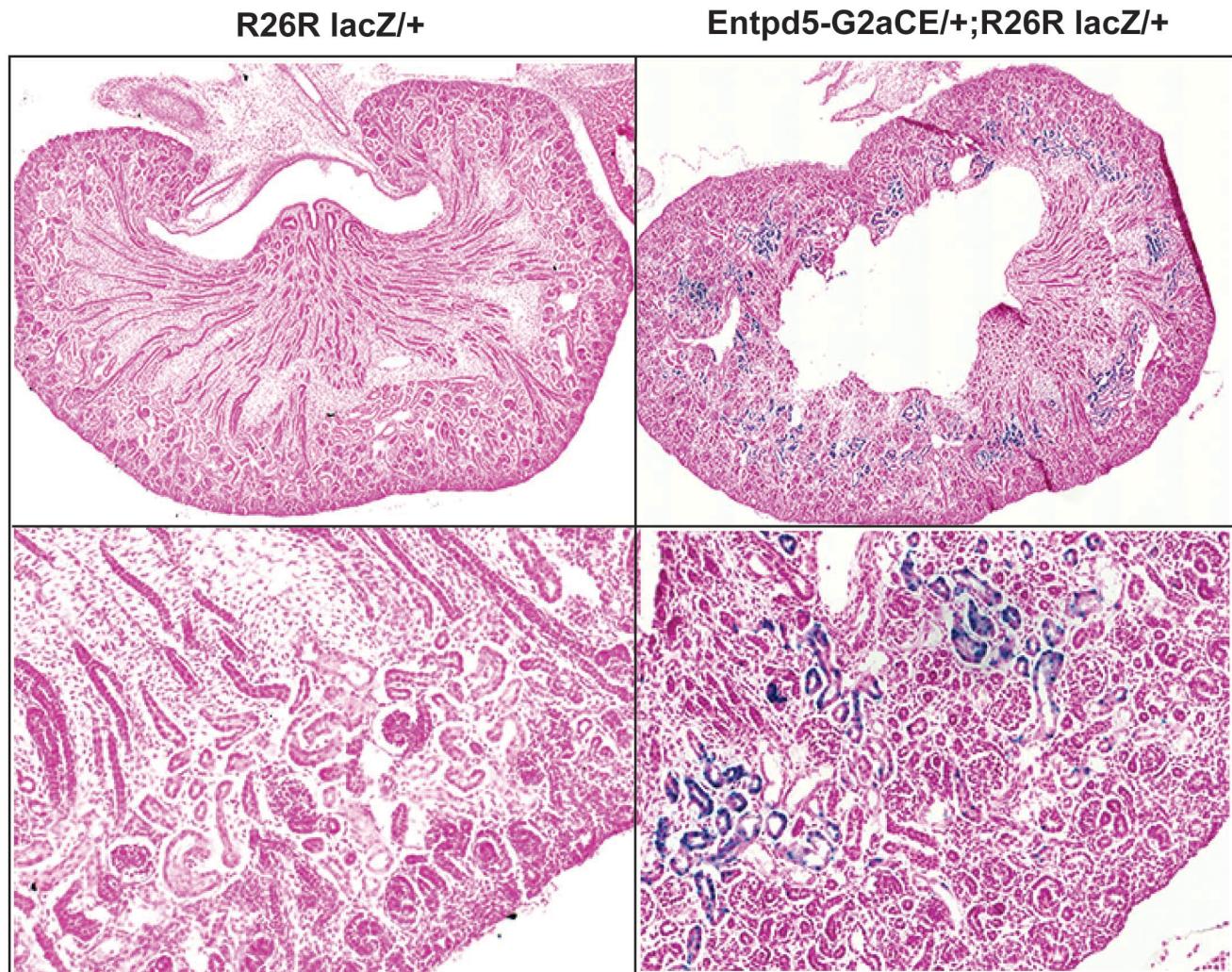


Figure 4. Cre-dependent β -gal activity in Entpd5^{G2aCE/+}; R26R^{lacZ/+} P0 kidney.
β-gal activity was detected within the proximal tubules in P0 kidneys following tamoxifen injection at 17.5dpc.

Immunohistochemistry

Whole UGSs were fixed in 4% paraformaldehyde at 4°C 2 hours, washed 3 times in PBS, equilibrated in 30% sucrose overnight and 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 $\text{Enpd5}^{\text{G2aCE}/+}; \text{R26R}^{\text{tdTomato}/+}$

17.5 dpc UGS sections.

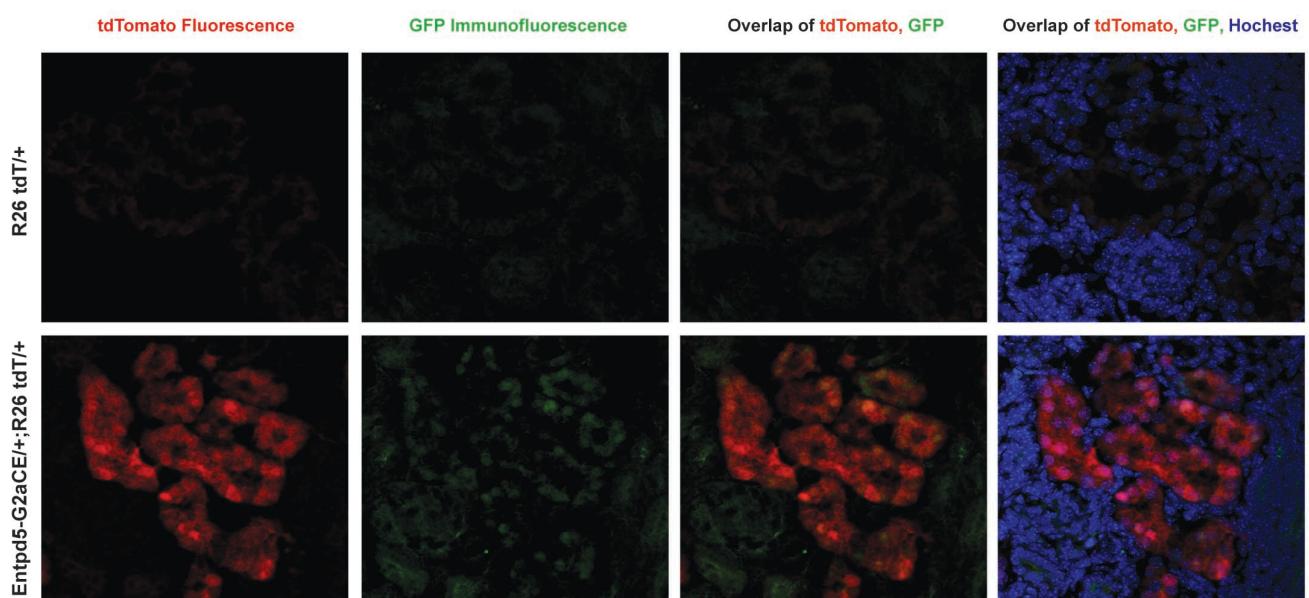


Figure 5. Tamoxifen dependent Cre activity in $\text{Enpd5}^{\text{G2aCE}/+}; \text{R26R}^{\text{tdTomato}/+}$ P0 kidneys colocalized with eGFP expressing cells. TdTomato fluorescence was observed in a percentage of eGFP⁺, proximal tubules within the P0 kidney following tamoxifen injection at 17.5dpc.

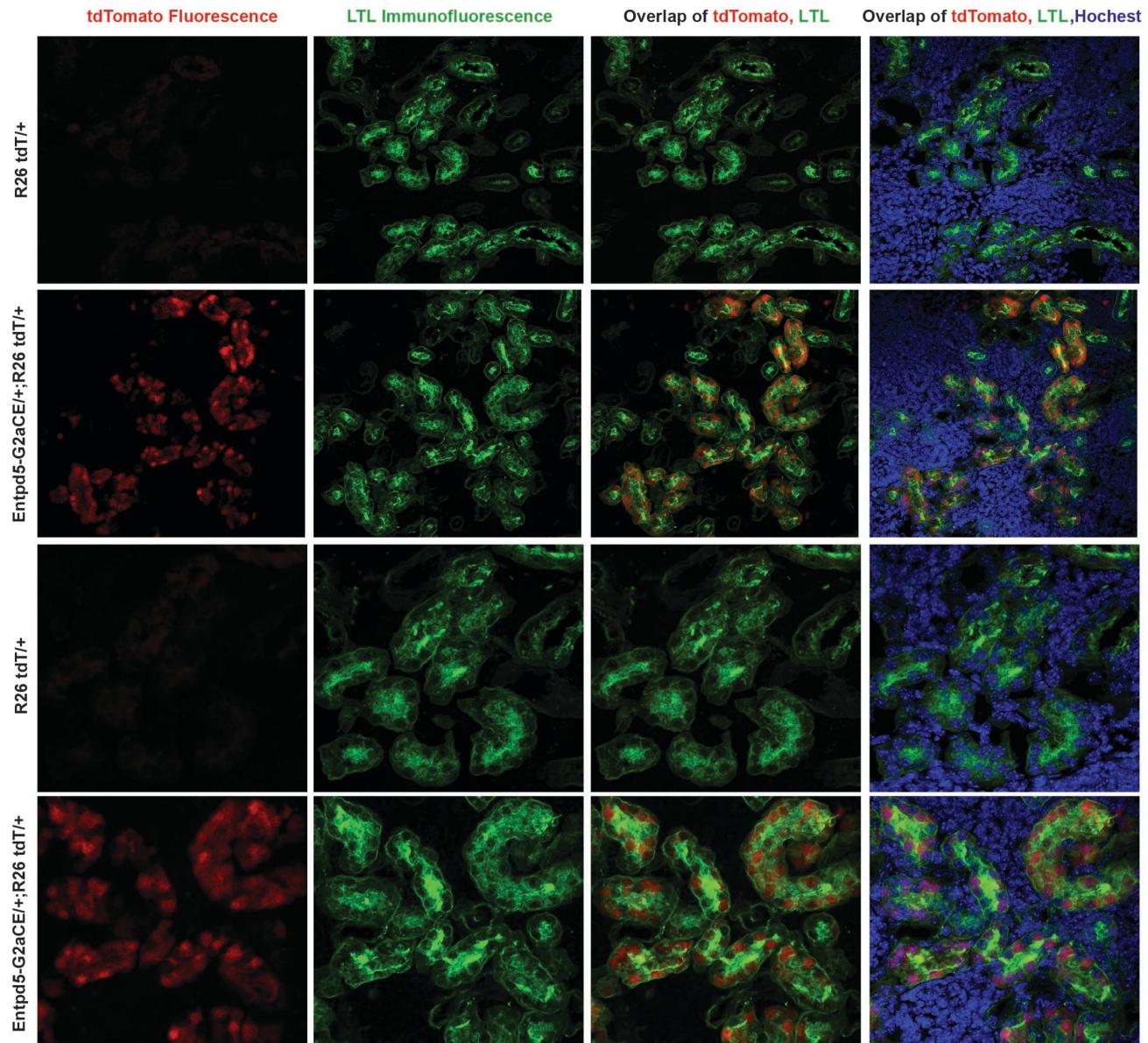


Figure 6. TdTomato fluorescence was detected in LTL⁺ proximal tubule cells in Entpd5^{G2aCE/+}; R26R^{tdTomato/+} P0 kidneys upon tamoxifen-mediated Cre recombination..
Co-localization of LTL⁺ and tdTomato⁺ cells in proximal tubules of the P0 kidney following tamoxifen injection at 17.5dpc.

Figure 7. Cre dependent tdTomato expression was detected in proximal tubule cells following tamoxifen induction in *Entpd5*^{G2aCE/+}; 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.

