Umod $^{IRES\ CRE\text{-}ERT2}$ **Allele Characterization**

Authors: Jinjin Guo, Jing Liu, Jill McMahon and Andrew P. McMahon

Findings: VALIDATED

Our analysis confirms activity of CRE::ERT2 under the regulation of the *Umod* gene. Cre dependent tdTomato activity was observed upon tamoxifen induction at P21 and analysis at P23 in the ascending limb of the loop of Henle. tdTomato positive cells co-localize with uromodulin positive cells but not with differentiated proximal tubule cells highlighted by LTL lectin FITC binding. A subset of tdTomato positive cells coactivate Slc12a3 at the distal tubule boundary.

Data:

Crosses

The Umod$^{IRES\ CRE\text{-}ERT2}$ strain is a CRISPR/ Cas9 mediated knock-in of IRES-CRE-ERT2 into the 3' UTR near the stop codon of the *Umod*, (Uromodulin) gene in JM8.N4 ES cells. The targeted *Umod* gene encodes a GPI-anchored glycoprotein, also known as Tamm–Horsfall glycoprotein, that is localized in the epithelial cell of the renal ascending limb of Henle. gRNAs were designed with the program: http://crispr.mit.edu. Annealed oligos containing the gRNA sites were cloned into BbsI sites of plasmid pSpCas9(BB)-2A-puro (Ran FA et al. Nature Protocol, 2013). The donor targeting construct was generated using Gibson Assembly with four PCR fragments: 5' 1-kb homologous arms (HA), IRES-CRE-ERT2-bGHpA DNA and 3' 1-kb homologous arms (HA), and linear pBluescript vector. The IRES-CRE-ERT2-bGHpA was inserted in the middle of the gRNA recognition site so that the final donor construct will not be cut by the gRNA. 5ug gRNA-Cas9 construct and 25ug donor targeting construct were transfected into C57BL/6 JM8.N4 ES cells (KOMP) with FugeneHD (Promega). The cells were kept in 2i media on gelatin coated plates during transfection for 48h followed by 48h 1.75ug/ml puromycin selection on MEF plates.

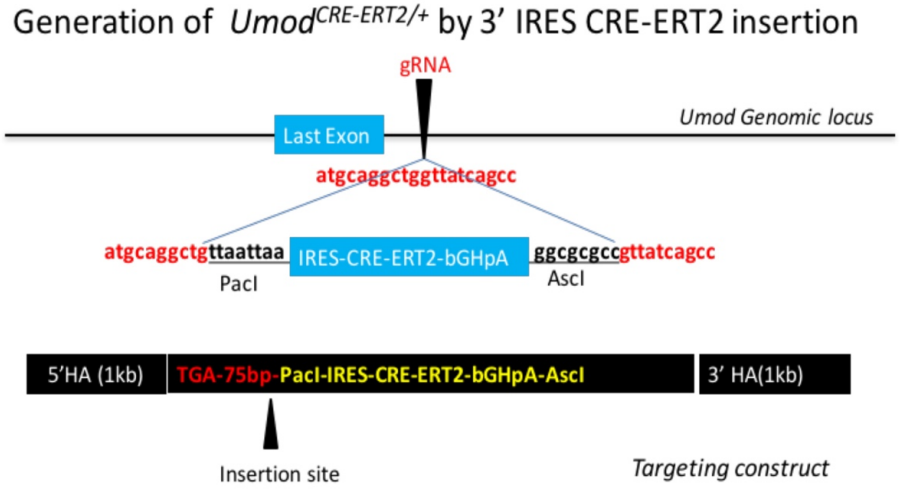


Figure 1. Diagram of the strategy adopted to generate CRISPR/Cas9 mediated knock-in of IRES-CRE-ERT2-bGHpA into the Umod locus of JM8.N4 ES cells.

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

number of chromosomes were injected at Jackson Labororatory into albino B6(Cg)-Tyr<c-2J>/J donor blastocysts. Male chimeras were mated to albino B6(Cg)-Tyr<c-2J>/J female mice to determine coat color transmission and heterozygous progeny were confirmed by PCR. F1 males were sent to the McMahon Lab for characterization. Umod$^{IRES\ CRE\text{-}ERT2/+}$ males were mated to R26R $^{tdTomato/tdTomato}$ female mice and the urogenital system (UGS) was collected from P23 day pups post Tamoxifen induction. Three F1 males were tested, (M1, M2, M3) and all transmitted the transgene (Table 1).

Line	Clone	GLT	Cre activity
Umod$^{IRES\ CRE\text{-}ERT2}$ M1	27	Yes	Yes
Umod$^{IRES\ CRE\text{-}ERT2}$ M2	27	Yes	Yes
Umod$^{IRES\ CRE\text{-}ERT2}$ M3	27	Yes	Yes

Table 1. Transmission analysis of founders

Genotyping

Tail samples of the embryos 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 (Figure 2).

Oligonucleotides: for targeted/transgenic allele Size: 459bp
DNA sequence (forward): 5'-CTCCCACCCAAAACAGACAC-3'
DNA sequence (reverse) 5'-GGAAAGACCCCTAGGAATGC-3'

Rxn Buffer and Conditions: (25μl reaction)

10X PCR Buffer	2.5ul
1.25mM dNTP	4ul
10uM primer F	1ul
10uM primer R	1ul
5x cresol red dye	5ul
Taq polymerase	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

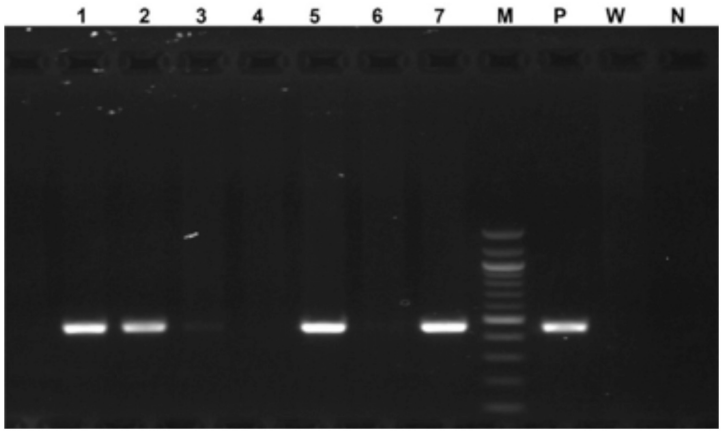


Figure 2: Lanes 1, 2, 5 & 7: show pups that display the expected diagnostic PCR product of 459 bp for the targeted allele. **M:** DNA Marker**, P:** Positive control**, Wt:** Wildtype. N: Negative control.

Cre-recombinase Activity

Umod$^{IRES\ CRE\text{-}ERT2/+}$ male chimeras were mated to R26R $^{tdTomato/tdTomato}$ females to generate Umod$^{IRES\ CRE\text{-}ERT2/+}$; R26R$^{tdTomato/+}$ pups. In order to activate tdTomato reporter expression, P21 pups were injected intraperitoneally with tamoxifen in corn oil (1X 2mg to 40g body weight) and the tissues were assayed at P23. Tamoxifen dependent Cre activity was detected in the Loop of Henle cells of the kidney in Umod$^{IRES\ CRE\text{-}ERT2}$/+; R26R$^{tdTomato/+}$ samples (Figure 3).

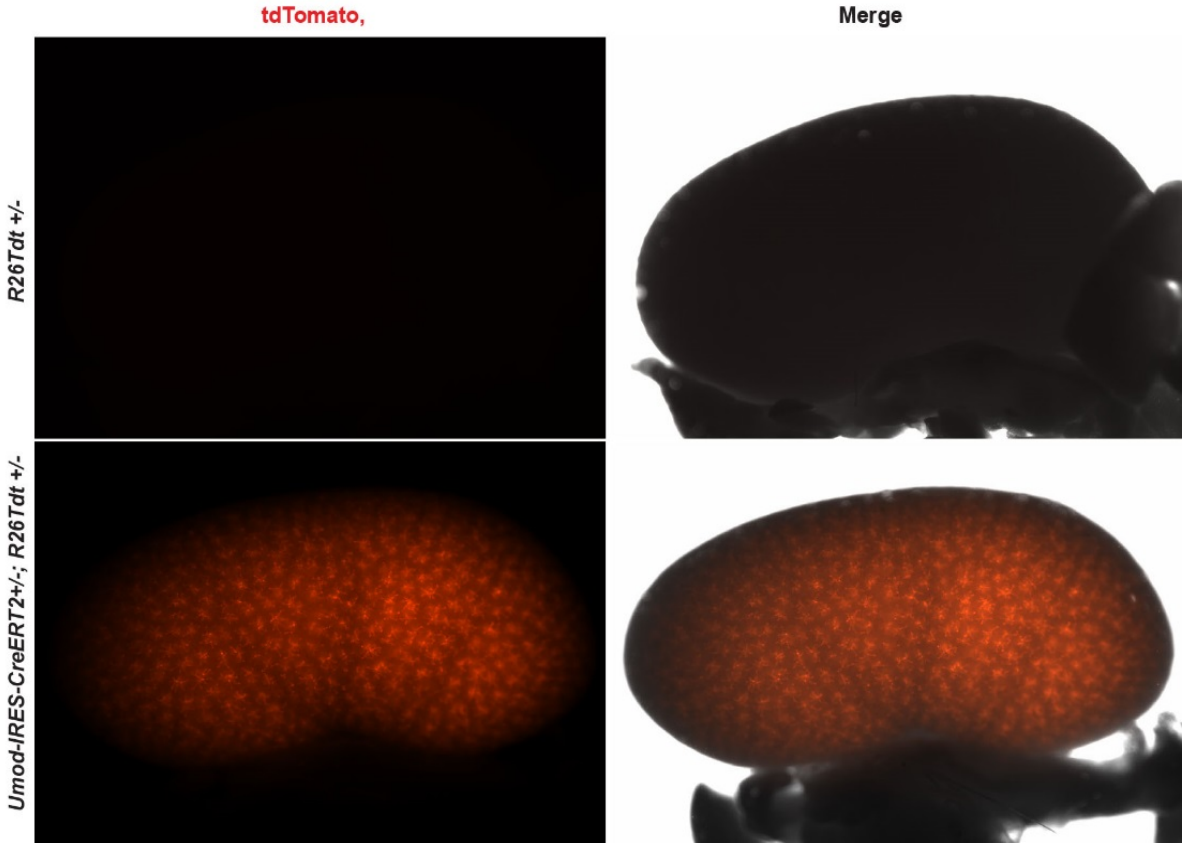


Figure 3. Tamoxifen dependant tdTomato positive cells observed in the kidney of Umod$^{IRES\ CRE\text{-}ERT2/+}$; R26R$^{tdTomato/+}$ of P23 pups following a single injection of tamoxin at P21 (2mg/40g body weight).

Immunohistochemistry

Whole UGSs were fixed in 4% paraformaldehyde at 4ºC for 1 hour, 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
Rabbit-anti-Slc12a3	Sigma	HPA028748	1/500	Donkey anti-rabbit A647	Invitrogen	1/500
Rabbit anti-Tamm-Horsfall (Uromodulin)	Alfa Aesar	J65429	1:250	Donkey anti-rabbit A647	Invitrogen	1/500
LTL lectin-FITC conjugate	Vector Laboratories	FL-1321	1/100			

Table 2. Summary of antibodies used to screen Umod$^{IRES\ CRE\text{-}ERT2}$/+; R26R$^{tdTomato/+}$ P23 kidney sections.

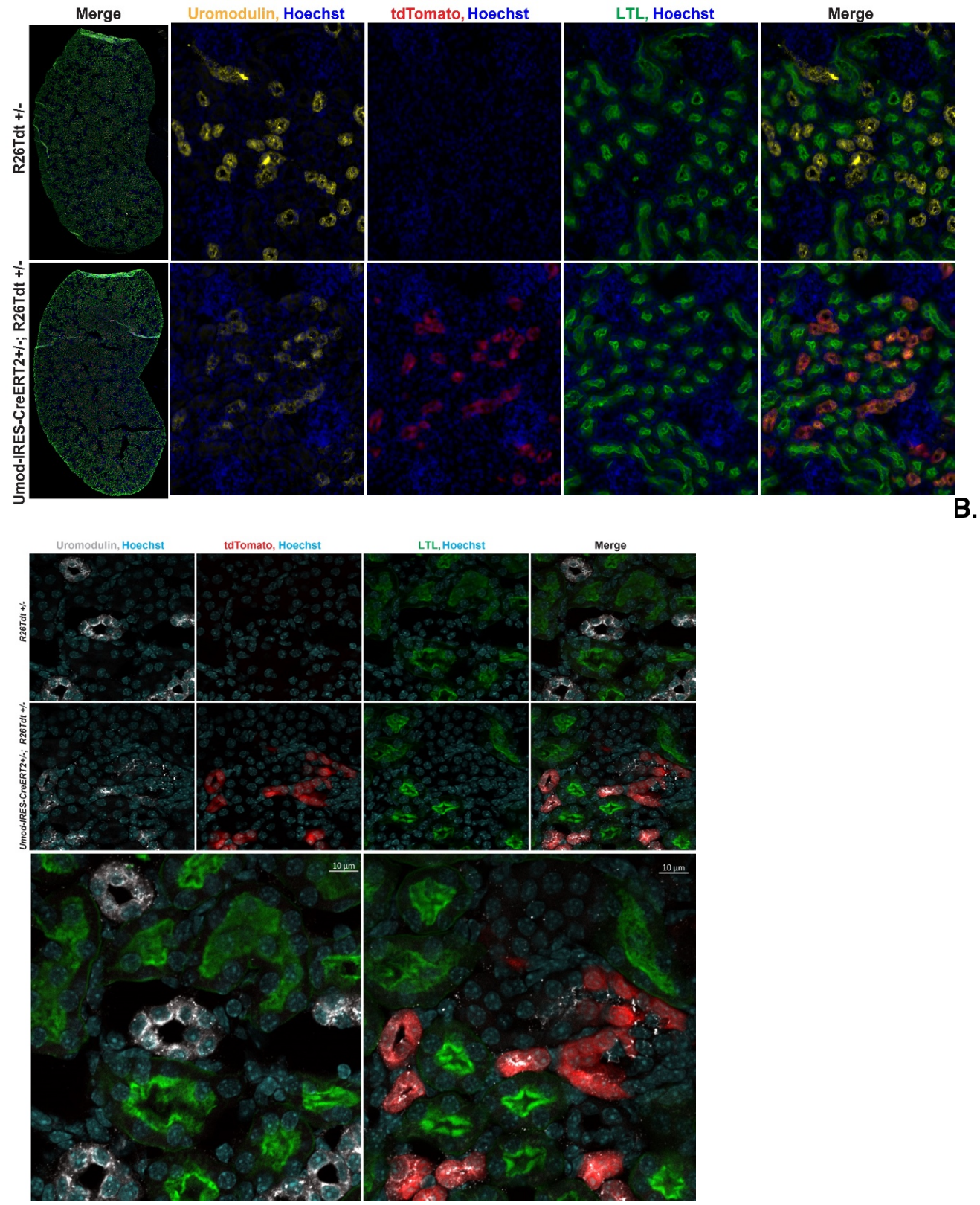
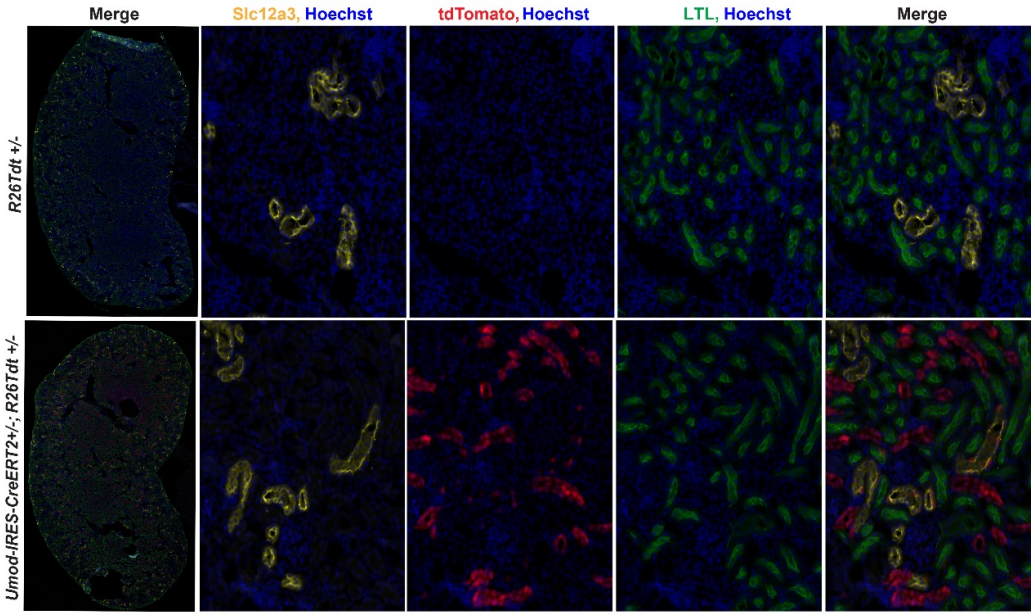


Figure 4. Tamoxifen dependent tdTomato positive cells co-localize with uromodulin in the loop of Henle but not with LTL positive proximal tubule cells in Umod$^{IRES\ CRE\text{-}ERT2}$/+; R26R$^{tdTomato/+}$ P23 kidneys (A&B), after a single injection of tamoxifen at P21(A&B).

A.



B.

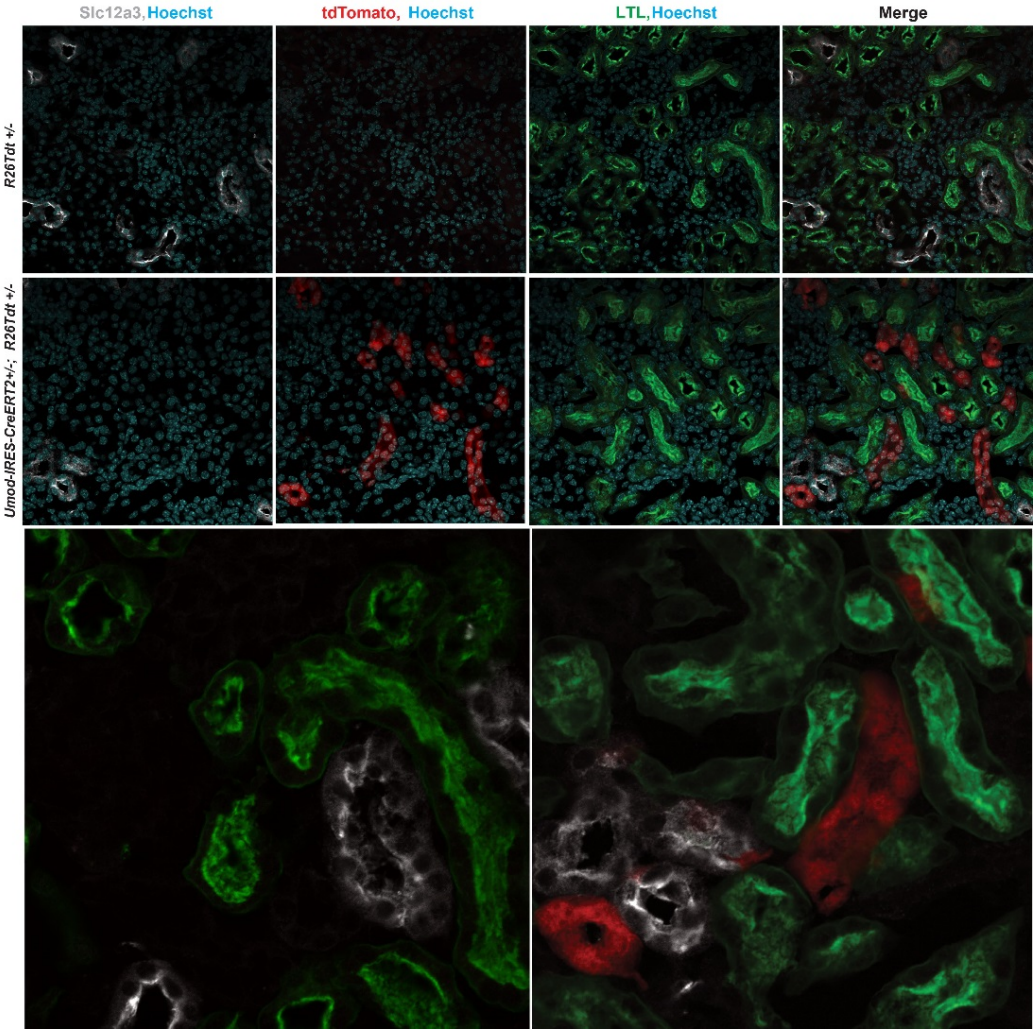


Figure 5. A percentage of Tamoxifen dependent tdTomato positive cells in Umod$^{\text{IRES CRE-ERT2}}$/$^{+}$; R26R$^{\text{tdTomato/+}}$kidney co-localize with Slc12a3 positive cells in P23 pups, after a single injection of tamoxifen at P21(A&B).

.

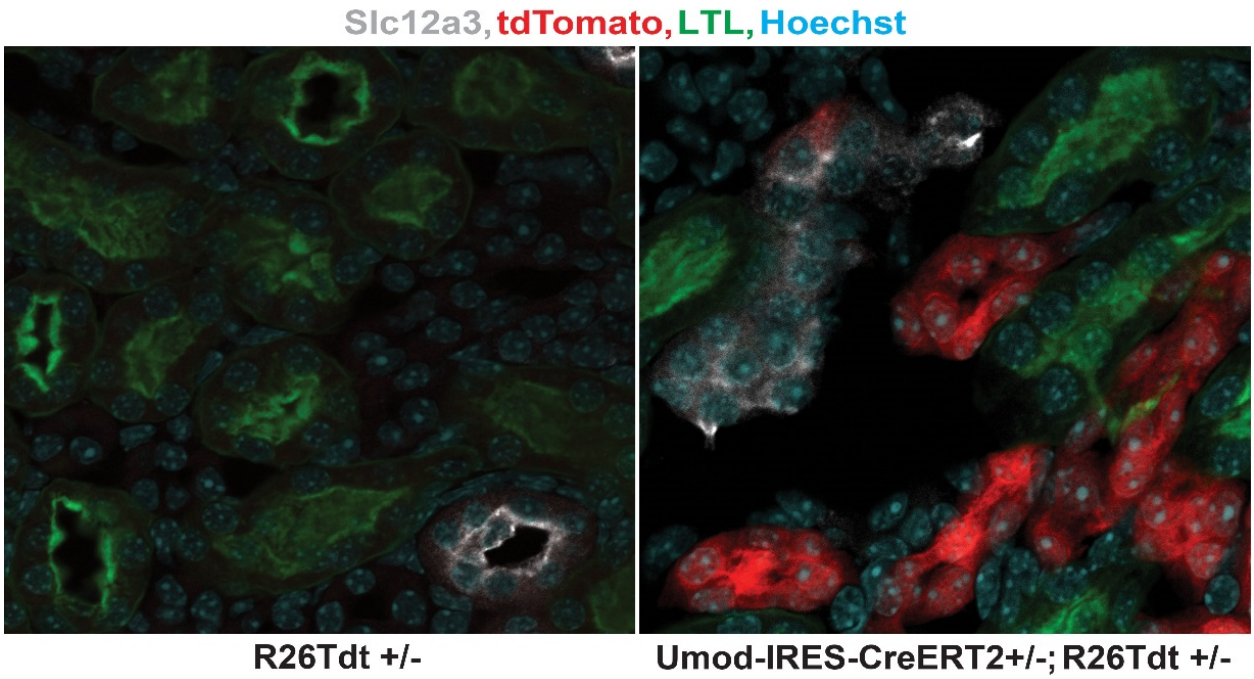


Figure 6. High magnification view showing a percentage of tdTomato positive cells of the ascending limb of the Loop of Henle in Umod$^{\text{IRES CRE-ERT2}/+}$; R26R$^{\text{tdTomato}/+}$kidney coexpress with Slc12a3 in a low number of cells of the distal convoluted tubules; suggests a small overlap of expression domains in P23 pups following injection of tamoxifen at P21.