

Slc12a3 IRES CRE-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 *Slc12a3* in the distal tubule segment of the nephron. Expression of Cre dependent tdTomato expression was observed upon induction in the kidney of postnatal day 21(P21) pups following tamoxifen injection at P19. Cre inducible expression in cells of the distal tubules was confirmed by immunohistochemistry. tdTomato+ cells co-localize with Slc12a3+ cells in the distal convoluted tubules, a small subset of cells also co-localize with Umod at the junction with the ascending limb of the loop of Henle (LOH).

Data:

Crosses

The *Slc12a3*^{IRES CRE-ERT²} strain is a CRISPR/ Cas9 mediated knock-in of IRES-CRE-ERT2 into the 3' UTR near the stop codon of the *Slc12a3* (Solute carrier family 12 member 3) gene in JM8.N4 ES cells. The targeted *Slc12a3* gene encodes a thiazide-sensitive sodium-chloride cotransporter expressed in the renal distal convoluted tubule. gRNA were designed through <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.

Generate *Slc12a3*^{CRE-ERT2/+} mice by 3' IRES (Jing Liu)

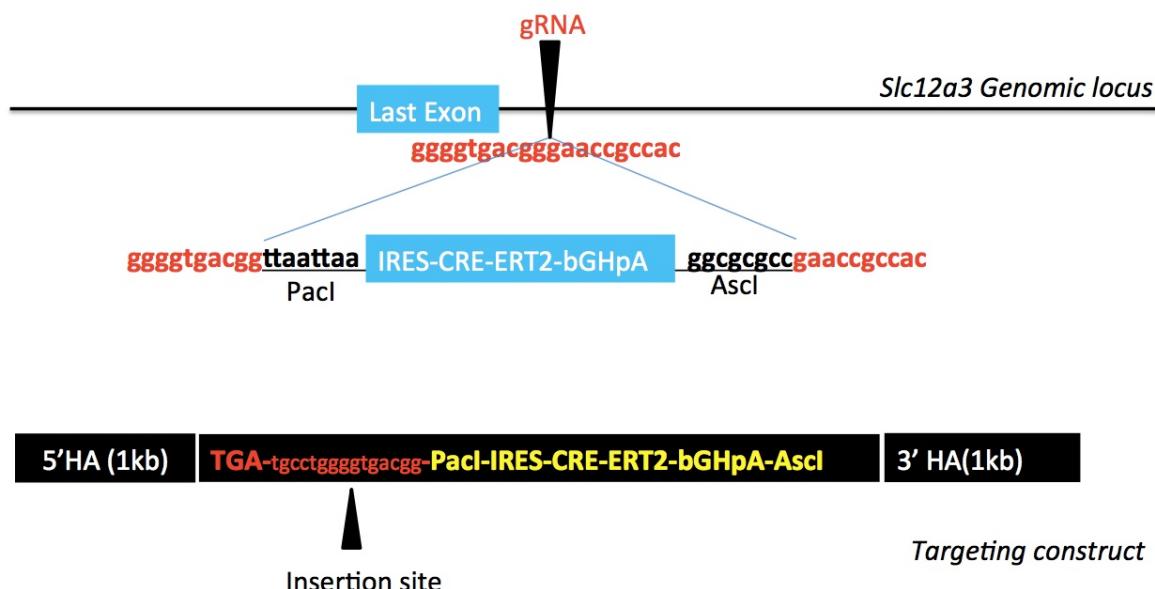


Figure 1. Diagram of the strategy adopted to generate CRISPR/Cas9 mediated knock-in of IRES-CRE-ERT2-bGHpA into the *Slc12a3* locus of JM8.N4 ES cells.

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 Laboratories 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. *Slc12a3*^{IRES CRE-ERT2/+} males were mated to R26R^{tdTomato/tdTomato} female mice and the urogenital system (UGS) was collected from P21 pups post tamoxifen induction. Three F1 males were tested (M1, M2, M3) and transmitted the transgene (Table 1).

Line	Clone	GLT	Cre activity
<i>Slc12a3</i> ^{IRES CRE-ERT2/+} M1	4	Yes	Yes
<i>Slc12a3</i> ^{IRES CRE-ERT2/+} M2	4	Yes	Yes
<i>Slc12a3</i> ^{IRES CRE-ERT2/+} M3	4	Yes	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: 498bp

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

DNA sequence (reverse): 5'-AGACCCCTAGGAATGCTCGT-3'

Amplifies 5' arm into IRES sequence within IRES region.

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	20sec		
10uM primer R	1ul	60°C	20sec	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				

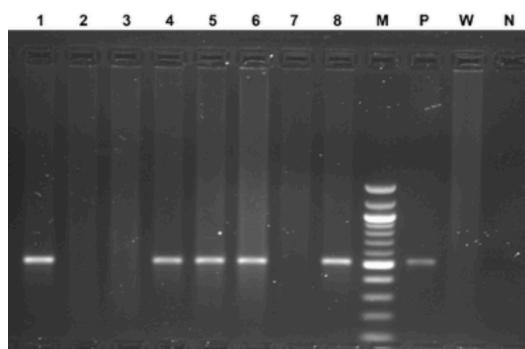


Figure 2: Lanes 1, 4, 5, 6 & 8 show pups that display the expected diagnostic PCR product of 498bp for the targeted allele. **M:** DNA Marker, **P:** Positive control, **Wt:** Wildtype. **N:** Negative control.

Cre-recombinase Activity

Slc12a3^{IRES CRE-ERT2/+} male chimeras were mated to R26R^{tdTomato/tdTomato} females to generate *Slc12a3*^{IRES CRE-ERT2/+}; R26R^{tdTomato/+} pups. In order to activate tdTomato reporter expression, P19 day pups were injected with tamoxifen in corn oil (1X 2mg to 40g body weight) and the tissues were assayed 48 hours after the injection. Tamoxifen dependent Cre activity was detected in the kidney in *Slc12a3*^{IRES CRE-ERT2/+}; R26R^{tdTomato/+} samples (Figure 3).

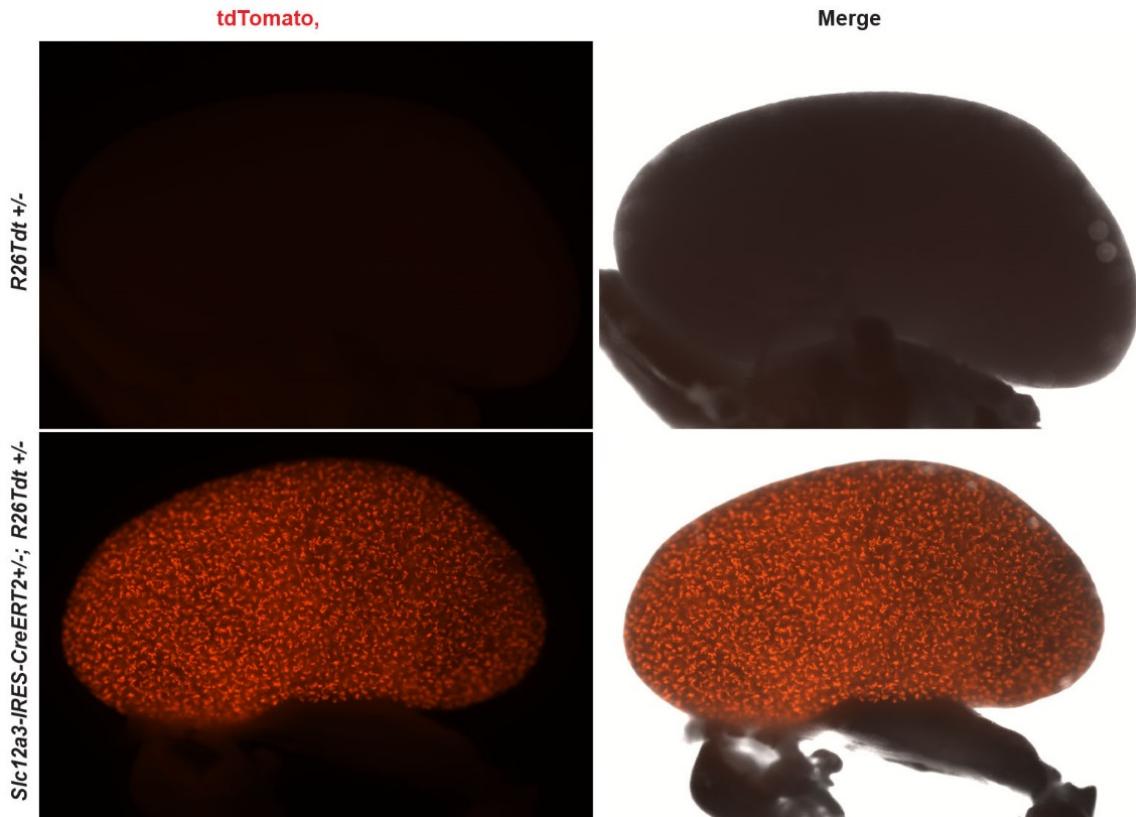


Figure 3. Tamoxifen dependant tdTomato positive cells observed in the kidney of *Slc12a3*^{IRES CRE-ERT2/+}; R26R^{tdTomato/+} P21 pups after a single injection at P19 (2mg/40g body weight).

Immunohistochemistry

The Urogenital system (UGS) was fixed in 4% paraformaldehyde at 4°C 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 kidney was sectioned at 12um and probed with the antibodies listed in (Table 2).

Primary Antibody	Company	Catalog #	Dilution	Secondary	Company	Dilution
Rabbit-anti-Slc12a3	Sigma	HPA0287 48	1/500	Donkey anti-rabbit A647	Invitrogen	1/500
Rabbit anti Tamm-Horsfall	Alfa Aesar	J65429	1:250	Donkey anti-rabbit A647	Invitrogen	1/500
LTL-FITC conjugate)	Vector Laboratories	FL-1321	1/100			

Table 2. Summary of antibodies used to screen *Slc12a3*^{IRES CRE-ERT2/+}; R26R^{tdTomato/+} P21 kidney sections.

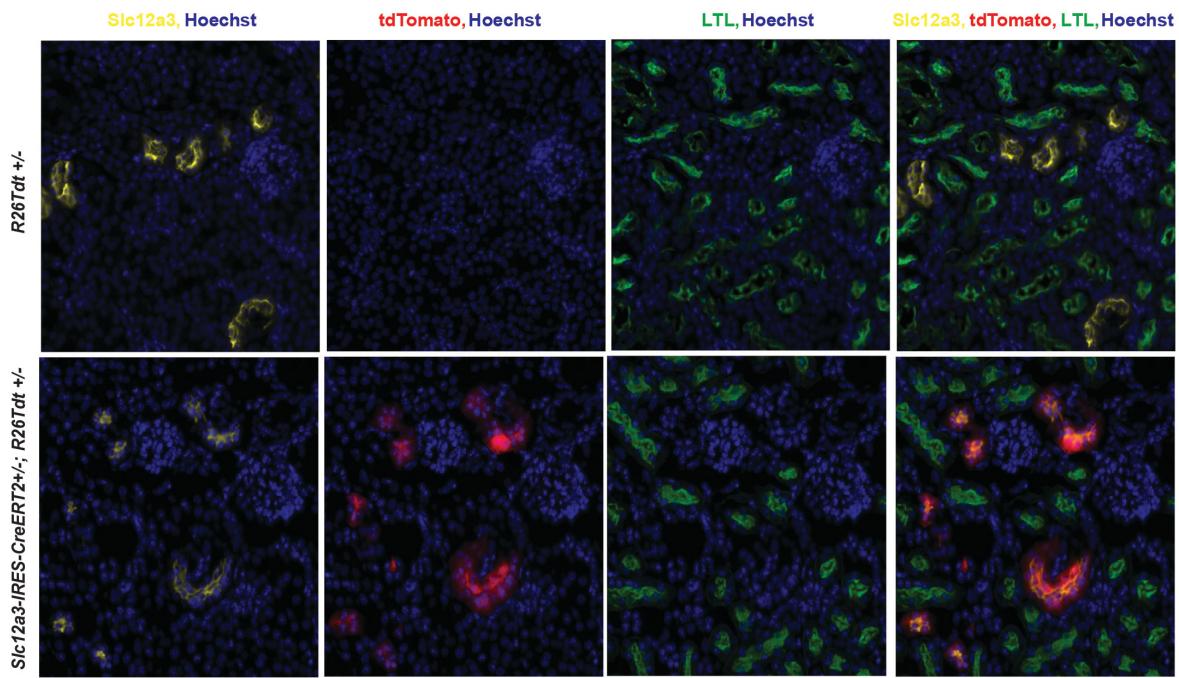
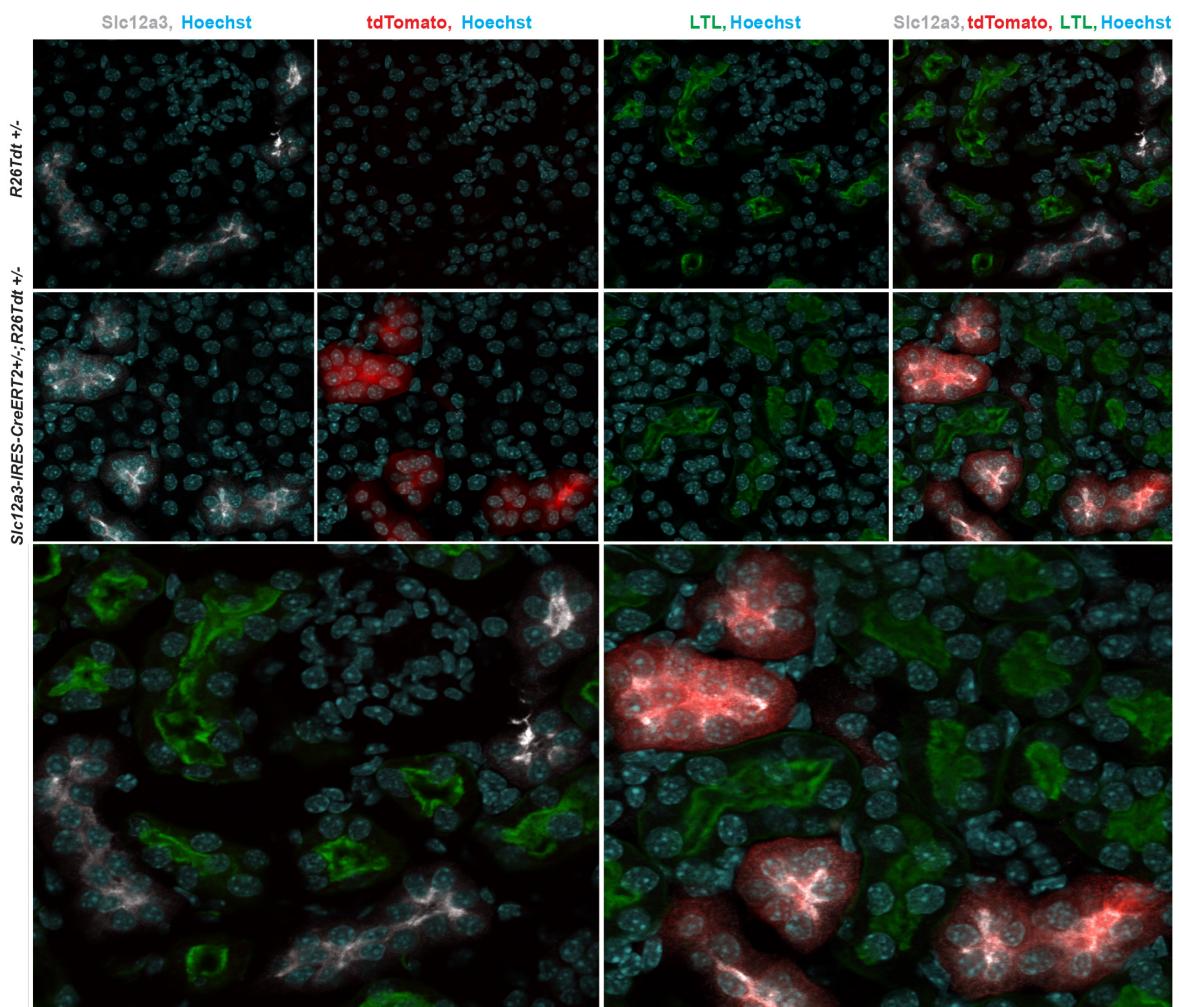
A.**B.**

Figure 4. Tamoxifen dependent tdTomato positive cells in *Slc12a3*^{IRES CRE-ERT2/+}; *R26R*^{tdTomato/+} kidney co-localize with *Slc12a3*+ cells in the distal convoluted tubules but not LTL+ proximal tubule cells in P21 day mice following tamoxifen injection at P19 (A&B).

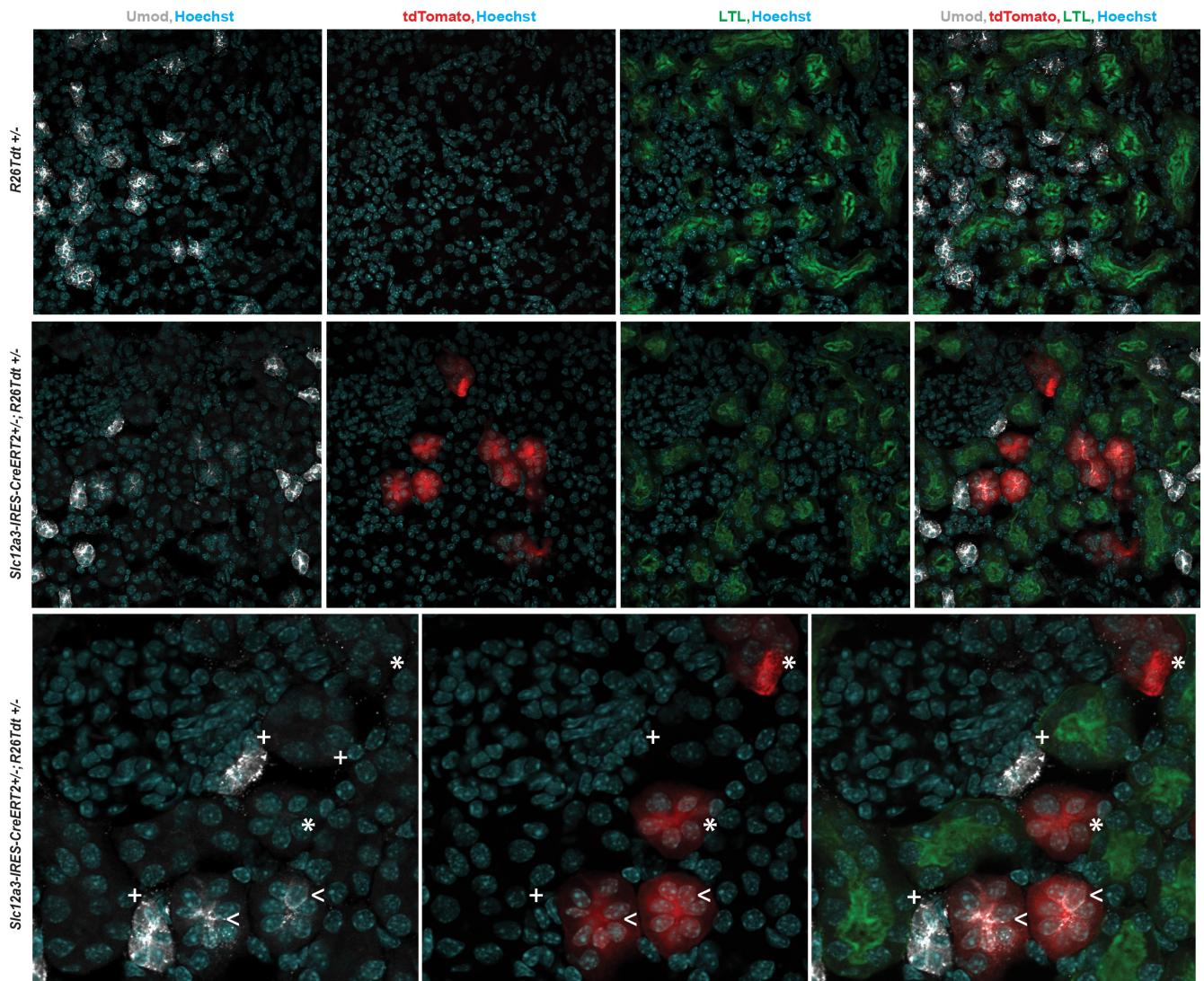


Figure 5. A small percentage of tamoxifen dependent tdTomato positive cells in *Slc12a3*^{IRES CRE-ERT2/+}, R26R^{tdTomato/+} kidneys are also Umod+ (arrow head) at the junction with the ascending limb of the loop of Henle (LOH). Tubule cells on either side of the junction express Umod (plus) or tdTomato (asterix) in P21 day mice following injection of tamoxifen at P19.