Trpv4$^{CRE-ERT2}$ Allele Characterization

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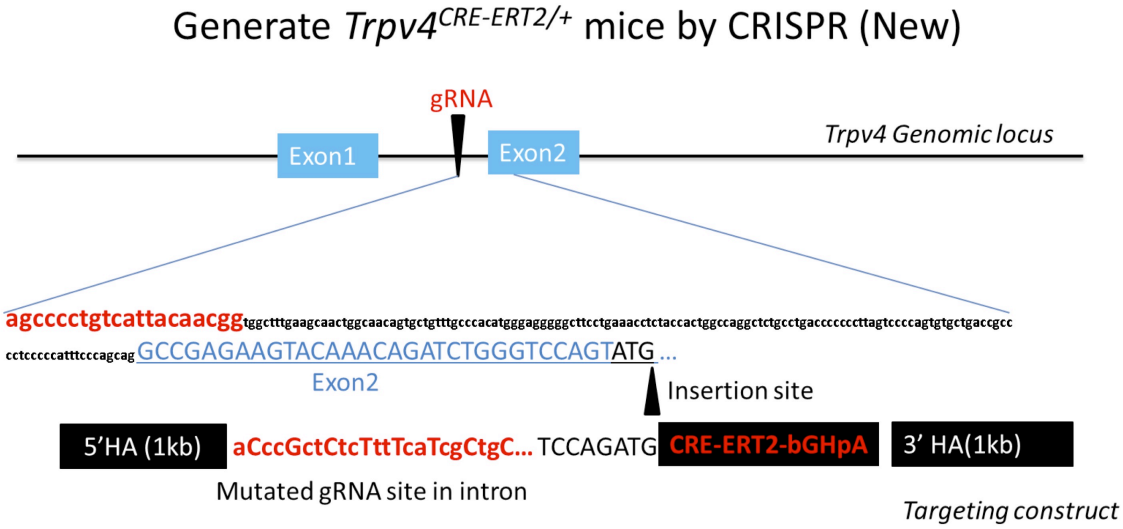
Findings: VALIDATED

Our analysis confirms activity of CRE::ERT2 under the regulation of *Trpv4*. Cre-dependent tdTomato activity was observed in the epithelium of the skin and chondrocytes of the rib in postnatal day 2 (P2) pups in the epithelium of the skin and chondrocytes of the rib following tamoxifen injection at P0. Cre inducible expression was confirmed by immunohistochemistry, tdTomato cells co-localize with a percentage of Trpv4+ cell types in the rib and skin. Expression of tdTomato and Trpv4 protein was examined in dorsal root ganglia at P2, but no expression was detected.

Data:

Crosses

The Trpv4$^{CRE-ERT2}$ strain is a CRISPR/ Cas9 mediated knock-in of CRE-ERT2 into the ATG site of the *Trpv4* (Transient receptor potential cation channel subfamily V member 4) gene in JM8.N4 ES cells. 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), CRE-ERT2-bGHpA DNA and 3' 1-kb homologous arms (HA), and linear pBluescript vector. A silent mutation was introduced into the 5' PCR fragment at 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



gRNA oligos for cloning:
Forward: caccgAGCCCCTGTCATTACAACGG
Reverse: aaacCCGTTGTAATGACAGGGGCTc

Figure 1. Diagram of the strategy adopted to generate CRISPR/Cas9 mediated knock-in of CRE-ERT2-bGHpA into the Trpv4 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. Trpv4 $^{CRE-ERT2/+}$ males were mated to R26R $^{tdTomato/tdTomato}$ female mice and the urogenital system (UGS) was collected from 3 wk old mice post Tamoxifen induction. All three of the F1 males (M1, M2, M3) transmitted the transgene (Table 1).

Line	Clone	GLT	Cre activity
Trpv4 $^{CRE-ERT2/+}$ M1	12	Yes	Yes
Trpv4 $^{CRE-ERT2/+}$ M2	12	Yes	Yes
Trpv4 $^{CRE-ERT2/+}$ M3	12	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: 466bp

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

DNA sequence (reverse) 5'-GCAAACGGACAGAAGCATTT-3'

Amplifies 5' arm into Cre sequence within Cre region.

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
Amplify Taq	0.2ul (5u/ul)
Genomic DNA	1ul
Total volume	**25 ul**

94ºC	3min	1 cycle
94ºC	20sec	35cycles
60ºC	20sec	
72ºC	45sec	
72ºC	10min	1 cycle

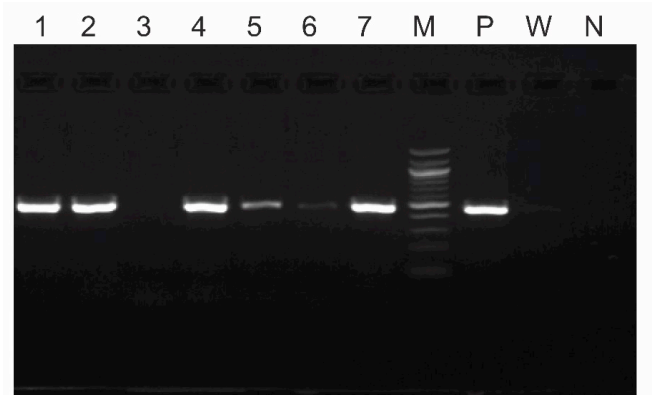


Figure 2: Lanes 1, 2, 4, 5, 6 & 7 show pups that display the expected diagnostic PCR product of 466 bp for the targeted allele. **M:** DNA Marker**, P:** Positive control**, Wt:** Wildtype.

Cre-recombinase Activity

Trpv4 $^{CRE-ERT2/+}$ male chimeras were mated to R26R $^{tdTomato/tdTomato}$ females to generate Trpv4 $^{CRE-ERT2/+}$; R26R$^{tdTomato/+}$ pups. In order to activate tdTomato reporter expression, P0 pups were injected intraperitoneally with tamoxifen in corn oil (1X 2mg to 40g body weight) and the tissues were assayed at 2 days after the tamoxifen injection. Tamoxifen dependent Cre activity was detected in the skin and toes as well as ribs of the Trpv4 $^{CRE-ERT2/+}$; R26R$^{tdTomato/+}$ samples (Figure 3-7).

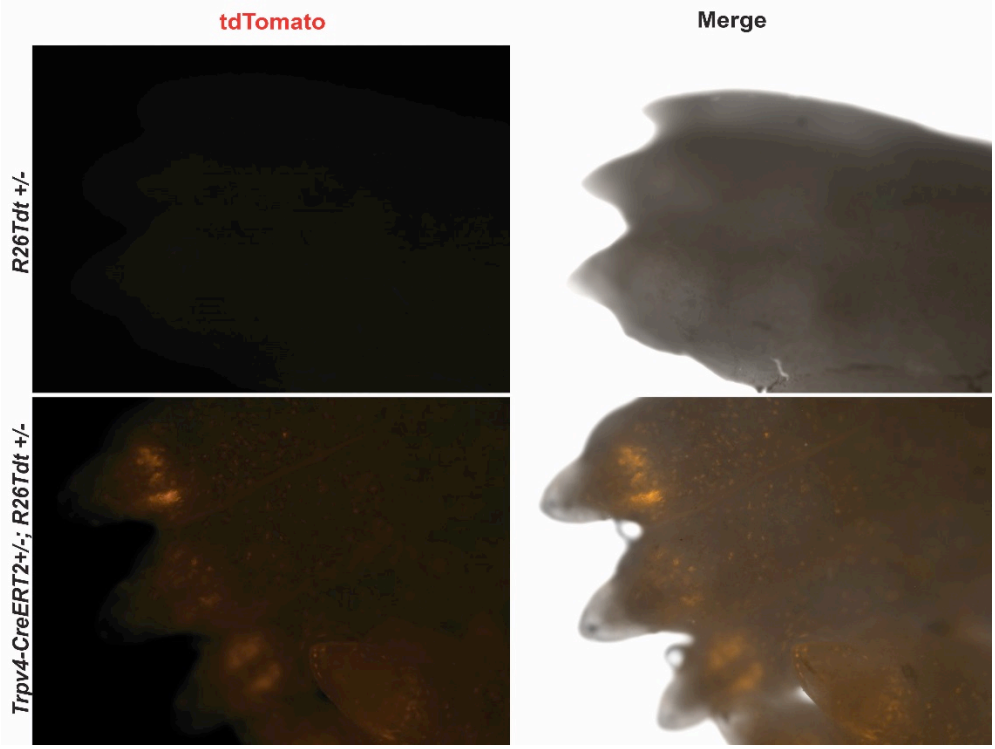


Figure 3. Tamoxifen dependant tdTomato positive cells observed in the skin and toes of Trpv4 $^{CRE-ERT2/+}$;R26R$^{tdTomato/+}$ P2 day pups after a single injection (2mg/40g body weight) at P0.

Immunohistochemistry

Eviserated P2 pup bodies were fixed in 4% paraformaldehyde at 4ºC for 1 hour, washed 3 times in PBS. The body was bisected equilibrated in 30% sucrose overnight and then embedded in OCT to provide the best orientation for the different tissues and flash frozen on dry-ice. The rostral and caudal segments were sectioned at 12um and probed with the antibodies listed in (Table 2).

Primary Antibody	Company	Catalog #	Dilution	Secondary	Company	Dilution
Mouse IgG1 anti Neurofilament	DSHB	GFP-1020	1/500	Goat anti-Mouse IgG1 A647	Invitrogen	1/500
Rabbit anti Trpv4	Alomone labs	ACC-034	1:1000	Donkey anti-rabbit IgG A488	Invitrogen	1/500
Rabbit anti-RFP	Rockland	600-901-375	1/2000	Donkey anti-rabbit IgG A488	Invitrogen	1/500

Table 2. Summary of antibodies used to screen Trpv4 $^{CRE-ERT2/+}$;R26R$^{tdTomato/+}$adult UGS sections.

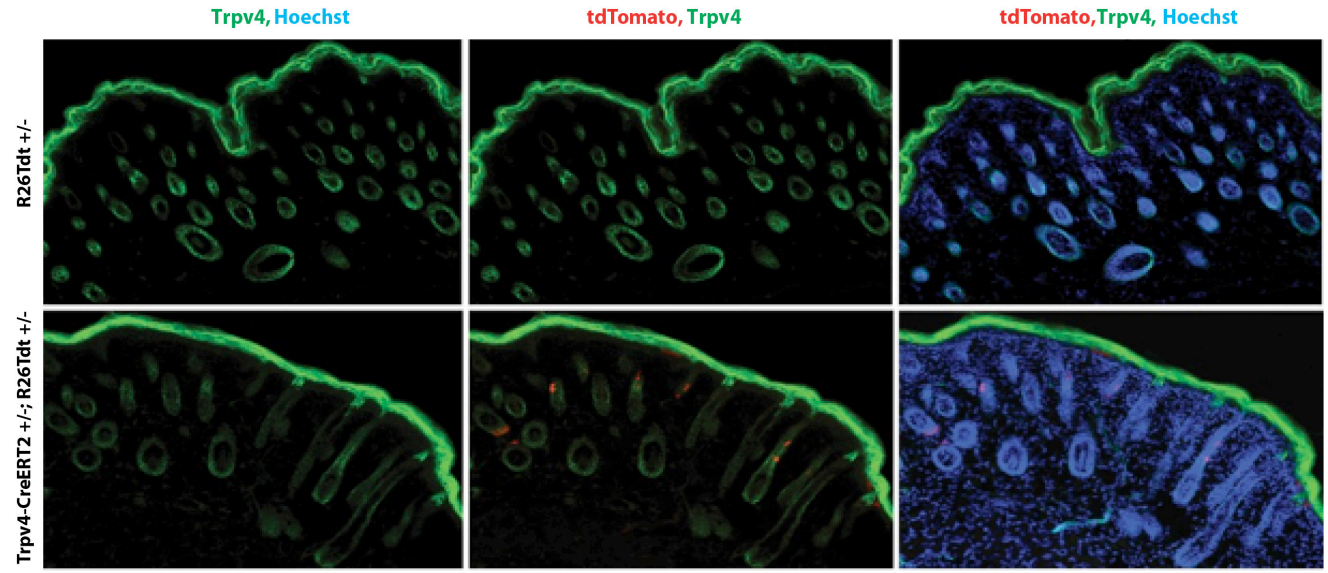


Figure 4. A percentage of tamoxifen dependant tdTomato positive cells co-localize with Trpv4+ cells in the epithelium of the skin of Trpv4 $^{CRE\text{-}ERT2/+}$;R26R$^{tdTomato/+}$ P2 pups after a single tamoxifen injection at P0.

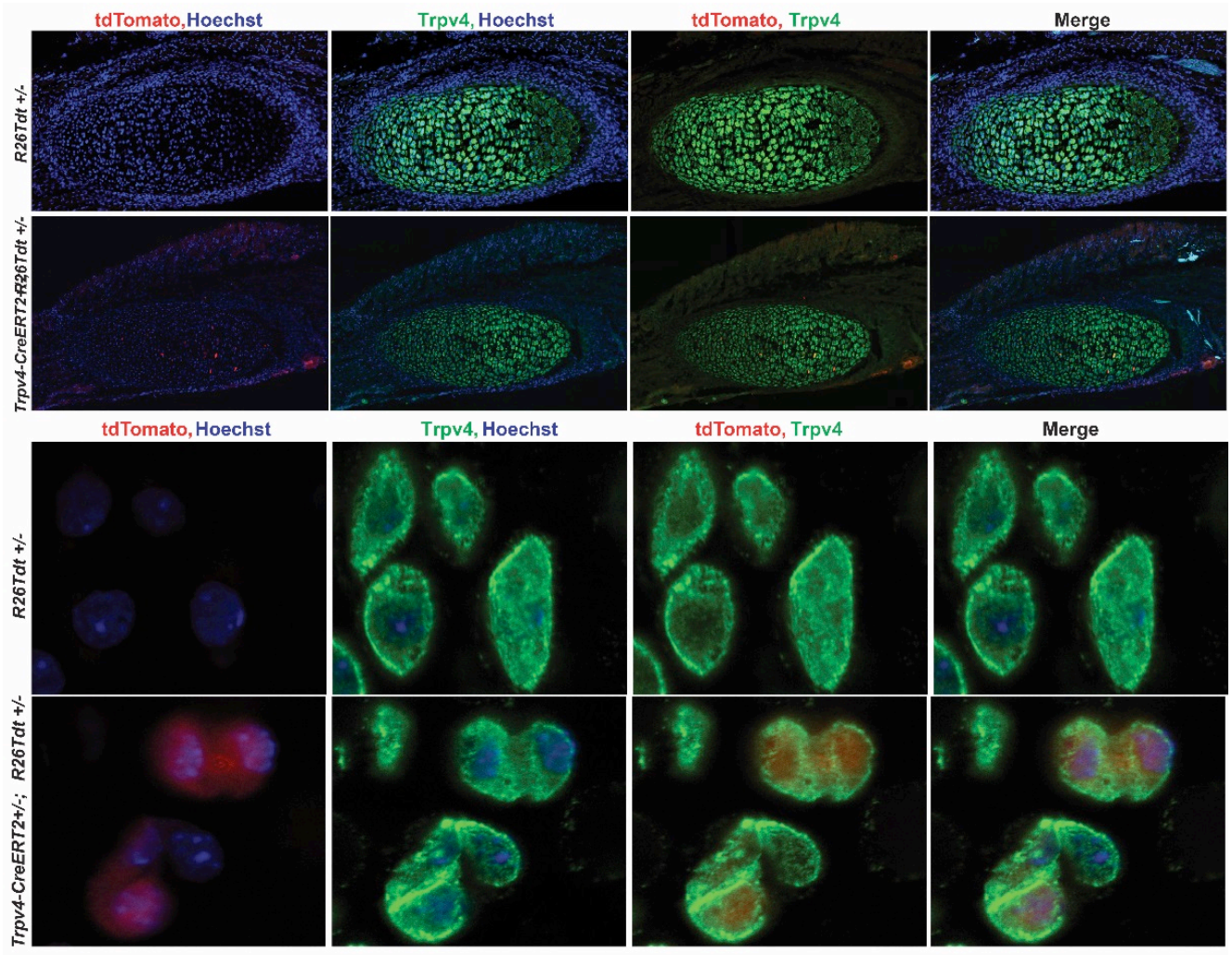


Figure 5. Tamoxifen dependant tdTomato positive cells present in the chondrocytes of Trpv4 $^{CRE\text{-}ERT2/+}$;R26R$^{tdTomato/+}$ co-localize with a percentage of Trpv4+ cells in P2 pups after a single injection of tamoxifen at P0.

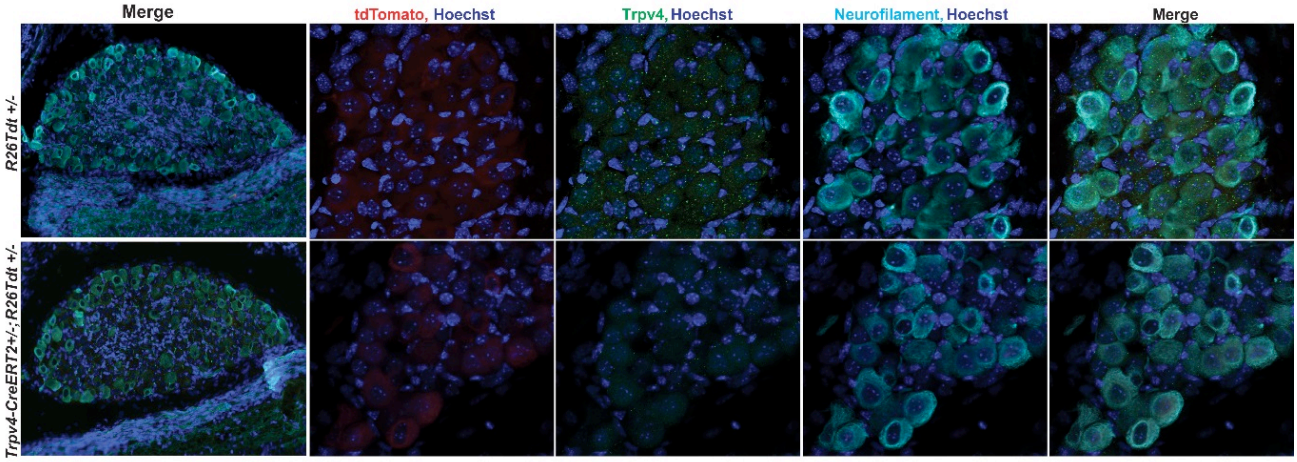


Figure 6. A single injection of tamoxifen was given to Trpv4 $^{CRE-ERT2/+}$;R26R$^{tdTomato/+}$ and R26R$^{tdTomato/+}$ at P0 (2mg/40g body weight) and pups analyzed at P2. Neurofilament positive neurons were observed in the dorsal root ganglion of both the Trpv4 $^{CRE-ERT2/+}$;R26R$^{tdTomato/+}$ and R26R$^{tdTomato/+}$ pups but no tdTomato+ or Trpv4+ cells were detected.