

Gfra3^{CRE-ERT2} Allele Characterization

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Findings: VALIDATED

Our analysis confirms activity of CreErt2 under the regulation of Gfra3. Expression of Cre dependent tdTomato expression was observed upon induction in bladder of postnatal day 7 (P7) pups following tamoxifen injection at P5. Cre inducible expression in the bladder was confirmed by immunohistochemistry, tdTomato cells co-localize with Gfra3+ cell types and in likely glial cells adjacent to Beta tubulin III+ neurons, labelled cells are distinct from Smooth muscle actin+ mesenchyme/muscle cells or Keratin 5+ bladder epithelial cells.

Data:

Crosses

The Gfra3^{CRE-ERT2} strain is a CRISPR/ Cas9 mediated knock-in of CRE-ERT2 into *Gfra3* (GDNF family receptor alpha-3) near the ATG site 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 by GIBSON assembly with four PCR fragments: 5' 1-kb homologous arms (HA), KOZAK-CRE-ERT2-bGHpA DNA and 3' 1-kb homologous arms (HA), and linear pBluescript vector. The KOZAK-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 *Gfra3*^{CRE-ERT2/+} mice by CRISPR (SH)

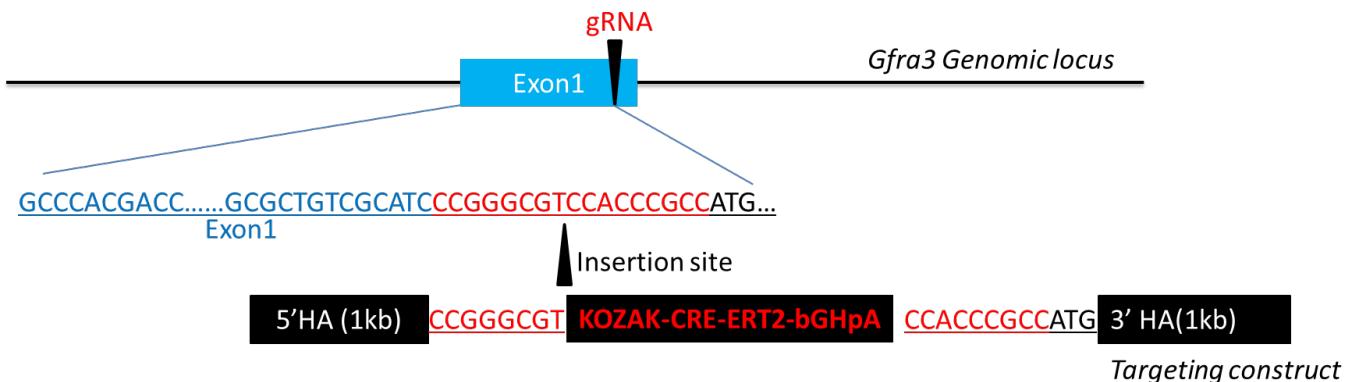


Figure 1. Diagram of the strategy adopted to generate CRISPR/Cas9 mediated knock-in of KOZAK-CRE-ERT2-bGHpA into the *Gfra3* locus of JM8.N4 ES cells.

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 Laboratory 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. Male chimeras were mated to B6 and R26R ^{tdTomato/tdTomato} female mice and the urogenital system (UGS) was collected from P7 pups post Tamoxifen induction. All three of the F1 males tested (M1, M2, M3) transmitted the transgene (Table 1).

Line	Clone	GLT	Cre activity
Gfra3 ^{CRE-ERT2/+} M1	3	yes	yes
Gfra3 ^{CRE-ERT2/+} M2	3	yes	yes
Gfra3 ^{CRE-ERT2/+} M3	3	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 (3' arm) Size: 496 bp

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

DNA sequence (reverse) 5-CGCGAACATCTTCAGGTTCT-3'

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 R	1ul	62°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					

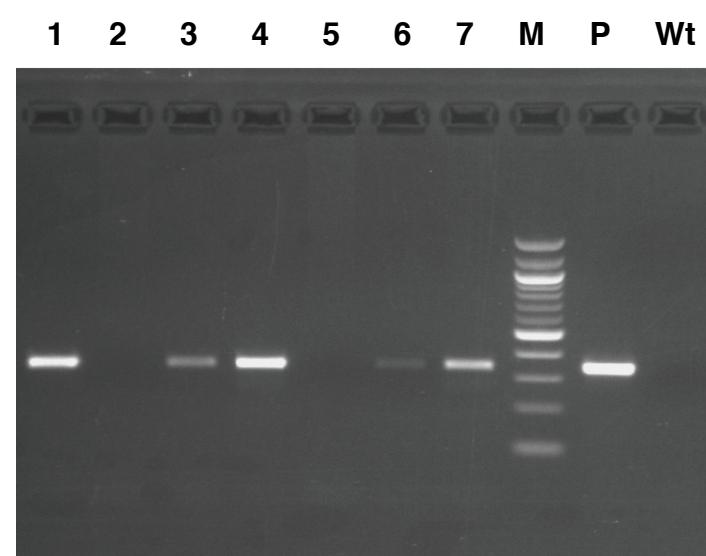


Figure 2: Lanes 1, 3, 4, 6 & 7 show pups that display the expected diagnostic PCR product of 496 bp for the targeted allele. **M:** DNA Marker, **P:** Positive control, **Wt:** Wildtype.

Cre-recombinase Activity

Gfra3^{CRE-ERT2/+} male chimeras were mated to R26R^{tdTomato/tdTomato} females to generate Gfra3^{CRE-ERT2/+}; R26R^{tdTomato/+} pups. In order to activate tdTomato reporter expression, P5 pups were injected intraperitoneally with tamoxifen in corn oil (1X 2mg to 40g body weight) and the tissues were assayed at P7. Tamoxifen dependent Cre activity was detected in the bladder of Gfra3^{CRE-ERT2/+}; R26R^{tdTomato/+} samples.

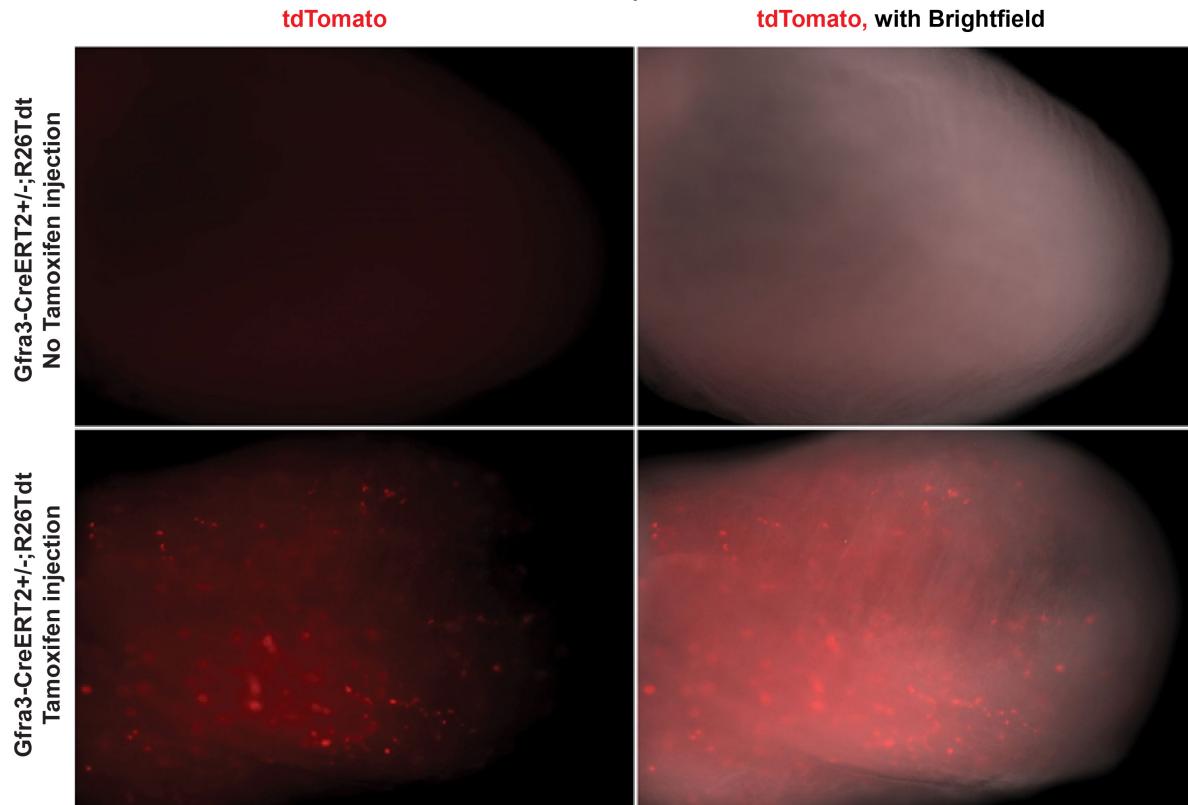


Figure 3. Tamoxifen dependant tdTomato positive cells observed in the bladder of Gfra3^{CRE-ERT2/+}; R26R^{tdTomato/+} P7 day pups after a single injection at P5 (2mg/40g body weight).

Immunohistochemistry

Bladders were 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 bladders were sectioned at 12um and probed with the antibodies listed in (Table 2).

Primary Antibody	Company	Catalog #	Dilution	Secondary	Company	Dilution
Rabbit anti-Beta tubulin neuronal class III (TUJ1)	BioLegend	MRB-435P	1/1000	Donkey anti-rabbit IgG A488	Invitrogen	1/500
Rabbit anti Keratin 5	Covance	PRB-160p	1:1000	Donkey anti-rabbit IgG A647	Invitrogen	1/500
Mouse IgG2a anti-Actin (α-Smooth Muscle)	Sigma	A5228	1/2000	Goat anti-mouse IgG2a A633	Invitrogen	1/500
Mouse anti- Beta tubulin III-TUJ1	Covance	MMS-435P	1/500	Donkey anti-rabbit IgG A488	Invitrogen	1/500
Goat anti-Gfra-3	R&D	AF2645	1/500	Donkey anti-goat IgG-A488	Invitrogen	1/500

Table 2. Summary of antibodies used to screen Gfra3^{CRE-ERT2/+}; R26R^{tdTomato/+} adult bladder sections.

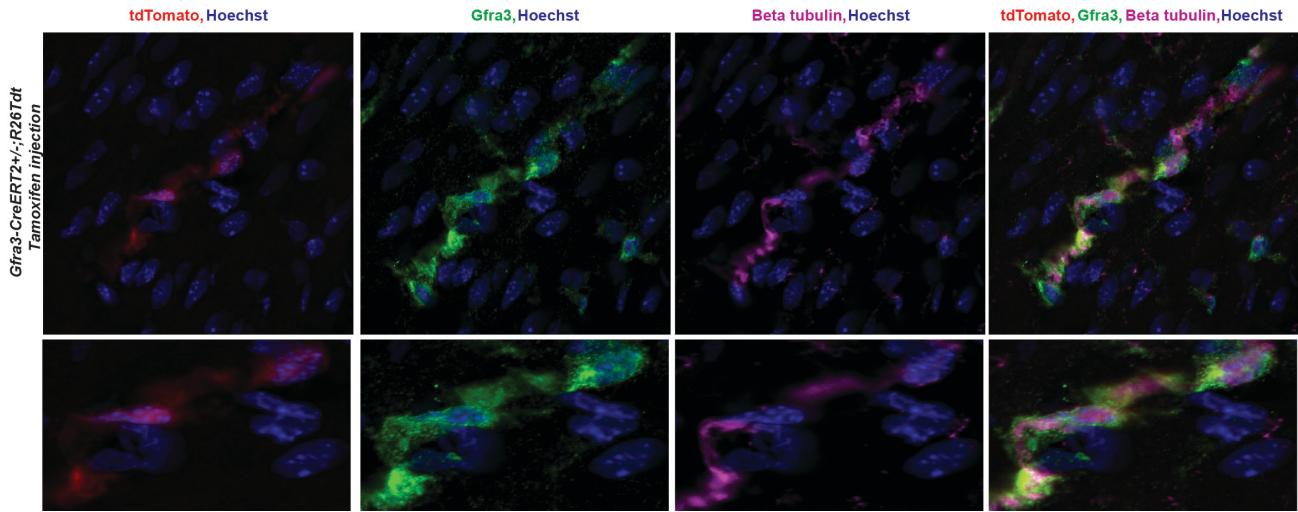
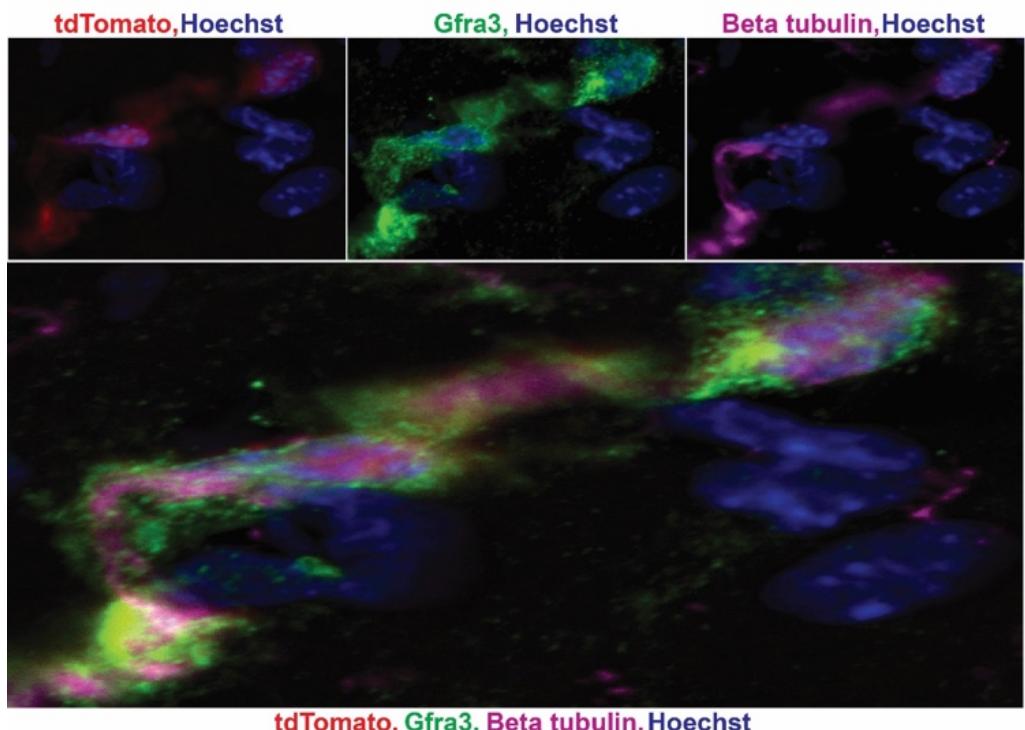
A.**B.**

Figure 4. A percentage of tamoxifen dependent tdTomato positive cells in $Gfra3^{CRE-ERT2/+}; R26R^{tdTomato/+}$ bladder co-localize with Gfra3+ and in likely glial cells adjacent to Beta tubulin III+ neurons in P7 pups following injection of tamoxifen at P5.

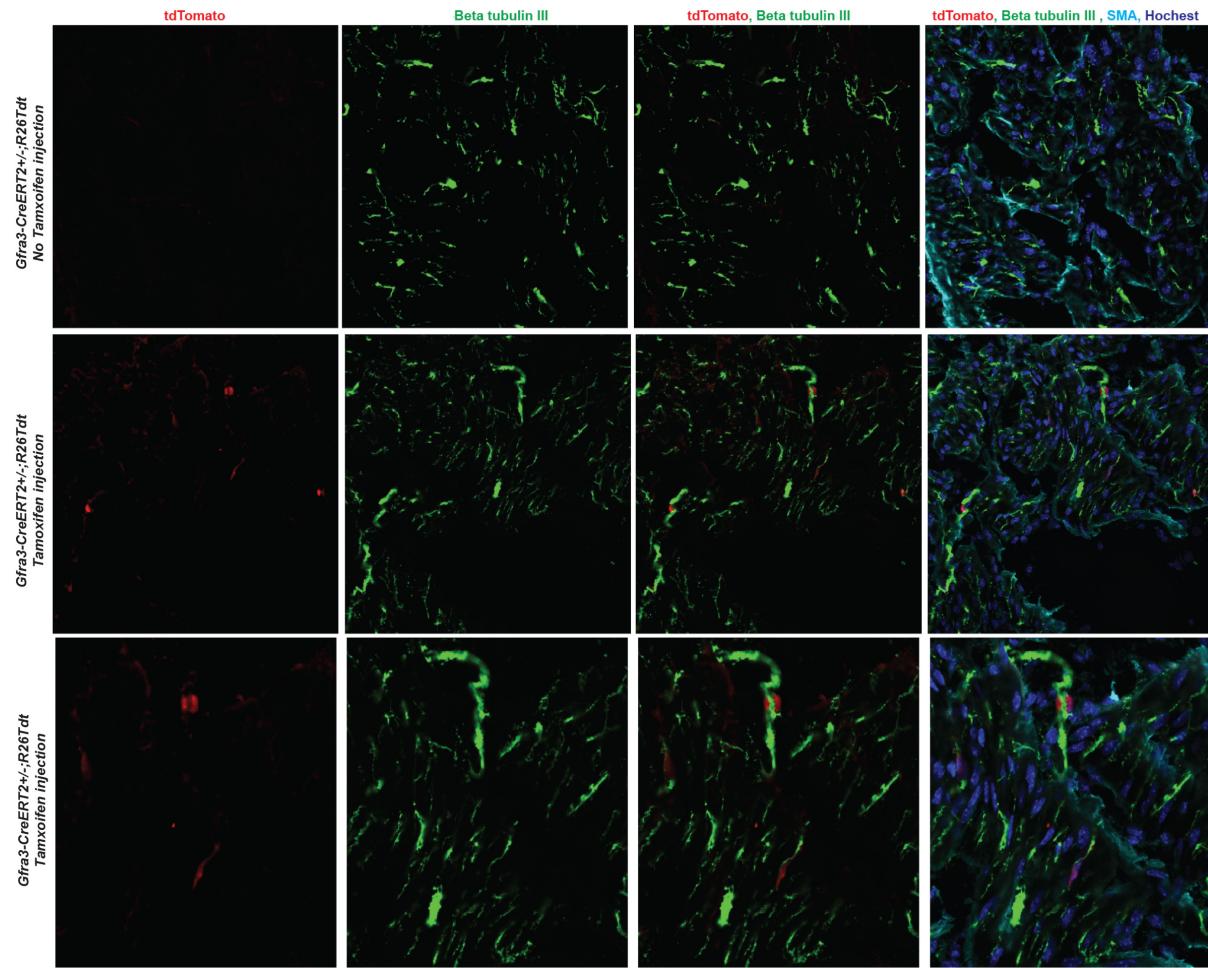
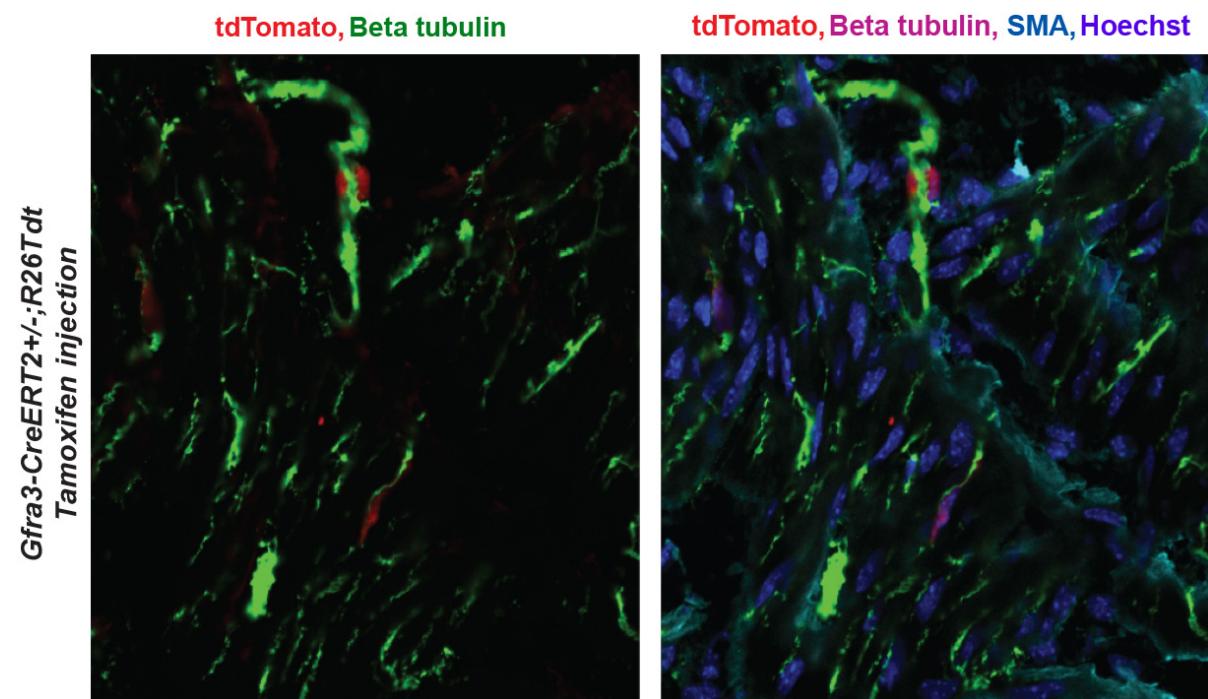
A.**B.**

Figure 5. A percentage of tamoxifen dependent tdTomato positive cells in Gfra3^{CRE-ERT2^{+/+}}; R26R^{tdTomato/+} bladder localize adjacent to Beta tubulin III+ cells that are distinct from α-Smooth Muscle Actin+ cells in P7 pups following injection of tamoxifen at P5.

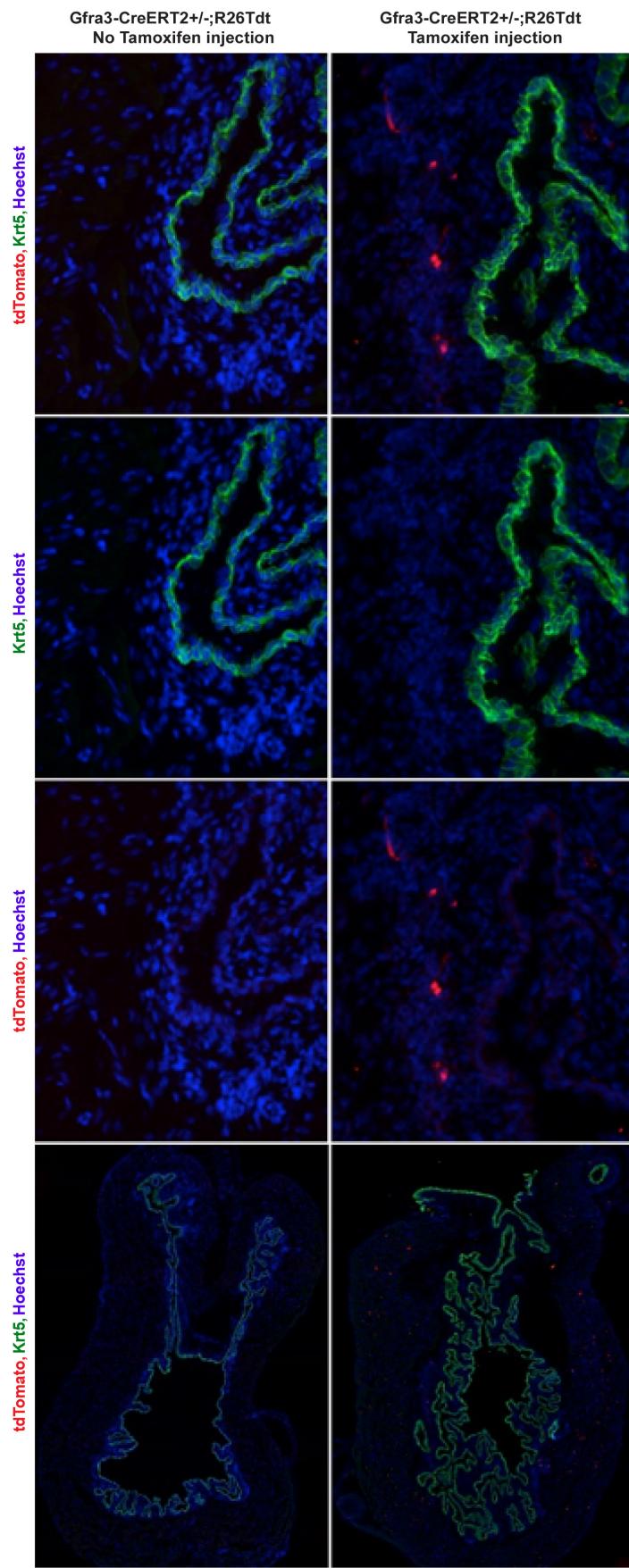


Figure 6. Tamoxifen dependent tdTomato positive cells in $\text{Gfra3}^{\text{CRE-ERT2}/+}$, R26R^{tdTomato/+} bladder do not co-localize with Keratin 5+ epithelial cells in P7 pups following tamoxifen injection at P5.