Upk3a-GCE Allele Characterization

Authors: Jinjin Guo, Jill McMahon, M. Todd Valerius, and Andrew P. McMahon

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Findings: VALIDATED

Our analysis confirmed the expression of eGFPCreER^{T2} (GCE) cells in the bladder urothelium and ureters in Upk3a$^{GCE/+}$ neonates. Cre dependent ß-galactosidase (ß-Gal) activity is observed in the bladder upon tamoxifen induction. GFP positive cells represent a large percentage of the cells lining the lumen of the bladder and adjacent to Krt5 positive cells in the urothelium. Upk3a protein as visualized with Upk antibody, is on the luminal side of the GFP positive cells.

Data:

Crosses

The Upk3a-GCE strain is a BAC transgenic line with eGFPCreER^{T2} (GCE) expressed in the Upk3a domain. Pronuclear injection of the BAC construct DNA into C57Bl6/DBA F1 mouse embryos resulted in the birth of 45 pups of which 1 male and 10 females carried the transgene. The male did not transmit the transgene, however, 5 of 7 females tested did transmit the BAC transgene and exhibited similar expression patterns as determined by lacZ staining of 16.5dpc-P21 UGS. Subsequent analysis was carried out with female founders F26 and F17.

The female founders were crossed with Rosa26R$^{lacZ/+}$ (R26R) male mice to obtain Upk3a$^{GCE/+}$; R26R$^{lacZ/+}$ embryos. The urogenital systems (UGS) of dissected Upk3a$^{GCE/+}$ embryos were viewed with a fluorescent microscope; GFP was detectable in the bisected bladder in wholemount at 15.5dpc (F17). In order to activate ß-galactosidase (β-gal) reporter expression from the R26R$^{lacZ/+}$allele, an intraperitoneal injection of tamoxifen in corn oil (1mg to 40g body weight) was injected into pregnant 13.5dpc mice or 2 days prior to collection in neonates or weanlings. A control group was injected with the same volume of corn oil. UGS from tamoxifen induced females were dissected at 15.5-16.5dpc and pups were collected from P1-P19. Tamoxifen dependant β-gal activity was observed in the bladder urothelium and ureters in Upk3a$^{GCE/+}$; R26R$^{lacZ/+}$ (F26 and F17); a small number of X-gal positive cells were observed in the bladder of corn oil injected Upk3a$^{GCE/+}$; R26R$^{lacZ/+}$ (F26) pups injected at P2 and collected at P5, probably as a result of the strong expression of Cre from the Upk3a sequence in the BAC construct.

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 (Fig1).

Oligonucleotides: for targeted/transgenic allele Size: 371bp
DNA sequence (forward): 5'-aggagctttgcagaagacca-3'
DNA sequence (reverse 2) 5'-gaacttcagggtcagcttgc-3'
Amplifies 5' arm into GFP sequence within GFP-Cre region.

Rxn Buffer and Conditions: (25*µ*l reaction)

10X GSB	2.5ul
25mM dNTP	1ul
10uM primer F	1ul
10uM primer R1	1ul
DMSO	2.5ul
2-mercaptoethanol	0.125ul
	0.2ul
Amplify Taq	(5u/ul)
5x cresol red dye	5ul
Genomic DNA	1ul
Total volume	**25 ul**

94ºC	3min	1 cycle
94ºC	30sec	35cycles
56ºC	30sec	
72ºC	45sec	
72ºC	10min	1 cycle

10X Gitschier Buffer (GSB):
670 mMTris, pH 8.8
166 mM Ammonium Sulfate
65 mM MgCl2
0.1% gelatin

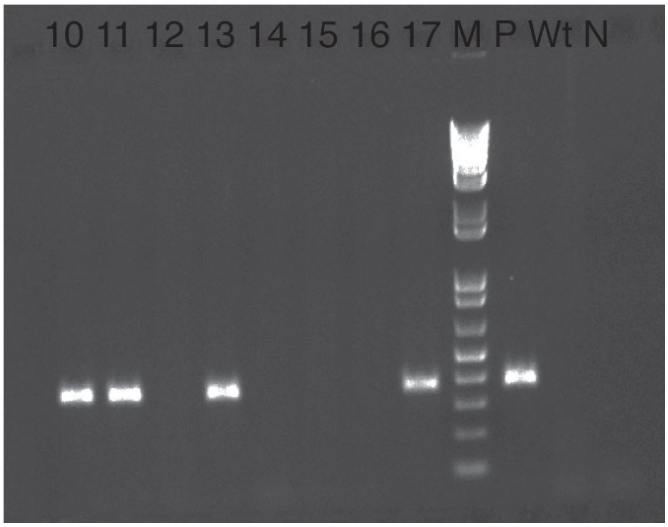

Fig 1: Numbers 10, 11, 13 and 17 Upk3a$^{GCE/+}$; Rosa26R$^{lacZ/+}$, numbers 12, 14-16 Rosa26R$^{lacZ/+}$**. P:** Upk3a$^{GCE/+}$ positive control; **W**: Wildtype control; **N**: Negative control.

Native Fluorescence

Dissected 15.5 dpc UGS were examined with a fluorescent microscope to view GFP expression. GFP was detected in bisected bladders in Upk3a$^{GCE/+}$ embryos (Fig 2).

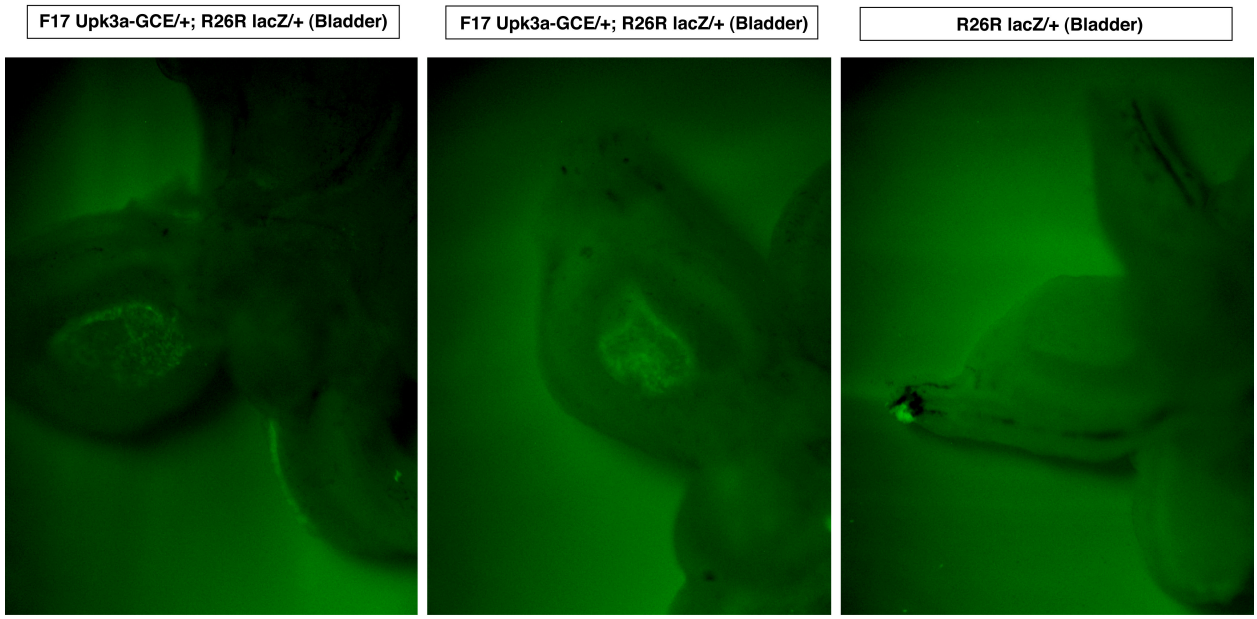

Fig 2. Wholemount GFP detection in 15.5dpc Upk3a$^{GCE/+}$ UGS.
GFP fluorescence was visible in the bladder urothelium at 15dpc.

Cre-recombinase Activity

Dissected UGS samples were stained with X-gal to assay for β-gal activity. Tamoxifen dependent Cre activity was detected in Upk3a$^{GCE/+}$, R26R$^{lacZ/+}$ samples (Fig. 3 and 4).

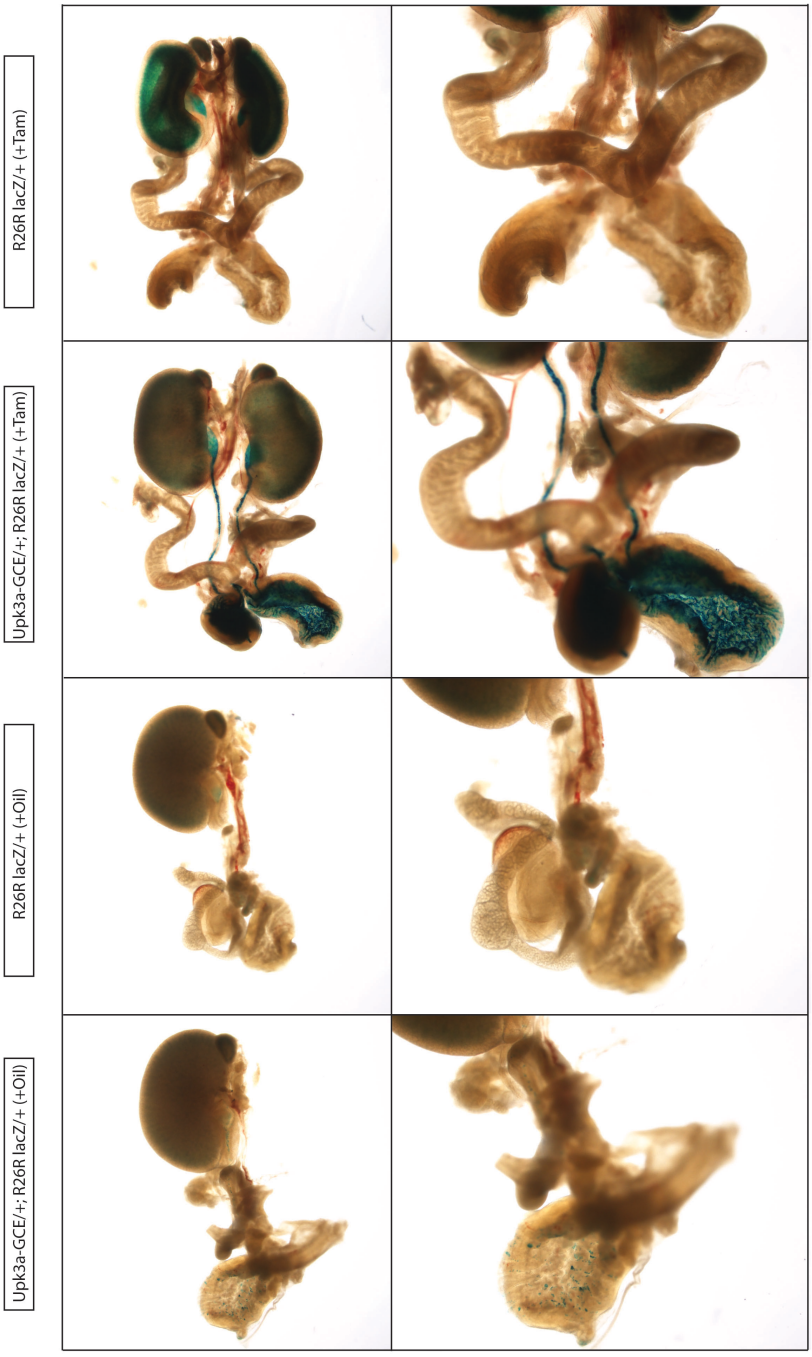

Fig 3. Cre-dependent β-gal activity in Upk3a$^{GCE/+}$; R26R$^{lacZ/+}$ UGSs at P5. An injection of tamoxifen given 3 days prior to collection at P5, resulted in X-gal staining of cells in the bladder and ureter in Upk3a$^{GCE/+}$; R26R$^{lacZ/+}$ but not R26R$^{lacZ/+}$ pups. An injection of corn oil results in a small number of cells with ß-gal activity in the bladder of Upk3a$^{GCE/+}$; R26R$^{lacZ/+}$ pups. (Note: the X-gal staining in the kidney is associated with endogenous enzyme activity and is not found in UGS collected from younger embryos).

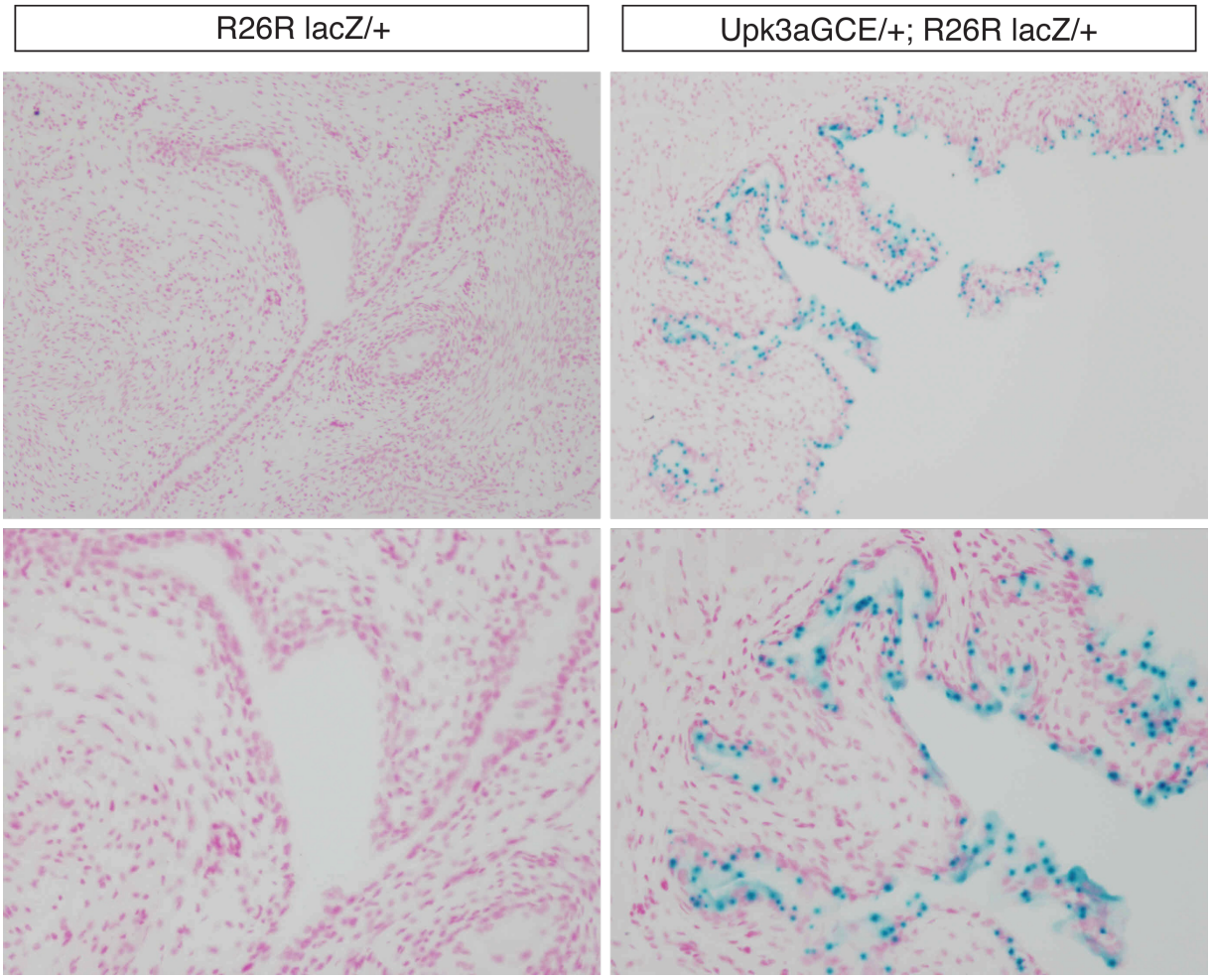

Fig 4. Cre-dependent β-gal activity in Upk3a$^{GCE/+}$; R26R$^{lacZ/+}$ UGS at P4. A single injection of tamoxifen at P2, resulted in a subset of cells proximal to the lumen of the bladder staining with X-gal in Upk3a$^{GCE/+}$; R26R$^{lacZ/+}$ but not R26R$^{lacZ/+}$ samples.

Immunohistochemistry

Immunohistochemistry was performed to examine whether the eGFPCreER^{T2} allele was expressed in the expected Upk3a domain. To test for Cre function, Upk3a$^{GCE/+}$; R26R$^{lacZ/}$ and R26R$^{lacZ/+}$ UGSs from tamoxifen injected mice were assayed. The location of GFP in the bladder urothelium with respect to the expression domains of Krt 5 and Upk and the colocalization of GFP and ß-gal expressing cells were examined in Upk3a$^{GCE/+}$; R26R$^{lacZ/+}$ P5 pups.

Whole UGSs were fixed in 4% paraformaldehyde at 4ºC 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 16um and probed with rabbit-anti-Keratin 5/chicken-anti-GFP/, mouse-anti-Actin (α-Smooth Muscle), rabbit-anti-Upk3a/chicken-anti-GFP/ mouse-anti-Actin (α-Smooth Muscle) and rabbit-anti-beta-gal/Chicken-anti-GFP/ mouse-anti-Actin (α-Smooth Muscle) respectively. GFP (Chicken,

Aves Labs, Inc, GFP-1020, 1:500); ß-gal (Rabbit, MP Biomedicals, LLC, 55976, 1: 10000), Keratin 5 (Rabbit, Covance, PRB-160p, 1:1000), Upk3a (Rabbit, 1:500 Kindly provided by Dr. Tung-Tien Sun), anti-Actin (α-Smooth Muscle) (Mouse IgG2a, Sigma, A5228, 1: 1000) were incubated overnight at 4ºC and detected with secondary antibodies Alexafluor 488, 555, 633, and 647 (Molecular probes) as indicated in the figure.

Immunohistochemistry data indicates GFP positive cells represent a large percentage of the cells lining the bladder, Upk3a protein as visualized with Upk pan antibody, is on the luminal side of the GFP positive cells. Krt5 positive cells closely oppose GFP positive cells with some apparent colocalization in a subset of the GFP cells in the bladder urothelium. (Fig. 5-9).

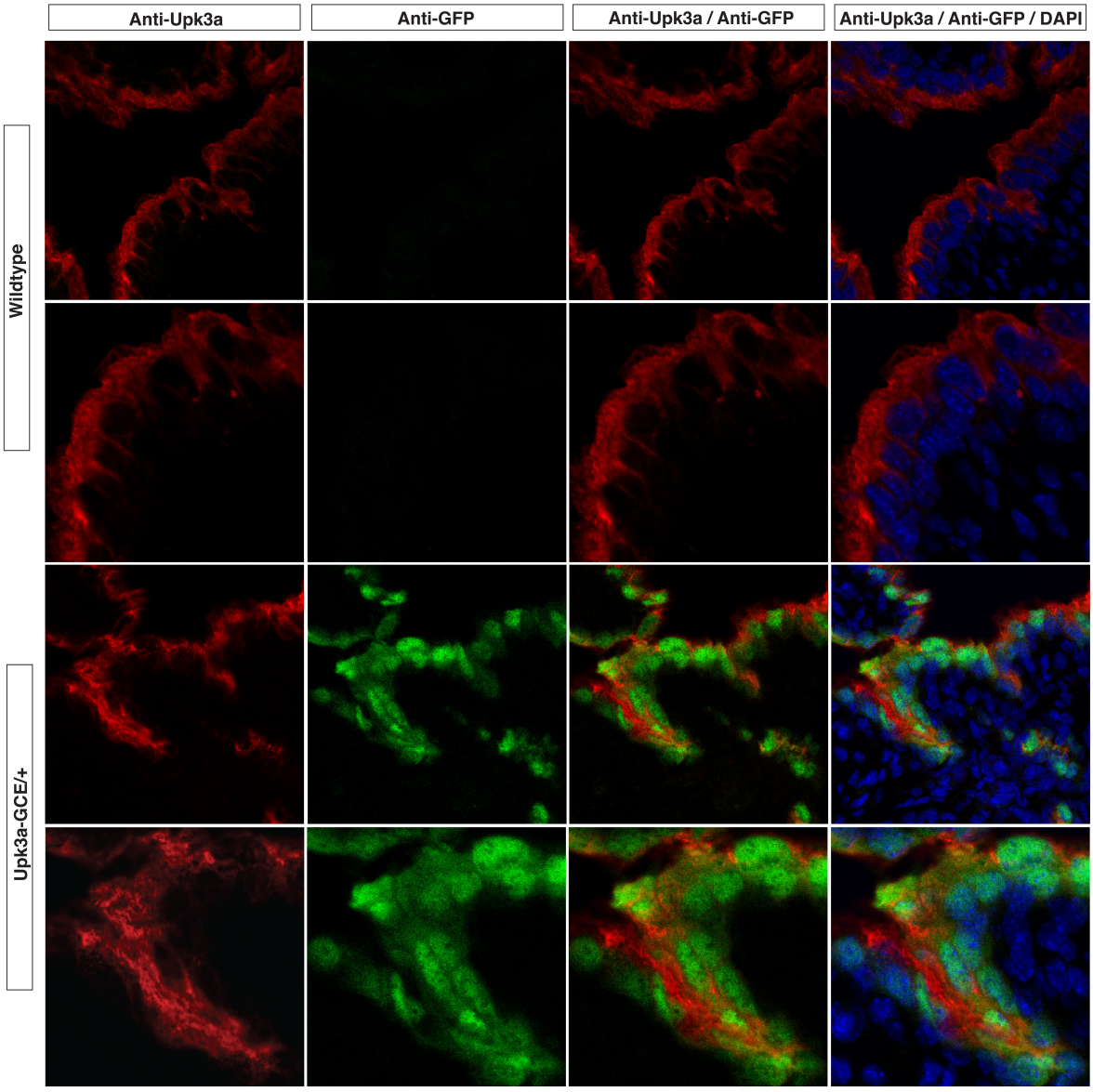

Fig 5. GFP is detected in cells lining the lumen of the bladder closely opposed to Upk positive cells in Upk3a$^{GCE/+}$; R26R$^{lacZ/+}$ P4 neonates.

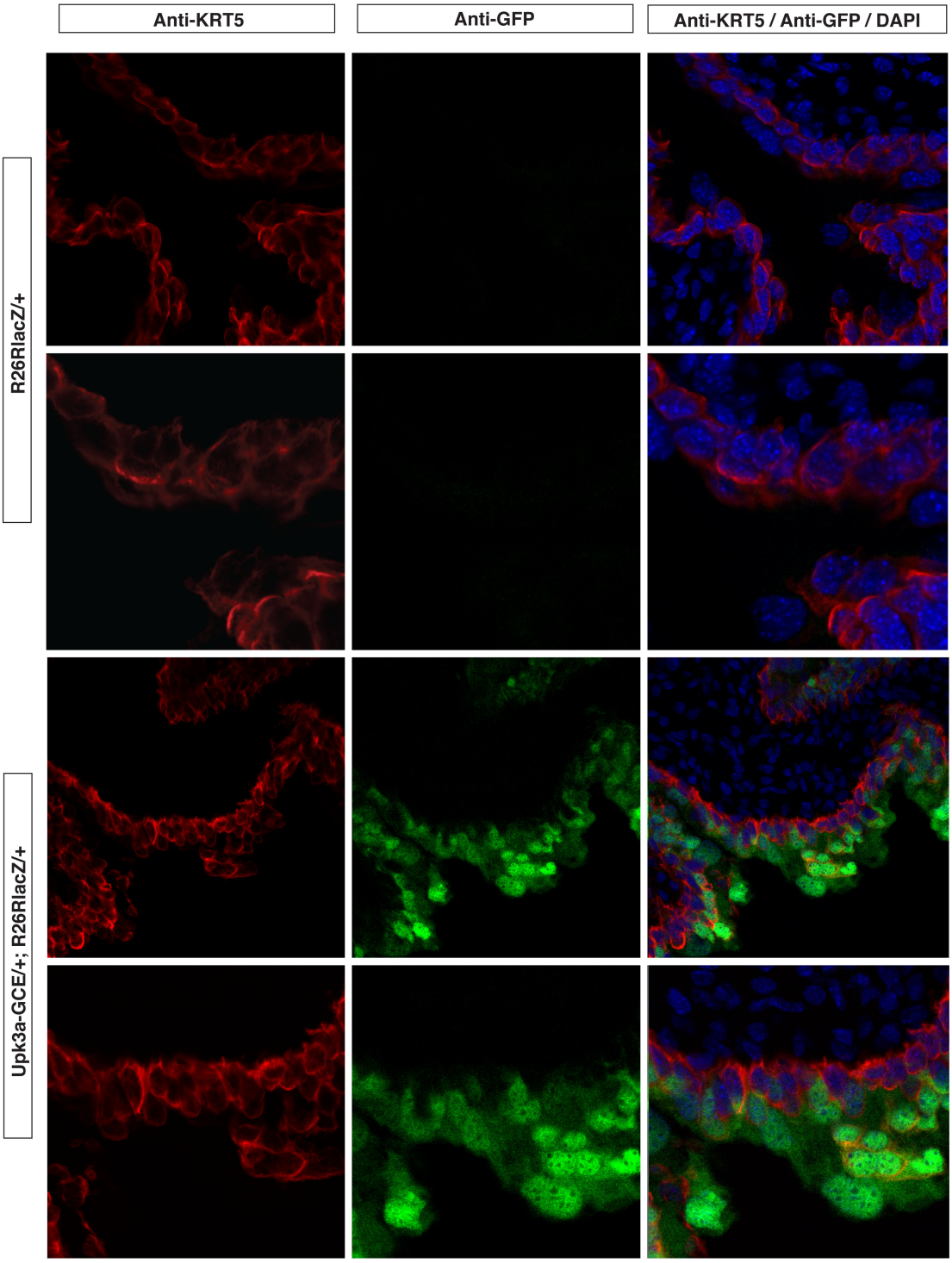

Fig 6. GFP is detected in cells lining the lumen of the bladder in close association with Krt5 positive cells in Upk3a$^{GCE/+}$; R26R$^{lacZ/+}$ P4 neonates. Krt5 positive cells closely oppose GFP positive cells with some apparent co-localization in a subset of the GFP cells in the bladder urothelium.