Inducible gene modification in the gastric epithelium of *Tff1-CreERT2;Tff2-rtTA;Tff3-Luc* mice

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Abbreviations: BAC, bacterial artificial chromosome ; β -Gal, β -Galactosidase ; DOX, doxycycline ; ER, estrogen hormone binding domain of the human estrogen receptor ; eGFP, enhanced green-fluorescent protein ; LSL, lox-stop-lox ; rtTA, reverse tetracycline transactivator ; TAM, tamoxifen ; Tff, Trefoil factor ; Tg, transgene ; TRE, tetracycline response element ; eYFP, enhanced yellow-fluorescent protein

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Abstract

Temporal and spatial regulation of genes mediated by tissue-specific promoters and conditional gene expression systems provide a powerful tool to study gene function in health, disease and during development. Although transgenic mice expressing the Cre recombinase in the gastric epithelium have been reported, there is a lack of models that allow inducible and reversible gene modification in the stomach. Here, we exploited the gastrointestinal epithelium-specific expression pattern of the three trefoil factor (*Tff*) genes and bacterial artificial chromosome transgenesis to generate a novel mouse strain that expresses the CreERT2 recombinase and the reverse tetracycline transactivator (rtTA). The Tg(*Tff1-CreERT2;Tff2-rtTA;Tff3-Luc*) strain confers tamoxifen-inducible irreversible somatic recombination and allows simultaneous doxycycline-dependent reversible gene activation in the gastric epithelium of developing and adult mice. This strain also confers luciferase activity to the intestinal epithelium to enable *in vivo* bioluminescence imaging. Using fluorescent reporters as conditional alleles, we show *Tff1-CreERT2* and *Tff2-rtTA* transgene activity in a partially overlapping subset of long-term regenerating gastric stem/progenitor cells. Therefore, the Tg(*Tff1-CreERT2;Tff2-rtTA;Tff3-Luc*) strain can confer intermittent transgene expression to gastric epithelial cells that have undergone previous gene modification, and may be suitable to genetically model therapeutic intervention during development, tumorigenesis and other genetically tractable diseases.

INTRODUCTION

The temporal and spatial regulation of genes in the murine stomach mediated by the combined use of gastric-specific promoters and conditional gene expression systems provide a unique opportunity to elucidate gene function in distinct stages of embryogenesis, adulthood and disease. In the adult stomach the rapidly renewing epithelium ensures a homeostatic equilibrium between the proliferative stem/progenitor cell compartment and apoptotic death of terminally differentiated cells (Karam and Leblond, 1993 ; Hoffmann *et al.*, 2008) to enable wound healing and prevent malignancies. While identification of molecular processes underpinning self-renewal and cell-fate decisions depend on the conditional modification of candidate genes in the stem cell compartment, identification of genes that contribute to gastric development necessitates a sensitive and inducible system for conditional gene alterations in the developing embryo. Experimental systems should therefore be endowed with independent regulation of DNA-recombinases as well as tetracycline-inducible gene expression to enable a versatile combination of irreversible gene modifications and reversible transgene expression.

Although several loci confer Cre-activity to the long-lived gastric stem and progenitor compartment, the *Lgr5* and *K19* loci also show considerable activity in other epithelia (Barker *et al.*, 2010, Brembeck *et al.*, 2001), while the stomach-specific *Atp4b* and *Troy* loci remain insufficient to reproducibly and reliably transduce transgene-mediated tumorigenesis (Syder *et al.*, 2004, Stange *et al.*, 2013). Therefore, we and others exploited the gastric epithelium-specific expression of the trefoil factor (*Tff*) genes *Tff1* and *Tff2* (Taupin and Podolsky, 2003; Karam *et al.*, 2004), and used bacterial artificial chromosome (BAC)-mediated transgenesis to confer genetic modification to stem/progenitor cells of the antrum and corpus region of the glandular stomach (Quante *et al.*, 2010; Thiem *et al.*, 2016).

Here, we describe a new transgenic mouse model, the Tg(Tff1-CreERT2, Tff2-rtTA, Tff3-Luc) strain, which enables tamoxifen-inducible, irreversible, Cre-mediated recombination and (simultaneous) doxycycline-mediated, reversible, induction of gene expression in the gastric epithelium. Therefore Tg(Tff1-CreERT2, Tff2-rtTA, Tff3-Luc) mice provide a genetic model to study developmental processes, tumorigenesis or other gastrointestinal diseases. Furthermore, Tff3-Luc-mediated reporter activity can trace the mucosa of the (small) intestine and might be a useful to monitor intestinal homeostasis and metaplasia.

RESULTS AND DISCUSSION

To confer expression of the tamoxifen-inducible CreERT2 recombinase and the doxycycline-inducible reverse tetracycline transactivator (rtTA) to the gastric epithelium without affecting endogenous gene expression, we took advantage of BAC transgenesis (Copeland *et al.*, 2001) and exploited the co-linear organization of the three murine *Tff* genes (Ribieras *et al.*, 2001) in a single transgenic vector. We inserted the *rtTA* open reading frame at the translational initiation site of the *Tff2* gene located on the *Tff1-CreERT2* transgenic BAC vector, in which the *CreERT2* recombinase cDNA replaced the *Tff1* coding sequence (Figure 1a; Thiem *et al.*, 2016). On the same BAC, we also replaced the *Tff3* coding sequence with a *firefly luciferase* (*Luc*) reporter function to enable *in vivo* bioluminescence of the intestinal mucosa (Fig. 1a-b). Following pronuclear injection of the resulting triple transgenic BAC, we derived four independent Tg(*Tff1-CreERT2*; *Tff2-rtTA*; *Tff3-Luc*) founder lines and confirmed retention of all three transgenes after germline transmission (Fig. 1c).

We first confirmed that the *Tff2-rtTA* transgene expression replicated expression of endogenous *Tff2* with a >10-fold higher expression in the corpus and antrum compared to the squamous fore-stomach and the proximal small intestine (Fig. 2a). We functionally validated the activity of the *rtTA* trans-activator in compound Tg(*Tff1-CreERT2*; *Tff2-rtTA*; *Tff3-Luc*) x *tetO-eGFP* mice, which showed widespread eGFP fluorescence signals throughout groups of adjacent epithelial cells above and below the gastric neck and isthmus regions (Fig. 2b-f). In the antrum and the corpus >40% of the glands contained GFP-positive cells (Fig. 2g), but entirely GFP-positive glands were only found in the antrum at a frequency of < 5%. Although entirely GFP-positive oxyntic glands in the corpus were absent, occasionally individual GFP-positive cells were found at the base of these glands (Fig. 2e,g). Using anti-eGFP immunohistochemistry, we found the strongest signal in deep antral glands (Fig. 2f), which harbour the long-lived stem cells in corresponding *Tff2-CreERT2* mice (Quante *et al.*, 2010) and which strongly express endogenous *Tff2* mRNA and *Tff2* protein (Jeffrey *et al.*, 1994). Consistent with previous *Tff2* expression surveys (Taupin and Podolsky, 2003), we also detected eGFP-positive cells in the Brunner's glands (Fig. 2e-f). We neither identified eGFP-positives cells in the small and large intestine (data not shown), nor in the lungs or kidneys (Fig. 2h), where others have localized *Tff2* mRNA (Hertel *et al.*, 2004; Quante *et al.*, 2010). We detected eGFP expression up to three days after removal of DOX-containing food pellets, but were unable to detect an eGFP signal one week

after DOX removal, or in un-induced mice or mice lacking the *Tg(Tff1-CreERT2;Tff2-rtTA;Tff3-Luc)* transgene (data not shown).

Next, we monitored *Tff1-CreERT2*-dependent β -Galactosidase (β -Gal) activity from the conditional *lox-Stop-lox-LacZ13* (*LacZ*) allele to confirm identical staining pattern between the *Tg(Tff1-CreERT2;Tff2-rtTA;Tff3-Luc)* strain and our previously reported *Tg(Tff1-CreERT2)* strain (Thiem *et al.*, 2016). Briefly, three days after administration of tamoxifen (1mg/20g mouse, twice daily for five consecutive days) we detected widespread β -Gal staining throughout the glandular epithelium, which encompassed entire glands in the antrum at a frequency of 35% (Fig. 3a-c). After 60 days we observed in both strains a frequent (>50%) appearance of entirely stained glands in the antrum (Fig. 3b-c, Thiem *et al.*, 2016), while less than 40% of the oxyntic glands in the corpus showed β -Gal staining (Fig. 3b-c). In line with our previous description of the *Tff1-CreERT2* transgene, we also detected β -Gal staining in Brunner's glands and in single airway epithelial cells (Fig. 3d and Thiem *et al.*, 2016), but not in the small and large intestine (data not shown).

We finally assessed the extent of *in vivo* bioluminescence conferred by the *Tff3-Luc* transgene, and found strong and consistent luciferase activity in the small intestine and a markedly weaker signal in the colon (Fig. 3e). Albeit at lower levels, this resembles endogenous *Tff3* expression (Fig. 3f), in particular the previously noted reduced expression of *Tff3* in the colon (Fernandez-Estivariz *et al.*, 2003). While we did not detect a significant luminescence signal in the stomach of *Tg(Tff1-CreERT2;Tff2-rtTA;Tff3-Luc)* mice (Fig. 3e), we anticipate that *Tff3-Luc* mediated luminescence might increase in situations of intestinal-type metaplastic transformation of the antral epithelium during tumorigenesis.

We then confirmed co-expression of the *Tff1-CreERT2* and *Tff2-rtTA* transgenes in *Tg(Tff1-CreERT2;Tff2-rtTA;Tff3-Luc);LSL-eYFP;TRE-eGFP* reporter mice, which we treated with tamoxifen (TAM) for five consecutive days followed by seven days of DOX-containing food (Fig. 4a). We observed an overlap between *CreERT2*-mediated eYFP-positive and *rtTA*-mediated induction of eGFP-positive epithelial cells located around the isthmus region of the corpus and antrum (Fig. 4b). We also detected double-positive cells in the putative stem cell compartment at the base of antral glands (Fig. 4b) where we previously had also reported *Tff1-CreERT2* activity (Thiem *et al.*, 2016). Consistent with this, we confirmed significant expression of putative gastric stem cell markers *Lgr5* (Barker *et al.*, 2010a), *Lrig1* (Noto *et al.*, 2013), *Troy* (Stange *et al.*, 2013) and *Mist1* (Qiao *et al.*,

2011) in FACS-sorted eYFP- and eGFP-positive cell populations, which we collected one day after three days of TAM or DOX-food administration respectively (Fig. 4c). As predicted from the more widespread expression of *Tff2* throughout the presumptive stem cell compartment at the base of the antral glands (Barker *et al.*, 2010a), stem cell marker expression was more abundant in the eGFP-positive population that arose from *Tff2-rtTA* activity. Finally, both populations revealed high expression of the reciprocal *Tff* gene, with more pronounced *Tff1* expression in the *Tff2-rtTA*-derived eGFP population than *Tff2* expression in the *Tff1-CreERT2*-derived eYFP population (Fig. 4d).

Notwithstanding the difference in kinetics and dynamics between the Cre-mediated lineage tracing and the rtTA-dependent reporter induction, these results may suggest that a larger proportion of *Tff2*-positive cells also express *Tff1* than the other way around.

Finally, we established whether the *Tff1-CreERT2* and *Tff2-rtTA* transgenes are suitable to modify gene expression in the stomach of developing mice. We analysed reporter activity in embryos from Tg(*Tff1-CreERT2*; *Tff2-rtTA*; *Tff3-Luc*); *TRE-eGFP*; *LSL-eYFP* mothers that had been treated with either TAM or were fed with DOX-food. Consistent with the confined gastric-specific expression pattern of *Tff1* and *Tff2* from embryonic day 19 (E19) onwards (Otto and Patel, 1999), we observed widespread eYFP and eGFP activity in the stomach of E19 embryos (Fig. 5a-b). Consistent with previous findings (Karam *et al.*, 2004), we observed eYFP and eGFP activity in most cells of the folded epithelial lining, predominantly towards the luminal edge of the perinatal stomach (Fig. 5c-d).

Here, we have characterized a novel tri-transgenic driver/reporter strain that enables for the first time sequential (irreversible and reversible) alterations of gene expression within the gastric epithelium of developing and adult mice, and also confers bioluminescent reporter activity to the intestinal epithelium. Accordingly, one area of application for the Tg(*Tff1-CreERT2*; *Tff2-rtTA*; *Tff3-Luc*) strain is cancer biology where the rtTA function can provide the means to genetically mimic the effect of therapeutic interference with an oncoprotein or the reversible suppression of tumor suppressor akin the recently published DOX-inducible short hairpin RNA against the *APC* intestinal cancer tumor suppressor gene (Dow *et al.*, 2015). Although the Tg(*Tff1-CreERT2*; *Tff2-rtTA*; *Tff3-Luc*) driver strain enables sequential somatic mutagenesis in the same subset of long-lived gastric epithelial cells, we surmise that it may be advantageous to use the rtTA-driver prior to the Cre-driver for two reasons. First, the more pronounced *Tff1* expression in *Tff2*-positive cells is poised to facilitate sequential

accumulation of Cre-mediated gene expression in rtTA-positive cells. Second, expression of the primary genetic lesion from the rtTA driver enables the establishment of models where the rtTA-driven oncogene becomes experimentally accessible to mimic the consequences of therapeutic interference with a “trunk” driver mutation (Swanton *et al.*, 2012). Meanwhile, the luciferase reporter activity not only directs bioluminescent reporter activity to the intestinal epithelium, but may also be helpful in monitoring intestinal, metaplastic transformation of the gastric epithelium as a process presumed to be required for inflammation-associated intestinal-type gastric cancer (Zullo *et al.*, 2012). Thus, our novel model system adds a unique experimental tool to the armament to study gene function in a tightly regulated fashion and at various time-points during development, adulthood and pathogenesis.

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METHODS

Mice and study approval

Heterozygous *LSL-LacZ13* (Thorey *et al.*, 1998), *LSL-eYFP^{Rosa26}* (Srinivas *et al.*, 2001) and *TRE-eGFP* (Tumbar *et al.*, 2004) mice were propagated on a mixed C57Bl6x129/Sv background. All mice were co-housed under specific pathogen-free conditions and age- and gender-matched heterozygous littermates were used for experiments. All animal studies were approved and conducted in accordance with the Animal Ethics Committees of the Ludwig Institute for Cancer Research/University of Melbourne Department of Surgery or the Olivia Newton-John Cancer Research Institute/School of Cancer Medicine, La Trobe University.

Generation of the *Tg(Tff1-CreERT2;Tff2-rtTA;Tff3-Luc)* strain

The generation of the *Tff1-CreERT2* BAC has been described previously (Thiem *et al.*, 2016). Briefly, to generate the *Tff1-CreERT2;Tff2-rtTA;Tff3-Luc* BAC transgene, we used a BAC of the clone *RP23-371G13* (~160kB, RPCI-23 BAC library, obtained from AGRF in Melbourne, Australia) which harbours the three *Tff* genes. We further used the open reading frames of the *CreERT2* recombinase (Metzger and Chambon, 2001) and the optimized reverse tetracycline/doxycycline-controlled transactivator *rtTA2s-M2* (*rtTA*) from plasmid *pUHRt62-1* (Urlinger *et al.*, 2000), as well as the coding sequence of the firefly luciferase (*Luc*) from plasmid *GL4.23* (Promega). We employed conventional cloning techniques to construct polyadenylation (pA)-signal-containing *CreERT2*-pA, *rtTA*-pA and *Luc*-pA expression cassettes that comprise a 5'-homology arm (200–300 bp) corresponding to the genomic sequence immediately upstream of the ATG start codon of the endogenous *Tff1*, *Tff2* and *Tff3* genes, respectively (Fig. 1a-b). Likewise, these cassettes also contained a 3'-homology arm corresponding to target regions further downstream in the respective genes (Fig. 1b). The latter homology arms were preceded by ampicillin or kanamycin resistance markers, which were flanked by one pair of either *frt*, *frt5* or *frt3* recombination sites as indicated (Fig. 1a-b). We employed a recombinase-mediated DNA approach in SW105 *E.coli* hosts (Parkitna *et al.*, 2009; Copeland *et al.*, 2001) to sequentially insert the resulting *Tff1-CreERT2*, *Tff2-rtTA* and *Tff3-Luc* expression cassettes into the *RP23-371G13* BAC, and subsequently excised the resistance marker through induction of the Flp-recombinase. We used PCR analysis to confirm all recombination events in the final *Tff1-CreERT2;Tff2-rtTA;Tff3-Luc* BAC, and we also determined the nucleotide sequence of the regions containing the ends of the homology

arms and the transgene encoding sequences. We injected the *NotI*-linearized BAC transgene into the male pronucleus of CBB6F1-derived one-cell embryos as described (Hammes and Schedl, 2000), and genotyped transgenic founder mice and their offspring by diagnostic PCR using genomic DNA extracted from tail biopsies.

Tamoxifen and doxycycline administration

Tamoxifen (TAM) (Sigma T5648) was dissolved in 100% ethanol (Sigma) and diluted 1:10 in sunflower oil (Sigma). Mice of approximately 20g body weight and between 6-8 week of age were injected twice daily intraperitoneally with 100µl of either the TAM-containing (corresponding to 1mg TAM per injection) or the vehicle solution. Treatment occurred during five consecutive days and TAM solutions were prepared freshly every three days. Doxycycline (DOX) was administered over periods of seven days in food pellets containing 200 mg DOX per kg food (Bio-Serv S3888).

Tissue collection

Resected stomachs were opened longitudinally, washed three times in PBS by vigorous shaking, and pinned-out on silicone-coated plates. Tissues were either snap-frozen or fixed as described below (Thiem *et al.*, 2016).

β-Galactosidase staining

Resected tissues were pre-fixed in 4% PFA (Sigma) for one hour at 4°C and the β-Galactosidase staining was performed as described previously (Thiem *et al.*, 2016).

Cryosectioning and fluorescence microscopy

Brightfield and fluorescence images of resected tissues were taken using a fluorescence dissection microscope. The tissues were then fixed in 4% PFA (Sigma) for one hour at 4°C, incubated in 50% sucrose/PBS overnight and cut into several strips that were embedded in a cryomold using OCT (Sakura) and were subsequently frozen on dry ice using isopentane (Sigma). Cryosections (3 µm) were counterstained with DAPI (Invitrogen) and analysed by confocal fluorescence microscopy.

Luciferin administration and IVIS imaging

Mice were analysed by live imaging using the Vivo Vision Lumina *In Vivo* Imaging System (IVIS, Xenogen) according to the manufacturer's instructions. To increase the penetrance of the luminescent signal, the belly hair was removed. Mice were then injected with luciferin (200 µl i.p.; 20 mg/ml;

Caliper Life Sciences) and were imaged 10 min later using standard settings with an exposure time of 2 min. Some mice were sacrificed to excise the gastrointestinal tract and other tissues for imaging.

Immunohistochemical analysis

Resected tissues were fixed in 10% neutral buffered formalin solution (pH 7.4) overnight, embedded in paraffin and sectioned (4 μ m). Immunohistochemical analysis was performed as described previously (Thiem *et al.*, 2013) using a primary antibody against GFP (Abcam ab290).

Isolation of YFP- and GFP-positive (YFP⁺ / GFP⁺) cells

Stomach dissection, dissociation and digestion were performed as previously described (Thiem *et al.*, 2016). Stomach cells from three Tg(*Tff1-CreERT2*; *Tff2-rtTA*; *Tff3-Luc*); *LSL-eYFP*; *TRE-eGFP* mice treated with Tamoxifen were pooled for sorting eYFP⁺ epithelium and cells of five stomachs from DOX-fed mice were pooled to isolate eGFP⁺ epithelium. Prior to sorting, the cells were stained with propidium iodide (live cell stain) and an antibody against Epcam (Biolegend, PE anti-mouse CD326, clone G8.8), and were sorted for alive (PI-negative), epithelial (Epcam -positive) and YFP⁺ or GFP⁺ populations using a BD FACS Aria III.

RNA isolation, quantitative RT-PCR and oligonucleotides

For gene expression analysis of tissues total RNA was extracted from frozen tissues using the RNeasy Plus Mini Kit (Qiagen) and cDNA was prepared from 2 μ g RNA using the High Fidelity cDNA synthesis kit (Applied Biosystems/Roche) according to the manufacturer's instructions. Quantitative RT-PCR analyses were performed on triplicate samples with SYBR green (Quantace) using the Rotor Gene 3000 (Corbett Research/ Qiagen) over 40 cycles (94°C/20s, 60°C/15s, 72°C/15s), following an initial denaturation step at 95°C/10min. Primers to specifically amplify *18S rRNA* were used to normalize cDNA concentrations of target genes. Primer pairs are listed in the table below and were tested for specificity and efficiency (Table 1).

For gene expression analyses of sorted epithelial cells total RNA was extracted from sorted cells using the RNeasy Plus Micro Kit (Qiagen) and cDNA was prepared using the SuperScript III First-Strand Kit (Invitrogen) according to the manufacturer's instructions. Quantitative RT-PCR analyses were performed with SYBR green (Quantace) using the ViiA7 (Thermo Fischer Scientific) over 40 cycles (94°C/20s, 60°C/15s, 72°C/15s), following an initial denaturation step at 95°C/10min. Primers to specifically amplify *Gapdh mRNA* were used to normalize cDNA concentrations of target genes.

Gene expression is presented relative to Gapdh expression and was obtained using the 2-dCT method.

Statistics

Unless otherwise stated, comparisons between mean values were performed by ANOVA or a two-tailed Student's *t*-test as appropriate using Prism5 software (GraphPad). A *P*-value (*P*) of less than 0.05 was considered statistically significant.

Declaration

The Tg(*Tff1-CreERT2*;*Tff2-rtTA*;*Tff3-Luc*) strain will be made available to the research community subject to completion of relevant Material Transfer Agreements.

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Figure Legends

FIG. 1. Generation of Tg(*Tff1-CreERT2*; *Tff2-rtTA*; *Tff3-Luc*) mice.

- (a-b)** Schematic illustration of the construction of the *Tff1-CreERT2*; *Tff2-rtTA*; *Tff3-Luc* BAC transgene using DNA recombineering technology to enable recombinase-mediated exchange between homologous regions (*red lines*) on cDNAs and endogenous loci on a BAC (**a**). The indicated *CreERT2*, *rtTA* and *Luciferase (Luc)* expression cassettes which included a polyadenylation (*polyA*) signal and an ampicillin (*Amp*) or kanamycin (*Kan*) resistance marker (*open box*) flanked by sets of incompatible *frt*, *frt3* and *frt5* recombination sites (*grey triangles*), were sequentially introduced into the *Tff1*, *Tff2* or *Tff3* locus of a BAC that carried the four indicated genes (*black boxes*). The insertion of each transgene resulted in a partial deletion of the corresponding gene on the BAC. Size (bp) of deleted fragments are indicated and homology arms flanking the transgenes and BAC DNA sequences are highlighted in red (**b**). Following recombination in *E.coli* and removal of the resistance markers, the *Tff1-CreERT2*; *Tff2-rtTA*; *Tff3-Luc* BAC was liberated by *NotI* restriction digest from its vector backbone harbouring the chloramphenicol (*Cam*, *grey box*) resistance marker.
- (c)** The *NotI*-liberated, linearized *Tff1-CreERT2*; *Tff2-rtTA*; *Tff3-Luc* BAC fragment was injected into the male pronucleus of fertilized CBB6F1 mouse oocytes. Zygotes were transferred to the oviducts of pseudo-pregnant females and the pups were analysed by diagnostic PCR for the integration of the transgenic BAC into the genome. DNA from tail biopsies of four independently derived founder mice (#1-4) was analysed by PCR using transgene-specific primers. Purified BAC DNA was used as a positive control (PC) and DNA from wild-type mice (WT) or water (NC) as a negative control. M, DNA standard/marker.

FIG. 2. *Tff2-rtTA* enables reversible gene activation in the glandular stomach.

- (a) qRT-PCR expression analysis for endogenous *Tff2* and transgenic *Tff2-rtTA* in three distinct regions of the stomach (forestomach, corpus, antrum) and the proximal small intestine (prox. SI) of Tg(*Tff1-CreERT2;Tff2-rtTA,Tff3-Luc*) mice ($n \geq 6$). Following normalization to 18S rRNA expression, results are shown as mean values $\pm$ SEM (compared to the forestomach samples: * $P < 0.05$, ns = not significant; # $P < 0.02$).
- (b) Schematic illustration of 7-day doxycycline (DOX) treatment period prior to collection of the stomachs for detection of fluorescence. Tg(*Tff1-CreERT2;Tff2-rtTA,Tff3-Luc*);TRE-eGFP reporter mice between 6-8 weeks of age were fed with DOX-containing (200 mg/kg) food pellets for seven days to induce DOX-dependent binding of rtTA to the *tetracycline response element* (TRE) and its subsequent activation which leads to expression of the enhanced green-fluorescent protein (eGFP) reporter.
- (c-d) Representative whole-mount stomach (overlay of brightfield and fluorescent images) from Tg(*Tff1-CreERT2;Tff2-rtTA,Tff3-Luc*);TRE-eGFP mice demonstrating the presence of green-fluorescent eGFP after DOX administration (c). Boxed regions are shown at higher magnification (d). Scale bar: 2 mm.
- (e) Corresponding cryosections were counterstained with DAPI (blue) and show green-fluorescent eGFP in epithelial cells of the corpus, antrum and Brunner's glands. Boxed regions are shown at higher magnification. Scale bars: 100 μ m.
- (f) Representative anti-GFP immunostainings on stomach paraffin-sections of Tg(*Tff1-CreERT2;Tff2-rtTA,Tff3-Luc*);TRE-eGFP mice which were fed with DOX food for seven days. Scale bar: 50 μ m.
- (g) Frequency of GFP positive glands was determined from well oriented cryosections from e. Two entire sections per mouse were counted for glands being either fully, partial or absent of GFP signal. Data is shown as mean values $\pm$ SEM, $n = 4$ mice.
- (h) Representative cryosections of kidney and lung counterstained with DAPI (blue) showing no specific green-fluorescent eGFP signal in epithelial cells. Boxed regions are shown at higher magnification. Scale bars: 100 μ m.

FIG. 3. CreERT2 and Luc activity in Tg(*Tff1-CreERT2*; *Tff2-rtTA*; *Tff3-Luc*) mice.

- (a) Schematic illustration of the 5-day tamoxifen (TAM) treatment protocol and follow-up period prior to collection of the stomachs for β -galactosidase staining. Mice were injected intraperitoneally with tamoxifen (1mg/20g body weight) twice daily for five consecutive days.
- (b) Representative whole-mount stomachs from mice of the indicated genotypes were stained for β -galactosidase activity after TAM administration and the indicated follow-up period. Corresponding paraffin-sections were counterstained with nuclear fast red. Boxed regions are shown at higher magnification. Scale bars: 100 μ m (*insets* 25 μ m).
- (c) Recombination frequency and LacZ lineage tracing was quantified by scoring glands as either absent, partial or fully traced by LacZ activity in well oriented section of **b**. At least two sections per mouse were analysed. Data is shown as mean values $\pm$ SEM, n= 4 mice per time point.
- (d) β -galactosidase activity was detected in single airway epithelial cells (*arrows*) of the lungs and in Brunner's glands of Tg(*Tff1-CreERT2*; *Tff2-rtTA*; *Tff3-Luc*); *LacZ* mice, collected three days after TAM administration. Boxed region are shown at higher magnification. Scale bars: 500 μ m.
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FIG. 4. *Tff1-CreERT2* and *Tff2-rtTA* reporter activity overlap in a subset of gastric epithelial cells.

- (a)** Schematic illustration of the tamoxifen (TAM) and doxycycline (DOX) treatment protocol. Mice (~20 g body weight, 6-8 weeks of age) of the indicated genotype were injected with TAM for five consecutive days to induce CreERT2-mediated activation of the yellow-fluorescent eYFP reporter. Mice were then fed with DOX-containing food pellets for one week resulting in rtTA-mediated activation of the green-fluorescent eGFP reporter.
- (b)** Representative cryosections from the antrum (*top rows*) and corpus (*bottom row*) of Tg(*Tff1-CreERT2;Tff2-rtTA;Tff3-Luc*);LSL-eYFP;TRE-eGFP mice. The merge demonstrates that the eYFP (*yellow*) and eGFP (*green*) reporters are both present in a subset of epithelial cells (*red*). The border of the gastric mucosa is outlined by white dotted lines (Lu, stomach lumen) and the arrows indicate reporter activity in basal cells of the antrum. Scale bars: 50 μ m.
- (c-d)** FACS-sorted *Tff1-CreERT2*-derived eYFP⁺ and *Tff2-rtTA*-derived eGFP⁺ epithelial cells were analysed for mRNA expression of the gastric stem cell markers *Lrg5*, *Lrig1*, *Troy* and *Mist1* (**c**) and the reciprocal Tff genes via qPCR (**d**). For three days either TAM (for eYFP⁺ sort) or DOX-food (for eGFP⁺ sort) was administered to Tg(*Tff1-CreERT2;Tff2-rtTA;Tff3-Luc*);LSL-eYFP;TRE-eGFP mice. Stomach cells from three TAM- and from five DOX-administered mice were pooled for a single sort for *Tff1-CreERT2*-derived eYFP⁺ epithelium and *Tff2-rtTA*-derived eGFP⁺ epithelium.

FIG. 5. Activation of *Tff1-CreERT2* and *Tff2-rtTA* during embryonic development.

Pregnant females were treated with 1 mg tamoxifen (oral gavage) on embryonic day (E) 16 and E17 or were fed with doxycycline-containing food pellets from E16 onwards.

(a,c) Brightfield and fluorescent (eYFP / eGFP: 100 ms / 50 ms exposure time) images of whole-mount stomachs resected from E19 embryos of the indicated genotypes.

(b,d) Corresponding cryosections were counterstained with DAPI (*blue*) and demonstrate the presence of eYFP (**b**) or eGFP (**d**) in gastric epithelial cells (Lu, stomach lumen). Scale bar: 50 μm .

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113x69mm (300 x 300 DPI)

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FIG. 4. *Tff1*-CreERT2 and *Tff2*-rtTA reporter activity overlap in a subset of gastric epithelial cells. (a) Schematic illustration of the tamoxifen (TAM) and doxycycline (DOX) treatment protocol. Mice (~20 g body weight, 6-8 weeks of age) of the indicated genotype were injected with TAM for five consecutive days to induce CreERT2-mediated activation of the yellow-fluorescent eYFP reporter. Mice were then fed with DOX-containing food pellets for one week resulting in rtTA-mediated activation of the green-fluorescent eGFP reporter. (b) Representative cryosections from the antrum (top rows) and corpus (bottom row) of *Tg(Tff1-CreERT2;Tff2-rtTA;Tff3-Luc);LSL-eYFP;TRE-eGFP* mice. The merge demonstrates that the eYFP (yellow) and eGFP (green) reporters are both present in a subset of epithelial cells (red). The border of the gastric mucosa is outlined by white dotted lines (Lu, stomach lumen) and the arrows indicate reporter activity in basal cells of the antrum. Scale bars: 50 μ m. (c-d) FACS-sorted *Tff1*-CreERT2-derived eYFP⁺ and *Tff2*-rtTA-derived eGFP⁺ epithelial cells were analysed for mRNA expression of the gastric stem cell markers *Lgr5*, *Lrig1*, *Troy* and *Mist1* (c) and the reciprocal Tff

genes via qPCR (**d**). For three days either TAM (for eYFP+ sort) or DOX-food (for eGFP+ sort) was administered to Tg(*Tff1-CreERT2*; *Tff2-rtTA*; *Tff3-Luc*); *LSL-eYFP*; *TRE-eGFP* mice. Stomach cells from three TAM- and from five DOX-administered mice were pooled for a single sort for *Tff1-CreERT2*-derived eYFP+ epithelium and *Tff2-rtTA*-derived eGFP+ epithelium.

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152x131mm (300 x 300 DPI)

A

Table 1. Oligonucleotides for PCR analysis.

Target (expected PCR size)	forward primer (5' > 3')	reverse primer (5' > 3')
Amplification of genomic DNA (diagnostic PCR)		
5'-end of <i>Tff1-CreERT2</i> (648 bp)	CTGTCTGAGCAGGCAGTGTAAAG	GGGACACAGCATTGGAGTCAGA
3'-end of <i>Tff1-CreERT2</i> (825 bp)	GTGGAGGAGACGGACCAAAGC	GATGAAGGCTAGGGAGATAACAC
5'-end of <i>Tff2-rtTA</i> (530 bp)	GCACAAACCTTACCCAGAGTAAC	GGTATGATGCCTGTCCAGCATC
3'-end of <i>Tff2-rtTA</i> (676 bp)	CTCCCCGGGTAACTAAGTAAGG	CCTTCTGTAAAGAGGGTGACTCT
5'-end of <i>Tff3-Luc</i> (592 bp)	GTTCTCGGTGAGGTGGTTGAGA	GATCCGATGGTTTGATTTCAGCC
3'-end of <i>Tff3-Luc</i> (644 bp)	GCCGCTTCGAGCAGACATGATA	GCAAGCCCCAGGGATCTGCC
Amplification for quantitative RT-PCR analysis		
<i>18S rRNA</i>	GTAACCCGTTGAACCCATT	CCATCCAATCGGTAGTAGCG
<i>Gapdh</i>	CACTGAGCATCTCCCTCACA	GTGGGTGCAGCGAACTTTAT
<i>Tff1</i>	AGCACAAGGTGATCTGTGTCC	GGAAGCCACAATTTATCCTCTCC
<i>Tff2</i>	TGCTCTGGTAGAGGGCGAG	CGACGCTAGAGTCAAAGCAG
<i>Tff2-rtTA</i>	GCAGGGAGCCGAACCTGCCT	TCAAGAGCGTCAGCAGGCAGC
<i>Tff3</i>	TGGTCCTGGTTGCTGGGTCCT	AGTCAAAGCAGCAGCCACGGT
<i>Tff3-Luc</i>	GCAGTCGTCGTGCTGGAACAC	GAATCTCGCGGATCTTGCGGG
<i>Lrg5</i>	CCTTGCCCTGAACAAAATA	ATTTCTTTCCAGGGAGTGG
<i>Lrig1</i>	ACTCAAGAGTCTGCGGGTCT	TCCTCGATTGTACCCGAGAT
<i>Troy</i>	CCAAGGAATGTGGCTTCG G	TGGTGTGTGAGCAGTTGG
<i>Mist1</i>	TGGTGGCTAAAGCTACGTGT	CATAGCTCCAGGCTGGTTTT

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Title:

Inducible gene modification in the gastric epithelium of Tff1-CreERT2, Tff2-rtTA, Tff3-luc mice

Date:

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