METHODS ONLINE

Animals. All experiments were conducted according to the UK Home Office Project Licenses 70/7543, P14FED054 or PF4639B40. Male and female adult mice were used for *in vivo* experiments. Animals were housed in individually ventilated cages and fed on standard chow. Double mutant *Ahcre^{ERT}Rosa26$^{flEYFP/wt}$, R26^{M2rtTA}/TetO–HGFP, Ahcre^{ERT}Rosa26$^{flConfetti/wt}$ and AhCre^{ERT}Rosa26$^{flDNM–GFP/wt}$* animals on a C57BL/6N background were generated as described previously [1-4]. Triple mutant *AhCre^{ERT}R26$^{flDNM–GFP/Confetti}$* mice were generated by crossing *Ahcre^{ERT}, R26$^{flConfetti/wt}$* and *R26$^{flDNM–GFP/wt}$* mice. C57BL/6N wild type mice were also used as indicated.

Chemically induced mutagenesis. To generate mutations in the esophageal epithelium, mice were treated with Diethylnitrosamine (DEN, Sigma Cat# N0756) in sweetened drinking water (40 mg per 1,000 ml) for 24 hours 3 days a week (Monday, Wednesday and Fridays) for 8 weeks [2]. After each dosage mice received sweetened water until the next DEN treatment. Control mice received sweetened water as vehicle for the length of the treatment. After the 8 weeks, all mice were administered normal water until the collection date.

Whole mount sample preparation. Mouse esophagus was dissected, cut longitudinally and the muscle layer removed by gently pulling with forceps. The entire tissue was then incubated for 2–3 h in 5 mM EDTA at 37°C before separating the epithelium from the underlying submucosa with fine forceps. The whole epithelium was then flattened and fixed in 4% paraformaldehyde for 30 min at room temperature. Tissues were then washed in PBS and stored at 4°C.

Tissue immunostaining. For tissue immunostaining, wholemounts were blocked for 1 hour in 800µl of staining buffer (0.5% bovine serum albumin, 0.25% fish skin gelatin, 0.5% Triton X-100 in PBS and 10% donkey serum). Where needed samples were incubated with primary antibodies (anti GFP/YFP, Thermo Fisher Scientific Cat# A10262; anti Active Caspase 3, Abcam Cat#Ab2302; Alexa Fluor® 647 anti-mouse CD45 Antibody, Biolegend Cat# 103124; anti Cytokeratin 14, Covance Cat# PRB-155P) in staining buffer overnight at room temperature, followed by 4 washes of 20min with 0.2% Tween-20 in PBS. Samples were then incubated with secondary antibodies (Alexa Fluor 488 Donkey Anti-Chicken, Jackson ImmunoResearch Cat# 703-545-155; Alexa Fluor 555 Donkey Anti-Rabitt, Thermo Fisher Scientific Cat# A-31572) in staining buffer for 3h at room temperature and washed as above. Finally, tissues were incubated overnight at room temperature with 1 µg/ml DAPI or 0.4µM TO-

PRO™-3 Iodide solution (Thermo Fisher Scientific, Cat# T3605) to stain cell nuclei and mounted using VECTASHIELD Mounting Media.

Confocal microscopy. Images were acquired on a Leica TCS SP8 (Leica Microsystems) confocal microscope using ×10, ×20 or ×40 objectives. Typical settings for acquisition of z stacks were optimal pinhole, line average 3–4, scan speed 400-600 Hz and a resolution of 512 x 512 or 1,024 × 1,024 pixels. Visualisation and image analysis were performed using IMARIS (bitplane), ImageJ or Volocity 3D Image Analysis Software (Perkin Elmer).

Histology. The esophagus from control and DEN-treated mice (12 months post-DEN) were dissected, fixed in 10% formalin for at least 24h and stored at 4°C. Tissues were then embedded in paraffin and cut at 5 µm thickness. Sections were stained with hematoxylin and eosin and scanned.

Basal cell density. The basal cell density of the esophageal epithelium was measured at different time points in control and DEN-treated mice. Whole-mounted tissues were analysed by confocal imaging and the number of $DAPI^{+}$ basal cells per field of view was quantified from 7-10 random images per animal (2-3 animals per condition and time point).

Number of surrounding basal cells. Confocal images of mouse esophageal epithelium stained with Dapi and Cytokeratin 14 were used to measure the number of neighbouring cells per basal layer cell. For this, 100 basal cells per mouse were randomly selected from 10 different images, and the number of neighboring cells manually counted. A total of 400 cells from 4 mice were measured.

***In vivo* clonal lineage tracing.** To genetically label clones we crossed the appropriate floxed reported mouse lines (*Rosa26*$^{flEYFP/wt}$, *Rosa26*$^{flConfetti/wt}$, *Rosa26*$^{flDNM-GFP/wt}$ *or* *Rosa26*$^{flDNM-GFP/Confetti}$) with conditionally inducible *AhCre*^{ERT} mice. In these strains, the relevant fluorescent reporters can be genetically induced following treatment with ß-napthoflavone (BNF, MP Biomedicals Cat# 156738) and tamoxifen (TAM, Sigma Aldrich Cat# N3633). Specifically, transcription of the *Cre* mutant estrogen receptor fusion protein (CreERT) is induced following intraperitoneal (i.p) BNF injection. A subsequent i.p injection of TAM is necessary in order for the CreERT protein to gain access to the nucleus and excise the loxP flanked “STOP” cassette resulting in the expression of the relevant reporter. As the switch occurs at the gene level, the descendants of the originally labelled cell (clones) will also constitutively express the reporter and can be visualised by fluorescent microscopy. The dose of BNF and TAM can be titrated to label only a small percentage of cells (clonal labelling) to

avoid fusion events when the clones expand over time (see details for each strain below). 10-16 week old mice were used for the lineage tracing experiments.

<u>*YFP clones*</u>. *Ahcre^{ERT}R26$^{flEYFP/wt}$* (*YFP-Cre*) mice were used for clonal labelling of the EE with *YFP* fluorescent protein (**Fig. 3a**). YFP expression was clonally induced by a single injection of 80 mg kg BNF and 1 mg TAM to mice control or previously treated with DEN for 2 months. Esophagus from induced mice were collected at different time points (10 days, 1, 3, 6 and 12 months) post induction, peeled, fixed and stained with DAPI as described above (*Whole mount sample preparation* and *Tissue immunostaining*). Whole EEs were imaged by confocal microscopy and the number of clones as well as the projected YFP clone areas were measured from these images as described below (YFP *clones number and projected areas*).

<u>*Confetti clones*</u>. *Ahcre^{ERT}R26$^{flConfetti/wt}$* mice were used to clonally label cells with one of four different fluorescent proteins (YFP, GFP, RFP or CFP) (**Fig. 4a**). Animals were treated with DEN in drinking water for 2 months followed by a single i.p injection of BNF (80 mg kg) and TAM (1mg) to clonally induce cell labelling. 9 or 18 months later mice were culled and the esophagus dissected. Whole mount EEs were processed as described above (*Whole mount sample preparation*). Fluorescent clones were imaged and their areas measured using Volocity 3D Image Analysis Software (Perkin Elmer). Selected individual confetti clones were then extracted and processed for DNA whole exome sequencing as described below (*Confetti clone cutting and sequencing*).

<u>*DN-Maml1 clones*</u>. *Ahcre^{ERT}R26$^{flDNM-GFP/wt}$* mice were used for clonal induction of the dominant negative mutant of *Maml1* (*DN-Maml1*) **(Extended Data 8a)**. This mutant inhibits Notch intracellular domain induced transcription, therefore disrupting the Notch signalling pathway [4]. It is also fused to GFP, which allows for clonal labelling of the mutant. Clonal induction of *DN-Maml1* was achieved by a single injection of BNF (0.08 mg/Kg) and TAM (0.25mg) to control or DEN-treated mice. Esophagus were collected at different time points (10 days, 1, 3, 6 and 12 months) after induction. Tissues were processed, stained with anti-GFP antibody and imaged on a confocal microscope as described above (*Whole mount sample preparation, tissue immunostaining* and *Confocal microscopy*). The coverage (% of the total EE occupied by mutant clones) of *DN-Maml1* clones was measured using Volocity 3D Image Analysis Software (Perkin Elmer).

<u>*Confetti-MAML clones*</u>. *Ahcre^{ERT}R26$^{flDNM-GFP/Confetti}$* mice **(Extended Data 8d)** were generated to analyse the relative growth of Confetti clones located either at the edges of or enclosed within *DN-*

Maml1 mutant areas. For this purpose we took advantage of the higher recombination efficiency of *DN-Maml1* as compared to the Confetti reporter. *Ahcre^{ERT}R26$^{flDNM-GFP/Confetti}$* mice were induced with a single injection of 80 mg/kg BNF and 1 mg TAM, and esophagus collected 1 month later. This dose, higher than the one used for the clonal labelling of *Ahcre^{ERT}R26$^{flDNM-GFP/wt}$* mice, generates a large amount of *DN-Maml1* mutant clones, with only a small percentage of them also expressing the Confetti reporter. The possible outcomes following this high induction are as follows: either single induction of *DN-Maml1*, single induction of GFP, YFP, RFP or CFP or double induction of *DN-Maml1* with one of the 4 Confetti fluorescent proteins (*Confetti-DN-Maml1*). Whole tissues were processed and imaged as above (*Whole mount sample preparation* and *Confocal microscopy*). The area of Confetti clones enclosed or at the edges of *DN-Maml1* clones was measured using Volocity 3D Image Analysis Software (Perkin Elmer). Only red and yellow Confetti clones were quantified.

Whole tissue YFP clones number and projected areas. To measure the number and size of the YFP clones from the entire mouse esophageal epithelium we developed the following pipeline. Whole mouse esophageal epithelia were prepared as described above (*Whole mount sample preparation*). A high precision motorised stage coupled to a Leica TCS SP8 confocal microscope was used to obtain contiguous 3D images of all epithelial layers (basal + suprabasal) from the entire mouse esophagus, that were later merged using the mosaic function of the Leica Software. Typical settings for acquisition of multiple z stacks were 1µm z-step size, zoom x1, optimal pinhole, line average 4, scan speed 400 Hz and a resolution of 1,024 × 1,024 pixels using a 10X HC PL Apo CS Dry objective with a 0.4NA. The Leica LIF files containing the merged images were then processed using Volocity 3D Image Analysis Software. To identify individual clones and measure their projected surface area images were opened using the "extended focus" visualization mode on the Volocity 3D software. Clones were then identified with the "find objects" function using a lower and upper intensity threshold of 25 and 255, respectively, with a minimum object size of 50µm^2 and a restrictive radius of 10µm.

In vivo transgenic label-retaining cell assay. *Rosa26^{M2rtTA}/TetO-HGFP* mice were used to measure the rate of cell division in the EE following DEN treatment. These mice are double transgenic for a reverse tetracycline-controlled transactivator (rtTA-M2) targeted to the Rosa 26 locus and a *HIST1H2BJ/EGFP* fusion protein (Histone-Green Fluorescent Protein, HGFP) expressed from a tetracycline promoter element. Treatment of these mice with doxycycline (Doxy, Sigma Aldrich Cat# D9891) induces the transient expression of HGFP, resulting in nuclear fluorescent labelling throughout the entire epithelium. When Doxy is withdrawn, HGFP is no longer expressed and is

diluted lineally by half after every cell division cycle. Therefore, the decline in fluorescence intensity can be measured to calculate the cell division rate. *Rosa26^{M2rtTA}/TetO-HGFP* mice received DEN or sweetened water for 2 months as described above. 2 months after finishing the treatments all mice were administered Doxy (2mg/ml) in sweetened water for 4 weeks. Mice were culled and tissues collected either immediately (t = 0) or 7 days (t =7) after Doxy withdrawal (time post-DEN = 3 months). Esophagus were peeled, fixed and stained as detailed above and imaged on a confocal microscope using a 40x objective. Tissues were stained with CD45 antibody to label immune cells, which were excluded from the quantifications. The intensity of HGFP in individual basal cells was analysed using ImageJ. The average proliferation rate in control and DEN tissues was calculated using the ratios between the HGFP intensity of cells at times 0 and 7 days. Between 2599 and 4766 basal cells were analysed per condition and time point from 2-3 animals and 8 images per tissue.

EdU lineage tracing assay. EdU (5-ethynyl-2'-deoxyuridine) incorporates into dividing cells, present only at the EE basal layer (**Fig. 1a**). EdU labelled cells can then stay in the basal layer or stratify upwards into the suprabasal layer. The number of EdU positive cells can therefore be used to quantify proliferation and differentiation rates in the esophageal epithelium of DEN-treated mice. Wild type animals received DEN for 2 months as described above. 6 months after DEN treatment mice were administered 10µg of EdU (i.p.) and the esophagus were collected 48h later. Tissues were peeled, fixed and EdU detected in wholemounts using a Click-iT EdU imaging kit (Life technologies Cat# C10086) according to the manufacturer's instructions and imaged by confocal microscopy. The number of epithelial cells positively stained for EdU was quantified in the basal and suprabasal layers using Volocity 3D software. A total of 1873 and 2080 EdU positive cells (5 images per animal, 6 animals per group) were counted from control and DEN-treated mice, respectively. Proliferation was measured as the total number of EdU positive cells present in both basal and suprabasal layers, whereas the differentiation rate was calculated by dividing the number of EdU positive suprabasal cells by the total (basal + suprabasal) EdU positive cells.

Detection of apoptosis by activated caspase-3 staining. Mice were treated with DEN for 2 months and tissues collected 10 days after DEN withdrawal. Whole mounted esophageal epithelia were stained for activated caspase-3 and imaged by confocal microscopy. The number of caspase-3 positive cells in the basal layer was quantified with ImageJ. A total of 5355 cells were analysed from 11 images per mouse across the whole esophageal epithelium (n=2 mice).

Targeted sequencing of mouse esophageal epithelium grid samples.

Sample preparation. Mice esophagus were dissected and cut longitudinally before removing the muscle layer. The entire tissue was then incubated for 2–3 h in 5 mM EDTA at 37 °C before separating the epithelium from the underlying submucosa with fine forceps. The whole epithelium was then flattened, fixed in 4% paraformaldehyde for 30 min at room temperature and kept in PBS at 4 °C. For sequencing, the esophageal epithelium was mapped and cut in $2mm^2$ contiguous biopsies (**Fig. 1c**). Samples were digested and DNA extracted using the QIAMP DNA microkit (QIAGEN Cat# 56304) following manufacturer's instructions. DNA from the ears of the same mice was extracted with the same method and used as germline controls.

DNA sequencing and coverage metrics. We used an Agilent SureSelect custom bait capture comprising 192 genes designed to include frequently mutated genes in cancer (**Extended Data 1e**). Samples were multiplexed and sequenced on an Illumina HiSeq 2500 sequencer using paired-end 75-bp reads. Paired-end reads were aligned with BWA-MEM (v0.7.17, https://github.com/lh3/bwa) [5]with optical and PCR duplicates marked using Biobambam2 (v2.0.86, https://gitlab.com/german.tischler/biobambam2, https://www.sanger.ac.uk/science/tools/biobambam). The median coverage across all samples and genes after removing off-target reads, PCR duplicates and reads with mapping quality <25 and base quality <30 was 485.5x, ranging from 445-519x between individuals (**Extended Data 1f**).

Single clone isolation and whole exome sequencing.

Sample preparation and imaging. *AhcreERTR26*$^{flConfetti/wt}$ and *Ahcre*^{ERT}*R26*$^{flEYFP/wt}$ (*YFP-Cre*) mice were treated with DEN in drinking water 3 times a week for 8 weeks as described above. After DEN removal mice were induced by an intraperitoneal (i.p.) injection of 80 mg kg−1β-naphthoflavone and 1 mg tamoxifen. 9 or 18 months after induction animals were culled and tissues harvested. Esophagus were incubated for 2–3 h in 5 mM EDTA at 37 °C before removing the submucosa from the epithelium as described above. Confetti or YFP labelled clones were imaged on a fluorescent scope equipped with the appropriate filters. The projected area of the clones was measured using Volocity 3D Image Analysis Software.

Single clone isolation and sequencing. Clones were manually cut under a fluorescent micro-dissecting scope (Leica Microsystems) using ultra fine forceps and micro-scalpels. Individual clones were collected in low binding DNA tubes and digested in 3 µl RLT buffer (Qiagen Cat# 1048449) for

30min at room temperature. Digested samples were diluted 1:10 in water, separated in triplicates, transferred to 96-well plates and incubated 15 min at room temperature with Agencourt AMPure XP magnetic beads (Beckman Coulter Cat# A63881) at a 1:1 ratio. Beads with bound DNA were separated with a magnet and washed 3 times with 70% ethanol. DNA was resuspended in 10 µl elution buffer and transferred to a new plate. Whole genome DNA was amplified using 1 µl polymerase enzyme from the illustra GenomiPhi V2 DNA Amplification Kit (GE Healthcare Cat# 25-6600-32) and 9 µl of sample with the following conditions: 95 °C for 3 min, 4 °C for 5 min, 30 °C for 1.5 hours and 65 °C for 10min. DNA was then purified by mixing with beads at a 1:0.6 DNA/beads ratio followed by 3 washes with 70% ethanol and eluted with 30 µl of elution buffer (Qiagen Cat# 19086). Whole-exome sequencing was performed using the Mouse_Exome_Targets baitset from the Wellcome Sanger Institute pipeline. Captured material was sequenced on Illumina HiSeq 2500 sequencers using paired-end 75bp reads.

Mutation calling, sequence analysis and missense codon distribution in *Notch1*

Detailed bioinformatic methods are given in section 2 of the **Supplementary note**.

Statistical analysis. Data are expressed as mean values ± SEM unless otherwise indicated. No statistical method was used to predetermine sample size. The experiments were not randomized. The investigators were not blinded to allocation during experiments and outcome assessment.

Data availability. Accession numbers for the targeted sequencing of mouse gridded samples and WES of isolated single clones are ENA:ERP022921 and ENA:ERP015469, respectively. Individual data sets are available in Supplementary Tables 1-15.

Code availability. The code developed in this study has been made publicly available and can be found at https://github.com/gp10/ClonalCOMMUTE and http://doi.org/10.5281/zenodo.3648706.

Methods Online References

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