Appendix Material

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Appendix Methods

The Skinbow model

In this project, we used the *Rainbow3* transgenic mouse line (Tabansky et al, 2012), generated by microinjecting a *Brainbow-1.0* cassette (Livet et al, 2007) under the control of the ubiquitous *CAG* promoter (Niwa et al, 1991). In its basal form, this construct expresses the red fluorescent protein dTomato (Shaner et al, 2004). In the absence of recombination dTomato is the only fluorescent protein that can be visualized. Upon Cre recombination, either EYFP (Zacharias et al, 2002) or Cerulean (Rizzo et al, 2004) becomes expressed (Appendix Figure S1a). qPCR tests indicated insertion of 2 transgene copies in the *Rainbow3* line, in accordance with the fluorescent protein combinations observed (Tabansky et al, 2012). By crossbreeding these mice with the inducible *K14-Cre/ER* line (Vasioukhin et al, 1999), we generated double transgenic mice called here *"Skinbow"* allowing us to induce recombination in a dose dependant manner in basal keratinocytes regardless of their anatomical location (Appendix Figure S1a).

In the absence of tamoxifen, only dTomato could be visualized and we never observed any spontaneous recombination event (Appendix FigS3a). With a dose of tamoxifen (intraperitoneal injection of 1mg/d during 5 days per mouse) we induced Cre recombination at P21 after hair follicle formation was complete or at P42 after the first hair cycle. The dose has been chosen after testing different protocols as it appeared to be the maximal not lethal dose. Moreover, knowing that the renewal of the epidermis in a mouse is around 1 week, we injected tamoxifen for 5 consecutive days in order to target a maximum number of keratin 14 cells. We harvested back skin at various times post injection (3, 5, 12 and 24 weeks). The tissues were collected at least 3 weeks post injection as it allows the analysis to focus on the clonal fate of the actual cells that will help maintain the epidermis. Indeed, after 3 to 5 weeks, clones emanating from differentiated and non-progenitor basal cells would have produced corneocytes that further shed away. In whole mount IFE analysed with confocal microscopy (Appendix Figure S2b), we observed multiple clones of different colors, reflecting transgene expression in a large

fraction of keratinocyte (>70%). We detected clones expressing only one fluorescent protein (dTomato, YFP orCerulean) and, with lower frequencies others with colors resulting from FP mixtures: orange (tdTomato/YFP), pink (tdTomato/cerulean) and white (YFP/cerulean), in accordance with the coexpression of two *Brainbow* transgene copies in the *Rainbow3* line. Higher frequencies are expected for colors corresponding to expression of a single FP vs. multiple FPs due to reduction of the transgene array by intercopy recombination in a fraction of cells. Finally, the stability of colour combinations strongly argues against additional recombination events upon long periods of observation. This was essentially demonstrated by the stability or the trend towards the increase of the baseline red colour. For convenience and to best distinguish clones from each other, YFP was represented in yellow, dTomato in red and cerulean in blue (Appendix Figure S2a and S2b). Both male and female mice were used in the studies.

Flow cytometry

To calculate the respective frequency of the six colours upon recombination we conducted flow-cytometry analysis of adult back skin 3 weeks post tamoxifen injection. Back skin samples were harvested and incubated overnight at 4°C in Trypsin-EDTA Solution 0.25% (Sigma Aldrich, St. Louis, MO, USA) for preparing dissociated cells as previously described(Nowak & Fuchs, 2009). Flow cytometry data were acquired using a Gallios cytometer (Beckman Coulter, CA, USA) and subsequently analyzed with Kaluza software (Beckman Coulter, CA, USA). The flow cytometry analysis was performed on an ungated whole skin cell suspension. Most epidermal cells (85.3±4%) expressed the transgenes and a majority (71.7±6.6%) could be recombined. The frequency of each colour is displayed in Appendix Figure 2c. The high density of staining in Skinbow mice allows therefore for considering the fate of rare populations of stem cell as compared to previous studies using low density staining (1/600 cells)(Clayton et al, 2007).

Immunofluorescence

For staining of specific antigens, after fixation, whole mount back-skin samples were permeabilised and blocked at 4°C overnight for 24 hours with 1x phosphate buffered saline (PBS) + 0.5% TritonX (PBTX) and 20% Normal Goat Serum (NGS) or Normal Donkey Serum (NDS), 1% Dimethyl Sulfoxide (DMSO), 100mM Maleic Acid (7.5pH) before incubation with a primary antibody diluted in the blocking solution without serum and left to bind at 4°C 24 hours. Primary antibodies include: 1 in 50 rabbit anti-ki67 (Abcam), 1 in 300 goat anti-mLrig1 (R&D System). A secondary antibody conjugated with Alexa-fluor 647 (Invitrogen) was incubated at a 1:500 dilution in blocking solution, for 24 hours at 4°C. Nuclei were revealed using a DAPI conterstain, at 0.2µg/ml for 30 minutes, followed by mounting with fluorescent mounting media (DAKO, Glostrup).

Bromodeoxyuridine (BrdU) immuno-staining

Animals were injected intra-peritoneally with BrdU (0.1 mg/g body weight of 5-Bromo-2′-deoxyuridine, BrdU 10 mg/ml, Sigma-Aldrich) twice per day for 3 days. They were submitted to a 1 to 5 week chase and skin was collected for whole mount imaging. Samples were immersed in HCl 7% solution for 20 min at 37°C and blocked at 4°C overnight for 24 hours with PBTX, 20% Normal Goat Serum (NGS), 1% Dimethyl Sulfoxide (DMSO), 100mM Maleic Acid (7.5pH) before incubated with the primary antibody diluted in the blocking solution without serum (1 in 50 rat anti-BrdU, Abcam). A secondary antibody conjugated with Alexa-fluor 647 was incubated at a 1:500 dilution in blocking solution, for 24 hours at 4°C followed by mounting with fluorescent mounting media (DAKO, Glostrup).

TPA experiment

12-O-tetradecanoyl-phorbol-13-acetate (TPA) from Sigma has been diluted in acetone (final concentration 25 mg/ ml). Skinbow mice, injected with tamoxifen at P42, were topically treated with

25 nmol of TPA in acetone applied to the shaved back skin of mouse 3 weeks after the last tamoxifen injection. Mice were killed and processed to get whole-mounts of back-skin after either 48hrs or 5 weeks. At 48 hours animals were also pulse labelled with BrdU for 2 hours.

Scoring and sample analysis

Evaluation of cell fate

To analyse the fate of labelled cells as proliferative (CP) or post-mitotic (PM) entering differentiation, we performed analysis of clones that contained 2-4 cells and determined for each cell if they were basal and suprabasal. This allowed inferring on cell fates resulting from the first cell division. Single cells not dividing after labelling were considered PMs and were not taken into account as non-informative. Cells that were found to be suprabasal were considered deriving from a PM basal cell. Finally cell divisions were considered resulting from a CP. It was assumed that the proportion of divisions giving rise to 2CP or 2PM would have to be identical.

Evaluation of clone size

Every sample was assessed blindly to experimental conditions.

Cell count was performed on 100X Z image stacks acquired from whole mounted skin. Adjacent keratinocytes harbouring the same colour were counted.

Given that the lineage tracing was performed on all basal keratinocytes expressing Keratin 14 and those basal cells that differentiate move strictly upwards to incorporate the granular layer, the corneocyte count was considered as a proxy for the number of labelled keratinocytes in the entire depth of the epidermis. The dorsal epidermis is constituted by a smaller number of layers as compared to the tail or footpad epidermis. Careful examination of individual clones in tri-dimensional images of whole mount skin in sparse labelling conditions showed that surface corneocytes were systematically in relation with the underlying basal epidermal cells, excluding the possibility of lateral movement or expansion of suprabasal cells (Figure EV1a). Moreover, suprabasal cells, in particular corneocytes, do not

proliferate. Therefore clone size based on their total number directly reflected the proliferative activity of epidermal basal cells. Each corneocyte represented in average a group of 13 ± 3.2 nucleated keratinocyte from the basal layer to the stratum corneum. Using individual sections from Z stacks, limits of clones (lines) were identified and cells (dots) within each clone were counted (Figure EV1b). The bright signal of surface nucleated corneocytes allowed an easy and robust estimation of clone size at the terminal stage of Keratin14-expressing cells' differentiation. For verification purposes, on selected high power images, the total clone number was precisely estimated and validated the proxy count as exemplified in Figure EV1a.

Count of hair follicle density over time. Whole mount images of IFE were obtained at birth, 3, 6, 8 weeks, 3 months and 6 months of age. The distance between regularly disposed hair follicles was measured by the number of keratinocytes found between them (Appendix Figure S4).

Clones attached to hair follicles: clones were considered attached to a hair follicle if a cell carrying the colour of this clone was in contact with the hair shaft of the follicle at any level from the infundibulum (Figure EV1c). Of note, clones that were only in hair follicles and could not be visualized in the IFE were not considered. For all time points from 3 to 12 weeks, clones that were extending beyond the field of view were excluded. At 24 weeks, many clones had grown beyond the microscope field of view and were therefore considered in separate analyses.

Analysis

Clone size, attachment to hair follicles and cycling status were reported for each time point. Clone size was averaged and compared between groups using two-tailed t tests. Clone size distributions were compared between conditions using Chi square tests. A p value of less than 0.05 was deemed significant. No statistical method was used to predetermine sample size. The experiments were not

randomized; however the investigators were blinded to allocation during experiments and outcome assessment.

Mathematical modeling and simulation

To model the dynamics of epidermal basal cell population we used a two-dimensional lattice model similar to the one described in Klein et al (Klein et al, 2008). We extended and modified this model to match the high-density 6-color labelling used in the experiments and included the effect of the cyclical activity of hair follicles (HF) on the dynamics of the cell population.

In this model a rectangular lattice is occupied by either committed progenitor cells (**CP**), post-mitotic cells (**PM**), or vacant spaces (**Ø**) that appear subsequently to the migration of post mitotic cells from the basal layer to suprabasal layers. In addition, we considered hair follicles spaced on a rectangular grid overlaid to the lattice.

CP cells divide with rate λ_{cp} when adjacent to a vacant space; the division can be symmetric, producing two **CP** cells or two **PM** cells, or asymmetric, resulting in one **CP** and one **PM** cell, with corresponding probabilities: r, r and $1-2r$.

PM cells migrate to upper layers producing **Ø** vacant lattice sites at rate Γ. The cells in the lattice can exchange their position with a neighbouring vacant site with a diffusion rate σ. Following Klein et al(Klein et al, 2008) in the simulations we used the following parameters: Γ= 0.31/week, σ=200/week, λ_{cp}=1e4/week, and the lattice size was N=60x60 cells.

We assumed that the activity of the **CP** cells depends on a broader environment.

We also incorporated the cyclical activation of hair follicles in the model, assuming that the hair follicles have an active phase (anagen) of 1 week, followed by 3 weeks of inactivity (which includes catagen and telogen). When the hair follicles are in their anagen phase the rate of migration of **PM** cells out of the basal layer is increased to Γ_{active} = 3.0/week in an approximately circular neighbourhood consisting of 21 cells. Outside this neighbourhood and otherwise everywhere during the inactive

telogen phase the cell turnover is assumed to be slow: $\Gamma_{inactive} = 0.03$/week.

The stochastic evolution of the cell population was simulated using the Gillespie algorithm and the results were collected from 5000 independent realizations of the same stochastic process. To mimic the cell labelling experiments, at the beginning of the simulation we assign to each cell a label ("colour") that was transmitted to the progeny upon replication. In order to be consistent with the experiments we assume that 14.99% of the cells were unlabelled and the remaining are labelled with yellow, orange, white, blue, pink and red colours, respectively in the proportion indicated in supplemental figure2c. Thus the simulation reproduced the apparent clone size statistics taking into account that certain clones may appear larger than their real size due to the possible merging of clones of the same colour. At the same time we also kept track of the 'real' clone-sizes by assigning a second label that is unique to each cell in the initial distribution, and therefore keeps track of the real clone size distribution. This dual labelling procedure in the simulations allowed us to compare simulations to both experimental data and theoretical calculations.

Estimating the proportion of randomly distributed clones connected to hair follicles

Considering the basal layer as a square lattice, where each site represents a single cell and HFs overlaid on a square grid with separation of N lattice sites in both directions, we estimated the probability that a clone of size S (contiguous area covering S lattice sites) contains a HF if it was placed randomly on the lattice. For simplicity we consider square shaped clones ($S=M^2$), and due to translational symmetry we can restrict the analysis domain to a single square of size NxN with doubly periodic boundaries and a single HF at the lower left corner. Thus a clone can be placed at NxN different positions and by simple counting we obtain the probability of containing a HF for a randomly placed clone of size S as: $p(S) = (M/N)^2 = S/(N^2)$, assuming that the clone size was less than the area between HFs ($S < N^2$) or otherwise $p = 1$. This probability function is in good agreement with the simulated clone distributions when the activation of HFs is not considered.

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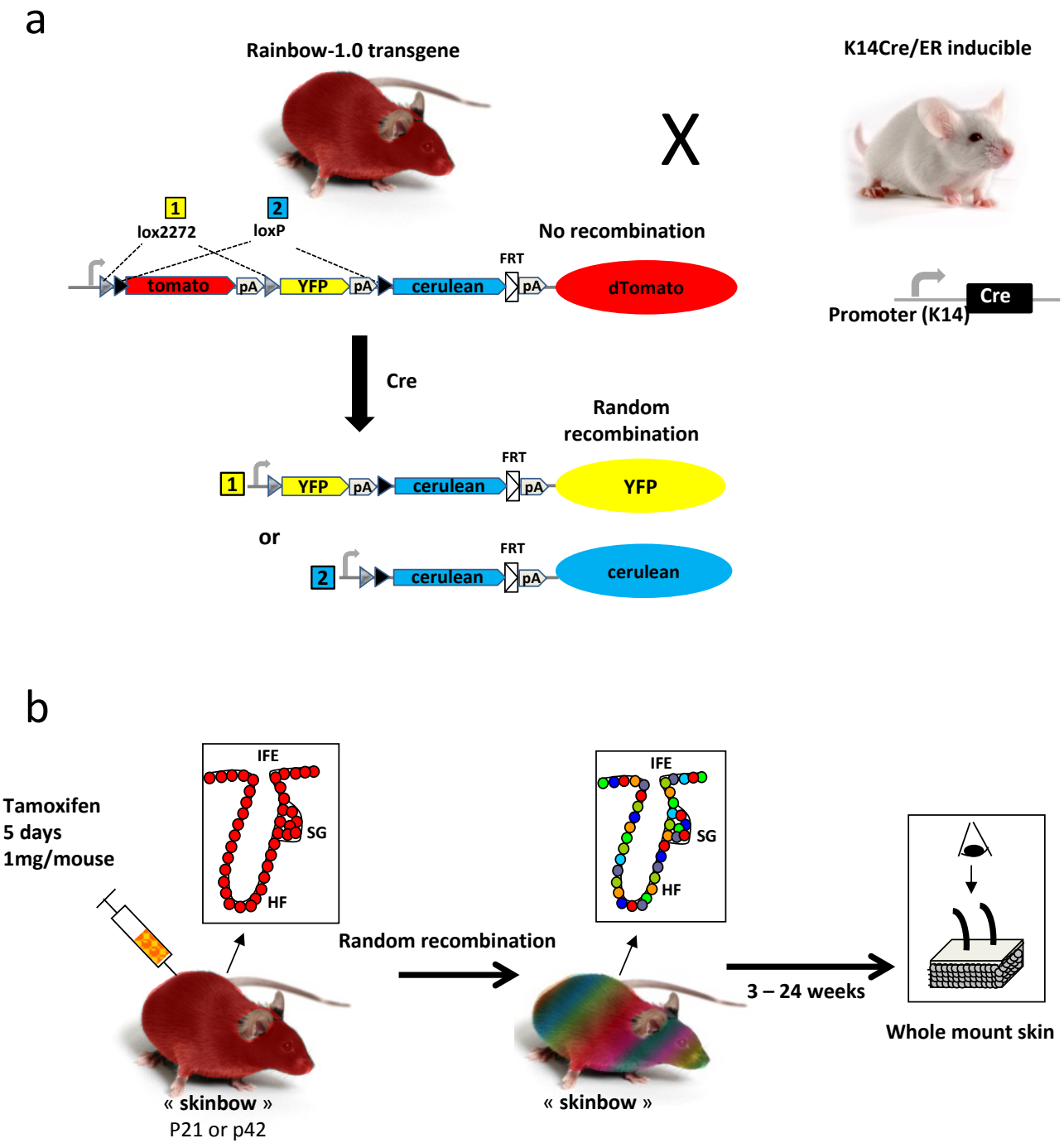
Appendix Figures

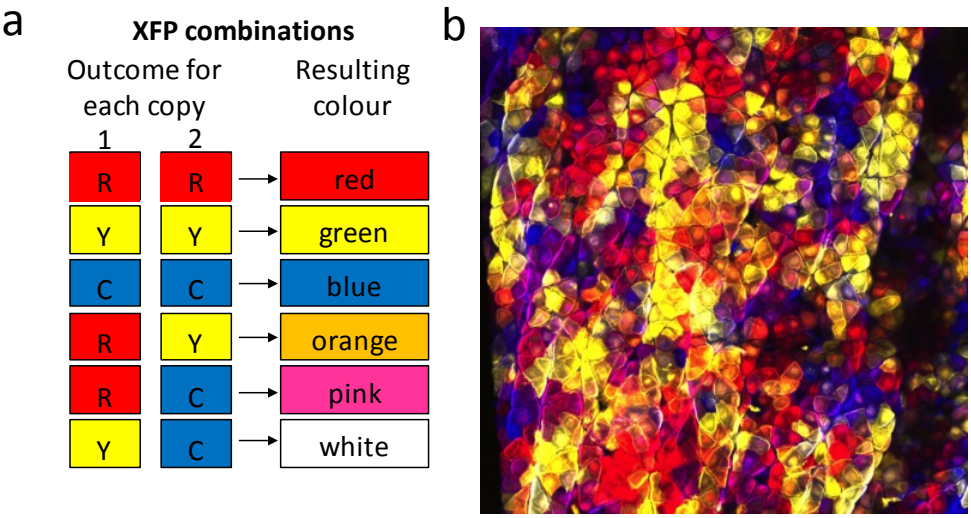
Appendix Figure S1

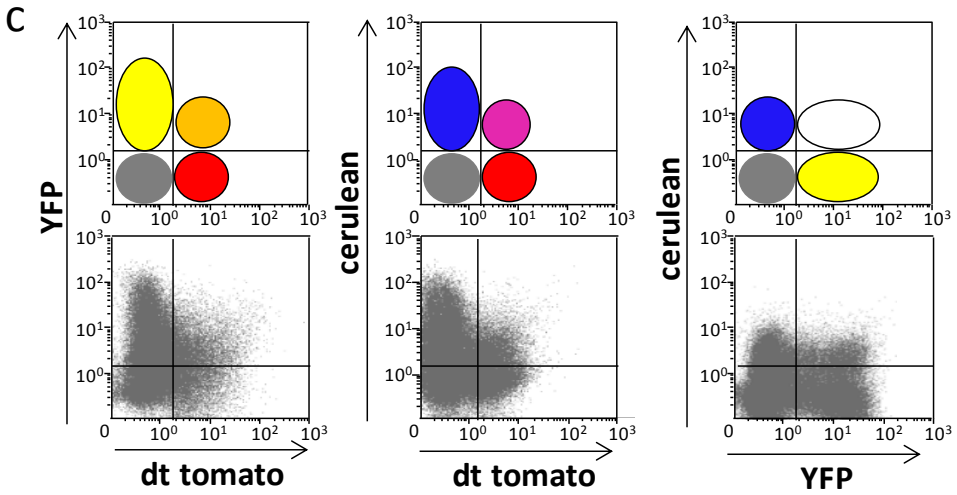


Appendix Figure S1: Skinbow experimental design.

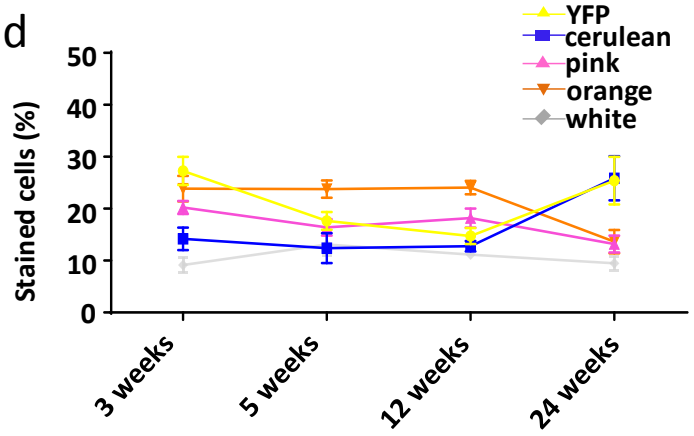
a) We used transgenic mice containing a *Brainbow-1.0* cassette under the control of a ubiquitous promoter *(CAG)* allowing random expression of d-Tomato (red), Cerulean (blue) or eYFP (yellow) upon Cre recombination. Crossing this line with *K14-Cre/ERT2* transgenic mice allowed recombination in all basal keratinocytes regardless of their anatomical location to track their fate over time. b) Experimental design. Before tamoxifen injection, skinbow cells express dTomato. Tamoxifen injections performed at P21 or P42 (1mg/mouse/day for 5 days for high density or 0.3mg only once for low density staining) triggered random recombination in keratin 14-expressing cells. Back skin was harvested at 3, 5, 12 and 24 weeks post injection and wholemount samples were analyzed by confocal microscopy.

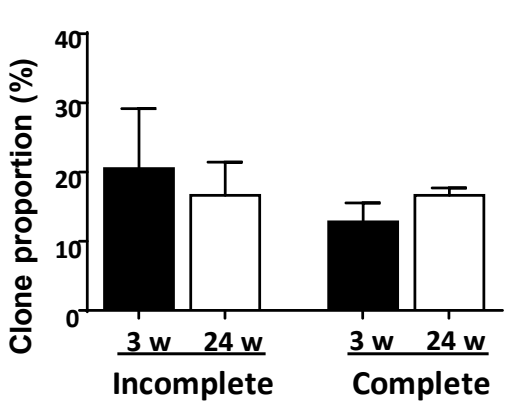
Appendix Figure S2





Colour distribution 3 weeks post injection	% dt tomato	% Cerulean	% YFP	% orange	% pink	% white
	28.24 ± 6.7	17.59 ± 3.2	25.56 ± 2.2	4.96 ± 2.0	12.11 ± 4.1	11.54 ± 2.5

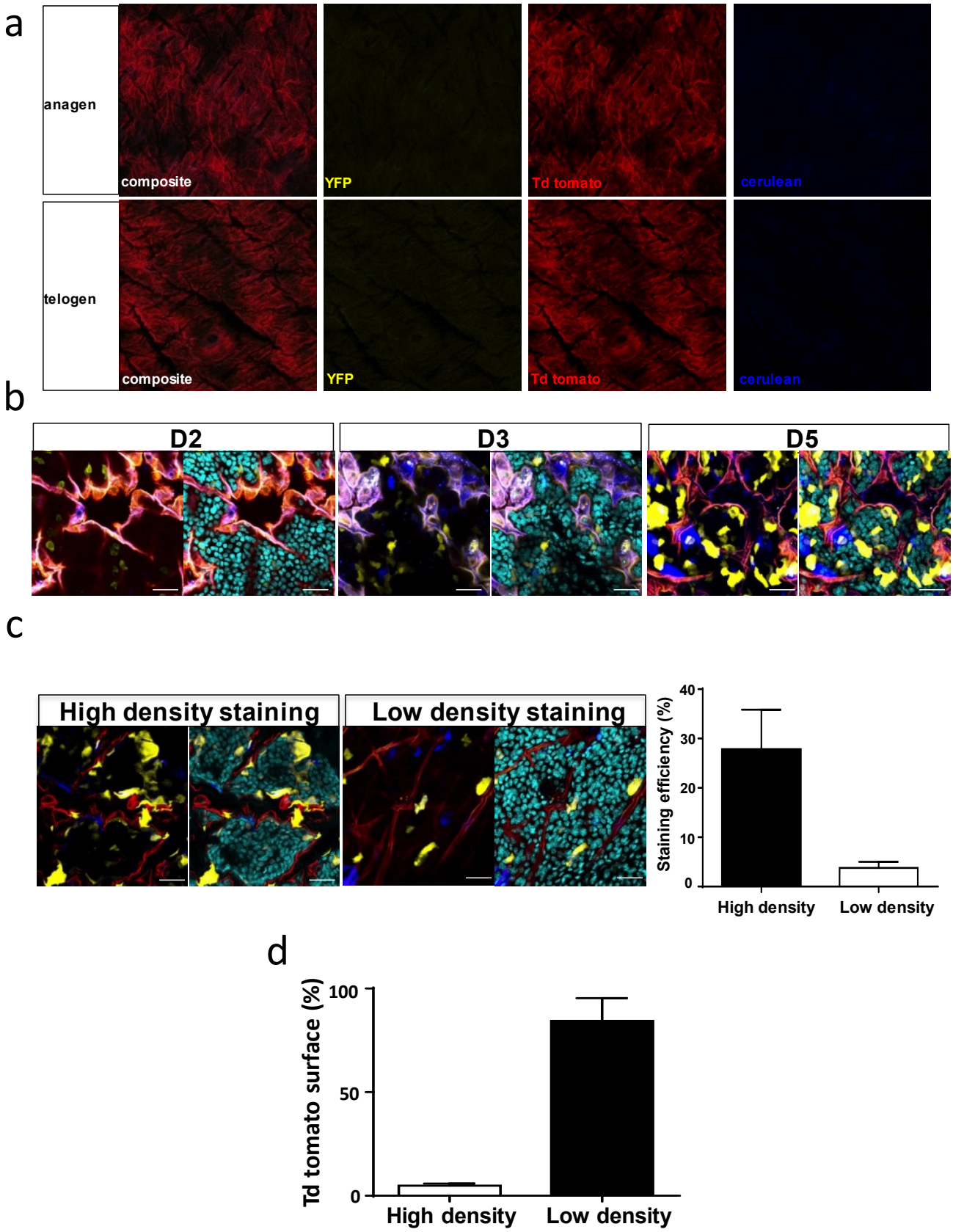




Appendix Figure S2: Frequency of recombination and stability of fluorescent protein expression over time

a) The *Brainbow* construct expresses three possible fluorescent proteins (dTomato, Cerulean and YFP), and independent recombination of two transgene copies can generate six distinct colour combinations. dTomato being the default colour it was not considered for analysis. b) Photomicrograph represents a 2D optical section from a Z stack acquisition of whole mounted skin displaying IFE clones of different colours (bar = 50 μm). c) Flow cytometry analysis of ungated whole back skin cell suspensions was used to determine the frequency of the six colours upon recombination. Representative dot plots show the frequency of the six colours 3 weeks after tamoxifen injection. The frequency of each colour is displayed in the table. d) Confocal microscopy analysis of the relative colour proportions in keratinocytes at the time points analysed. Besides the eYFP and the cerulean at later time points being overrepresented (likely because of their brightness), all other colours were relatively stable over time. e) Histogram chart comparing the change of clone proportion harbouring incomplete versus complete and terminal recombination of the both *Brainbow* constructs between 3 and 24 weeks. Incomplete group: clones harbouring 1 or 2 rainbow constructs not recombined (clone colours: red, orange, pink). Complete group: the two rainbow constructs are recombined and no further recombination is possible (clone colours: blue, yellow, white).

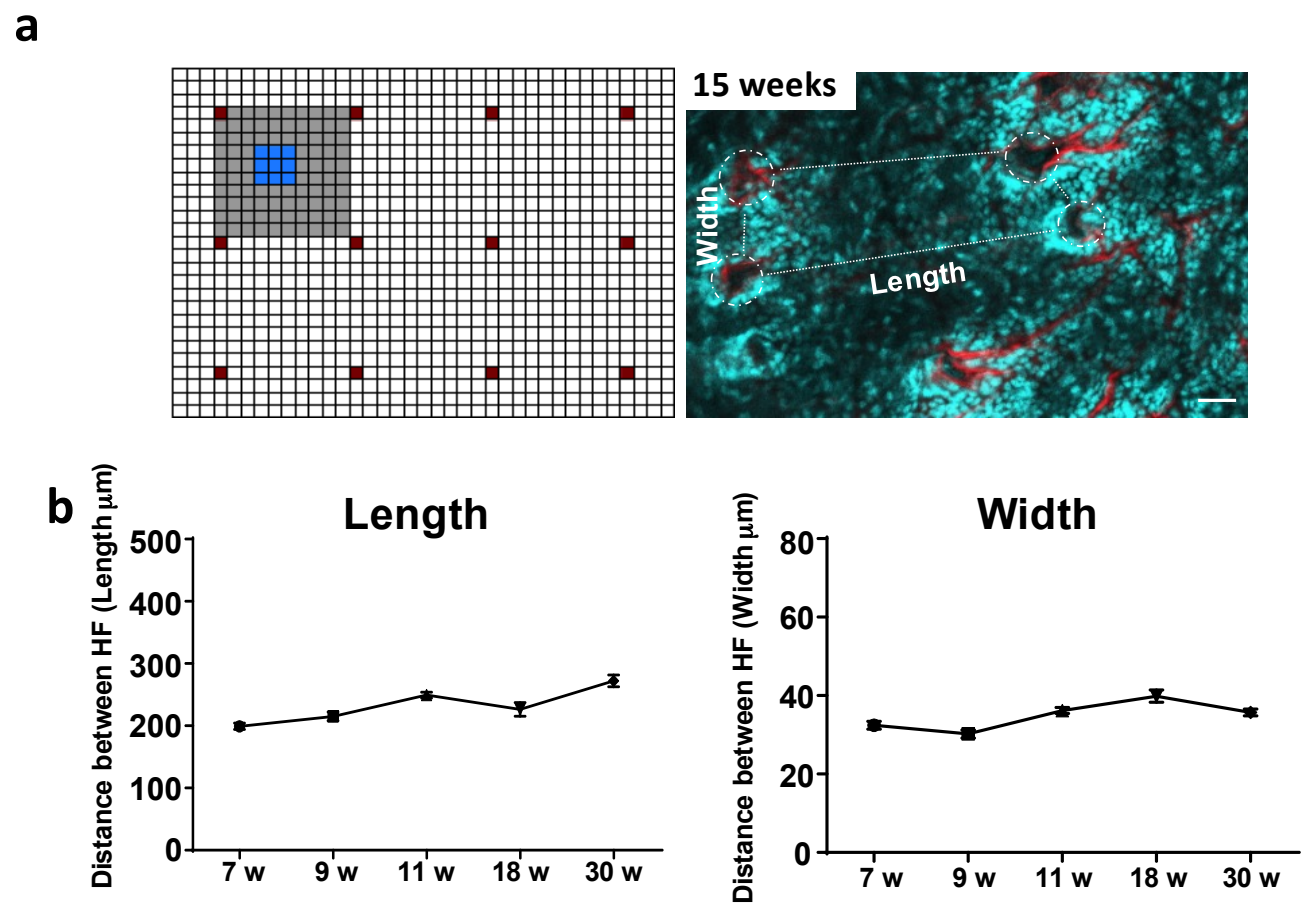
Appendix Figure S3



Appendix Figure S3: Skinbow characterisation

a) Photomicrograph represents a 2D optical section from a Z stack acquisition of whole mounted skin displaying the back skin from non-induced mice (age: 12 weeks) as controls to exclude spontaneous recombination in absence of tamoxifen. b) Photomicrograph of the back skin from mice injected at p21 (high density staining). The images represent the recombined cell fractions D2, D3 and D5 after tamoxifen injection. c) Photomicrograph of the back skin 1 week post tamoxifen injection from mice injected at p42 with high (high density staining) and low (low density staining) dose of tamoxifen. The graph represents the proportion of cells harbouring a recombination 1 week after tamoxifen injection. d) The graph represents the Td tomato surface 3 weeks post tamoxifen injection. Of note TdTomato colour represents the absence of recombination. The values are expressed in percentage +/- SD.

Appendix Figure S4



Appendix Figure S4: HF arrangement in the IFE.

a) Top left panel shows a schematic representation of skin tissue and hair follicles as a 2D lattice. In this rectangular grid, each square represents a basal keratinocyte; the hair follicles are shown as red units evenly spaced on the grid. Right panel displays a 2D samples from Z-stack images showing HF organization in whole mount preparation of back skin IFE at 15 weeks of age (DAPI staining). Dashed circles and lines represent skin units defined by four HF. All images are at similar magnification. (scale bars = 100 µm). b) Average distance (width and length of rectangles defined above) between hairs follicles at 7, 9, 11, 18 and 30 weeks of age showing stability in size in animals injected at P42 and collected at these time-points. Values are expressed in micrometre +/- SD.

Appendix Table S1

	No HF	HF region 21 cells	HF region 49 cells
3 weeks	0.0159	0.0114	0.0127
5 weeks	0.0220	0.0193	0.0248
12 weeks	0.0151	0.0139	0.0224
24 weeks	0.0164	0.0155	0.0233

Root-mean-square deviation between experimental data and different simulation scenarios: Modelling without any influence of hair follicles (HF), with HFs influencing 21 or 49 basal cells around them.