Developing and analysing novel tools to study endogenous WNT signalling in mice

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Chapter 3

Lineage Tracing of Mammary Stem and Progenitor Cells

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Abstract

Lineage tracing analysis allows mammary epithelial cells to be tracked in their natural environment, thereby revealing cell fate and proliferation choices in the intact tissue. This technique is particularly informative for studying how stem cells build and maintain the mammary epithelium during development and pregnancy. Here we describe two experimental systems based on Cre/loxP technology (Cre^{ERT2}/loxP and rtTA/tetO-Cre/loxP), which allow the inducible, permanent labelling of mammary epithelial cells following the administration of either tamoxifen or doxycycline.

Introduction

The mammary epithelium is a remarkably dynamic tissue, which undergoes extensive changes during the lifespan of an organism. These include rapid and invasive branching morphogenesis during puberty, additional side branching during consecutive reproductive cycles, massive expansion and terminal differentiation during pregnancy and, finally, complete remodelling during involution. Furthermore, the epithelium harbours extraordinary regenerative potential: an entire branched and fully functional mammary epithelium can grow out following transplantation of a small piece of epithelial tissue or epithelial cell suspensions into the cleared fat pad (Deome et al., 1959; Shackleton et al., 2006). Even a single mammary stem cell was able to generate a fully functional mammary gland in this transplantation assay.

Over the past few decades, it has become evident that in spite of its deceptively simple appearance, the bilayered mammary epithelium harbours multiple distinct cell populations in both the basal and the luminal layer. To fully understand how these different cell types are related, much effort has been dedicated towards unravelling the mammary gland stem and progenitor cell hierarchy (Visvader and Stingl, 2014).

A complete fate map of the mammary epithelium is not only of interest from a developmental perspective, but also for cancer research. Breast cancer is a heterogeneous disease comprising multiple distinct subtypes and this may at least in part be due to tumours arising from different cells of origin.

Much of our knowledge regarding the different cell populations in the mammary epithelium comes from cell sorting experiments using combinatorial cell surface markers, followed by either transplantation (to interrogate the regenerative potential of putative stem cell populations) or colony formation assays (to score the proliferative potential of progenitors) (Prater et al., 2013; Shackleton et al., 2006; Sleeman et al.,

2005; Smalley, 2010; Smalley et al., 2012; Stingl et al., 2006). Recently however, lineage tracing has emerged as a new gold standard for demonstrating stem cell activity within a tissue or organ and for tracking the fate of specific cells (van Amerongen et al., 2012; van Keymeulen et al., 2011; Prater et al., 2014; Rios et al., 2014; Šale et al., 2013; De Visser et al., 2012; Wang et al., 2015b). This technique relies on the lineage- specific expression of a DNA recombinase to activate expression of a reporter gene (Figure 1). Indeed, inducible lineage tracing analyses are a more recent addition to the mammary gland biology toolbox. They provide both spatial and temporal control and offer the possibility of permanently marking a cell population of interest in the intact mammary gland in order to track its developmental fate in vivo. Importantly, this circumvents disrupting the tissue and taking cells out of their natural environment, which is unavoidable for most other experimental analyses.

This chapter focuses on the two leading methods for in vivo lineage tracing, namely Cre^{ERT2}/loxP and rtTA/tetO-Cre/loxP. Both of these methods use genetically engineered mice to label a cell population of choice and both are inducible, thereby providing the investigator with full experimental control over the time point at which the trace is initiated.

Materials

2.1 Mouse Strains

1. Mice carrying a *Cre* reporter allele (*see* **Note 1**), allowing the inducible expression of a marker gene (e.g., lacZ or a fluorescent protein), to be combined with either a Cre^{ERT2} driver or an rtTA/ tetO-Cre system (**items 2** or **3** below, respectively, *see* **Note 2**).
2. Mice carrying a tamoxifen-inducible Cre^{ERT2} recombinase allele (*see* **Note 3**) under the control of a cell- or tissue-specific promoter (*see* **Note 4**).
3. Mice carrying both a reverse tetracycline-controlled transactivator allele (rtTA) under the control of a cell- or tissue-specific promoter (*see* **Note 4**) and a tetracycline-inducible Cre recombinase allele (*tetO-Cre*, *see* **Note 5**).

2.2 Administration of Tamoxifen to Activate the Reporter Gene

1. Gloves.
2. Microbalance.
3. Four 1.5 ml or 2 ml Eppendorf tubes.
4. Tamoxifen (see **Note 6**).
5. Corn oil (see **Note 7**).
6. Absolute ethanol.
7. Nutator mixer or rotator.

8. Two 3 ml syringes.
9. Two 22 μm syringe filters.
10. Two 1 ml BD™ slip-tip syringes with 26 G× 5/8 in. subQ needles (see Note 8).

2.3 Administration of Doxycycline to Activate the Reporter Gene

1. Gloves.
2. Microbalance.
3. Four 1.5 ml or 2 ml Eppendorf tubes.
4. Doxycycline (*see* **Note 9**).
5. PBS.
6. Two 3 ml syringes.
7. Two 22 μm syringe filters.
8. Nutator mixer or rotator.
9. Two 1 ml BD™ slip-tip syringes with 26 G× 5/8 in. subQ needles (*see* **Note 8**).

2.4 Components for Harvesting the Mammary Glands for Further Downstream Analyses

1. Euthanasia setup or equipment (*see* **Note 10**).
2. Dissection pad.
3. Spray bottle with 70 % ethanol.
4. 6–8 Pushpins.
5. Tissues.
6. Two pairs of surgical scissors.
7. Two pairs of fine (Iris or Graefe) forceps.
8. One pair of Dumont No. 5 forceps.
9. Razor blade (optional).

2.5 Whole-Mount Confocal Analysis

1. PBS.
2. 4 % Paraformaldehyde in PBS.
3. PBT (PBS + 0.1 % Tween-20).
4. Alexa Fluor 647 Phalloidin.
5. Glycerol.
6. Cover slips No 1.5.
7. Microscopy slides.
8. Dissecting fluorescence microscope.
9. Micro-dissecting scissors and forceps.
10. Tape

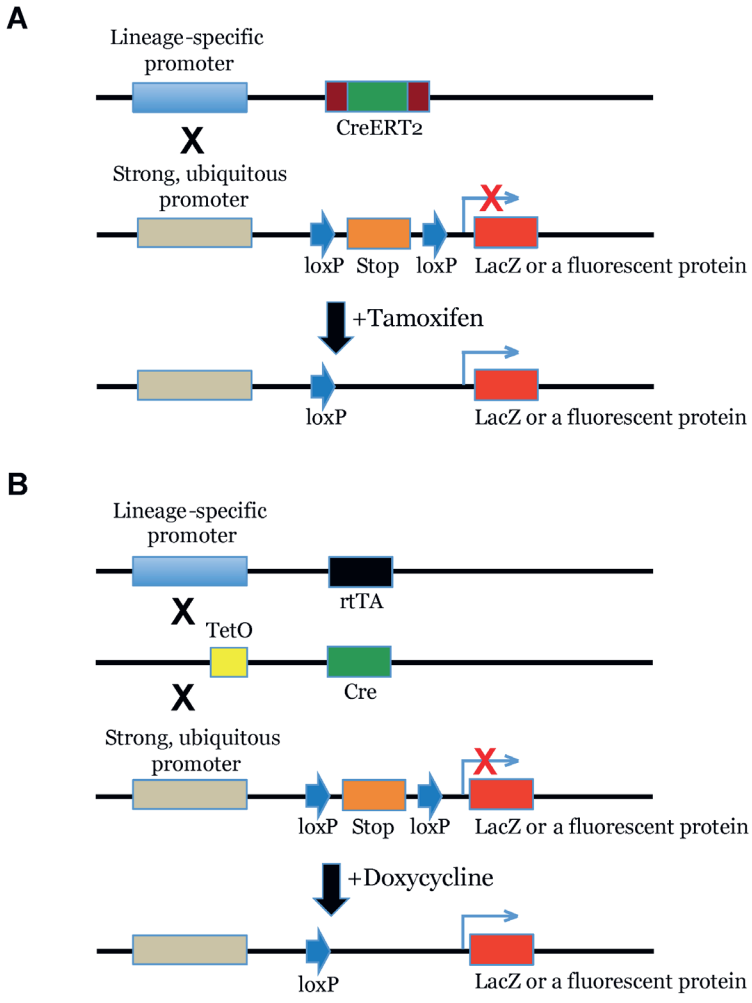

Figure 1: Overview of the two-component CreERT2/loxP system and the three-component rtTA/tetO-Cre/LoxP system. (a) The CreERT2/LoxP system consists of two transgenic alleles: one has *CreERT2* expression under the control of a lineage- specific promoter and the other has a marker gene (fluorescent or LacZ) under the control of a strong, ubiquitous promoter. Prior to Cre-mediated recombination, the presence of a stop sequence prevents expression of the marker gene. When tamoxifen is administered, *CreERT2* becomes activated in cells where the lineage-specific promoter is active. It excises the stop cassette by recombining the flanking loxP sites, resulting in expression of the marker gene. (b) The rtTA/tetO-Cre/LoxP system consists of three alleles. Instead of the Cre-recombinase, the rtTA transactivator is expressed under the control of a lineage-specific promoter. When doxycycline is administered, rtTA can bind and activate the *tetO* promoter on the *tetO-Cre* allele. The resulting *Cre* expression again causes excision of the stop cassette on the reporter allele, causing expression of the marker gene. In both systems, all progeny of an activated cell will keep expressing the marker gene under the control of the ubiquitous promoter, even when the pulse of Cre recombinase activity itself is transient.

Methods

3.1 Breeding of the Mice

1. Design the lineage-tracing experiment and determine the required time points for initiating (t_0) and analysing (t_X) the trace (Figure 2) (*see* **Note 11**).
2. For the Cre^{ERT2} system, cross heterozygous *Cre^{ERT2}* mice with homozygous reporter mice to generate double heterozygous transgenic mice. For the generation of *rtTA/tetO-Cre/loxP* mice, cross the double-heterozygous *rtTA/tetO-Cre* mice with homozygous reporter mice to generate triple heterozygous transgenic mice.
3. Genotype the double (for the Cre^{ERT2} system) or triple (for the rtTA/tetO-Cre/loxP system) transgenic mice by PCR (*see* **Note 12**).
4. Determine your experimental cohorts (*see* **Notes 13–15**).

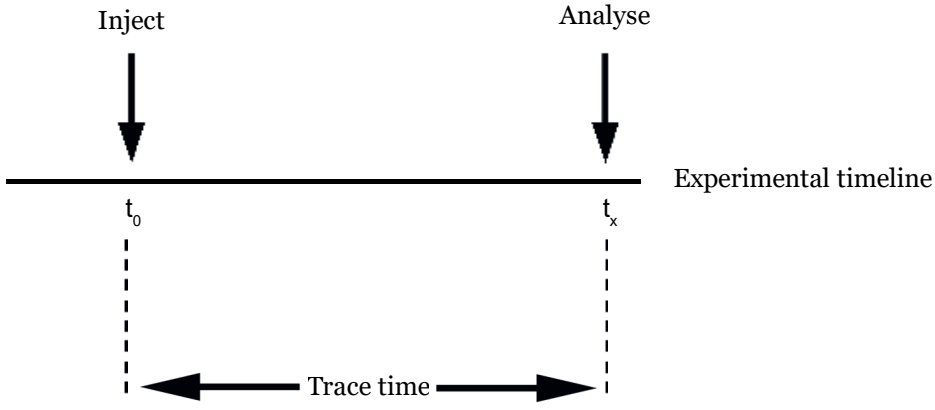

Figure 2: Experimental setup of a lineage tracing experiment. Overview of the experimental setup for a lineage tracing experiment. The trace can be started at any desired time point, even before birth (tamoxifen or doxycycline is administrated to the pregnant mother in that case). t0 depicts the start of the actual tracing experiment, when the cells are labelled through administration of tamoxifen (for the CreERT2/loxP system) or doxycycline (for the rtTA/tetO-Cre/loxP system). Analysis follows at t_x, which is any time point 24 h or later after cell labelling.

3.2 Induction of the Reporter Allele with Tamoxifen or Doxycycline

1. When using the Cre^{ERT2}/loxP system, proceed with **steps 2–4**. When using the rtTA/tetO-Cre/loxP system, proceed with **steps 5–7**.
2. Prepare the tamoxifen solution using the materials listed in Subheading 2.2. Remember to wear gloves. Weigh the required amount of tamoxifen and dissolve it at 5–20 mg/ml in 90 % corn oil and 10 % ethanol (for example, for 1 ml of 10 mg/ml tamoxifen solution, dissolve 10 mg of tamoxifen in 900 µl oil and 100 µl ethanol) (*see* **Note 16**).
3. Prepare the control oil solution. Mix oil and ethanol in a 90:10 ratio (for example, for 1 ml of control solution, mix 900 µl oil and 100 µl ethanol)
4. Incubate the solutions on a nutator or rotator to let the tamoxifen dissolve (*see* **Notes 17** and **18**). Continue with **step 8**.
5. Prepare the doxycycline solution using the materials listed in Subheading 2.3. Remember to wear gloves. Weigh the required amount of doxycycline and dissolve it at 20 mg/ml in PBS (for example, for 1 ml of 20 mg/ml doxycycline solution, dissolve 20 mg of doxycycline in 1 ml PBS).
6. Use PBS as control solution.
7. Incubate the solutions on a nutator or rotator to allow the doxycycline to dissolve. Continue with **step 8**.
8. Filter the solutions prepared in **steps 2–4** or **5–7** through a 22 µm syringe filter (*see* **Note 19**).
9. Aliquot the stock solution into 500 µl/vial and store at −20 °C.
10. Weigh the mice to determine how much of the stock solution should be injected (*see* **Notes 20** and **22**).
11. Fill a 1 ml syringe attached to a subQ needle with the tamoxifen, doxycycline, or control solution.
12. Hold the syringe vertically, with the needle pointing upwards, and remove air bubbles by flicking the syringe with your fingers, forcing the air to the top. Insert the plunger a little to let all air escape. Be careful not to spill any of the solution.
13. Put down the syringe and open the cage.
14. Lift the mouse that is to be injected out of the cage, holding it by its tail.
15. Check the identity of the mouse to be sure that you inject the correct one.
16. Place the mouse on the wire lid of the cage and let it grab the bars.
17. Fix the animal; make sure its head and tail are properly secured. Turn the mouse over and slightly tilt your hand, so that the head of the mouse is slightly lower than its abdomen. You now have one hand free to inject (*see* **Note 23**).
18. Pick up the syringe and gently insert the needle in the lower right or left quadrant at a low angle to prevent penetration of any organs (*see* **Notes 24** and **25**).

19. Inject the required amount of tamoxifen (for the Cre^{ERT2}/loxP system), doxycycline (for the rtTA/tetO-Cre/loxP) or appropriate control solution by pushing the plunger.
20. Carefully pull back the needle and put it down (*see* **Note 26**).
21. Transfer the mouse to a clean cage.
22. Repeat **steps 13–20** with the remainder of the mice that need to be injected (*see* **Note 27**)
23. Clean the workspace to remove any traces of tamoxifen or doxycycline (*see* **Note 28**).
24. Check the welfare of your animals daily in the first week following injection.

3.3 Dissection of Mammary Glands

1. At the required analytical time-point (t_x, Figure 2), euthanize the animal according to your institutional or national guidelines.
2. Secure the animal on a dissection pad with the belly facing up.
3. Spray the animal with 70 % ethanol (*see* **Note 29**).
4. Using a pair of scissors, make an incision in the skin along the vertical midline, moving from the groin up to the top of the sternum. Cut superficially, leaving the peritoneum intact and make sure not to hit any blood vessels.
5. Make a second incision towards the right knee of the animal. Start your incision at the point you began the incision from **step 4** and try to prevent hitting any blood vessels in the lower limb.
6. Make a third incision towards the left knee of the animal. Again, start your incision at the point you began the incision from **step 4** and try to prevent hitting any blood vessels in the lower limb (*see* **Note 30**).
7. Make a fourth incision towards the right shoulder of the ani- mal. Start your incision at the point you ended the incision from **step 4** and try to prevent hitting any blood vessels in the upper neck and limbs.
8. Make a fifth incision towards the left shoulder of the animal. Start your incision at the point you ended the incision from **step 4** and try to prevent hitting any blood vessels in the upper neck and limbs.
9. Using two pairs of tweezers, gently peel the skin sideways on either side of the animal, separating it from the peritoneum (which should still be intact).
10. Using pushpins, secure the skin flaps onto the dissecting pad (Figure 3).
11. At this point, you should be able to dissect the third and fourth mammary gland (*see* **Note 31**).
12. To remove the fourth mammary gland, gently grab the distal tip of the fat pad using a pair of forceps (*see* **Note 32**).
13. Hold onto the distal tip, gently pulling it up. Using a pair of scissors or a razor blade, gently remove the ligaments that attach the fat pad to the body wall, working your way towards the proximal end, where the nipple is located.

14. Process the mammary gland for further downstream analysis as required (*see* **Notes 33** and **34**).

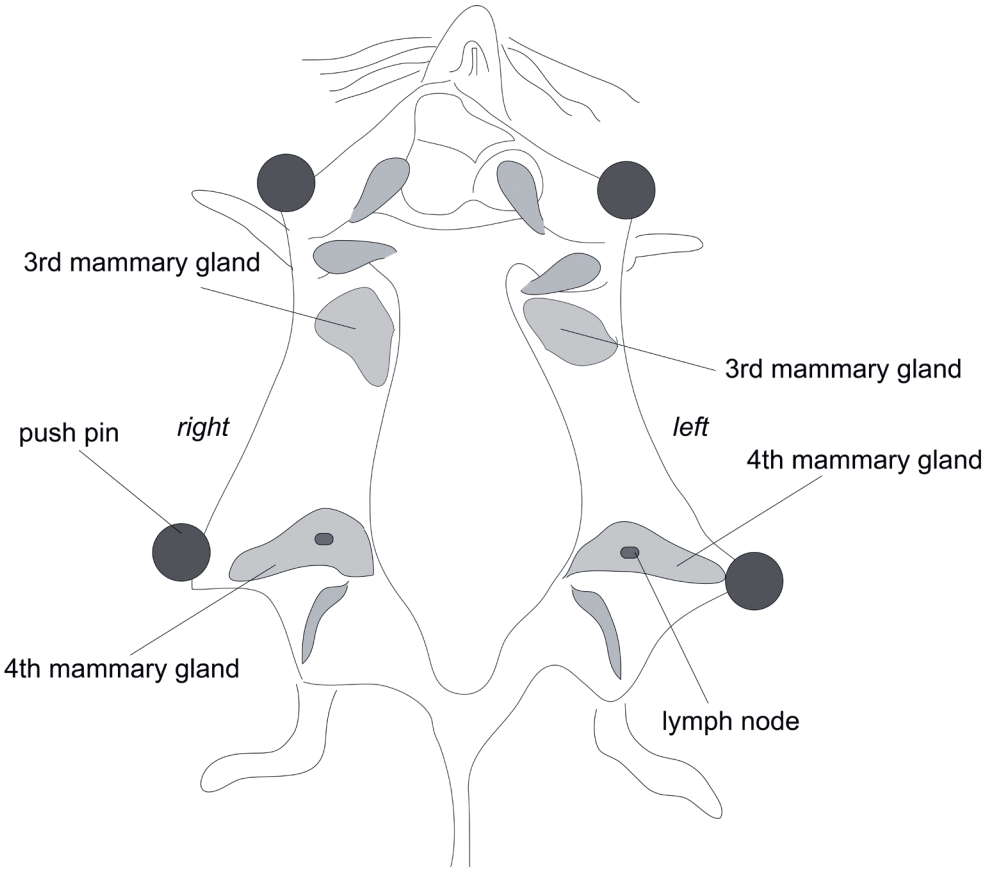

Figure 3 Anatomy of the mouse mammary glands. Schematic drawing to illustrate the location of the third and fourth mammary glands at the time of dissection. At this stage, the skin has been peeled away and has been pinned down onto the dissection pad. The peritoneum is still intact, hiding the abdominal and thoracic organs from view. The fourth mammary gland is clearly visible, as is the third mammary gland and both can be readily dissected.

3.4 Whole-Mount Confocal Analysis

1. Prepare cold fixation medium: heat 100 ml PBS to approximately 60 °C. Add 4 g paraformaldehyde powder to the solution and let dissolve on a heat stirring plate. After the solution becomes clear, put the solution on ice or store at −20 °C (*see* **Note 35**).

2. Rapidly dissect the third and fourth mammary glands as described in Subheading 3.3 and transfer directly into cold fixation medium (4 % PFA in PBS).
3. Incubate the tissues on ice for 30 min (*see* **Note 36**).
4. Prepare PBT: PBS + 0.1 % Tween-20. (v/v; for example, dis- solve 1 ml Tween-20 in 1000 ml PBS). Put this solution on ice.
5. Transfer the tissues to a 15 ml tube containing 10 ml of cold PBT.

6. Wash the tissues for 30 min on a rotator to remove residual PFA and allow gentle permeabilization.
7. Incubate the tissues in 500 µl PBT containing phalloidin (1:50; 500 µl PBT + 10 µl phalloidin). (*see* **Note 37**). Store them at 4 °C overnight.
8. On the next day, wash the tissues three times with ice cold PBS (20 min each time).
9. Prepare a clearing solution of 80% glycerol in PBT (for example 8 ml glycerol plus 2 ml PBT).
10. Transfer the tissues to a 15 ml tube containing 10 ml clearing solution and incubate at room temperature for 3 h (*see* **Note 38**).
11. Dissect the fluorescent ducts, using a dissecting microscope with a fluorescent light source. To obtain a good sample for confocal imaging, you need to trim the fat on the surface to reveal the ductal tree.
12. Mount the dissected tissues on slides and cover them with a cover slip. Tightly press down on the cover slip to flatten the tissue and let all air escape. Remove excess liquid (*see* **Note 39**).
13. Tape the cover slip to the slide on both sides of the sample.
14. Mount the slide on the stage of a confocal microscope and record Z-stacks (*see* **Note 40**) (Figure 4).

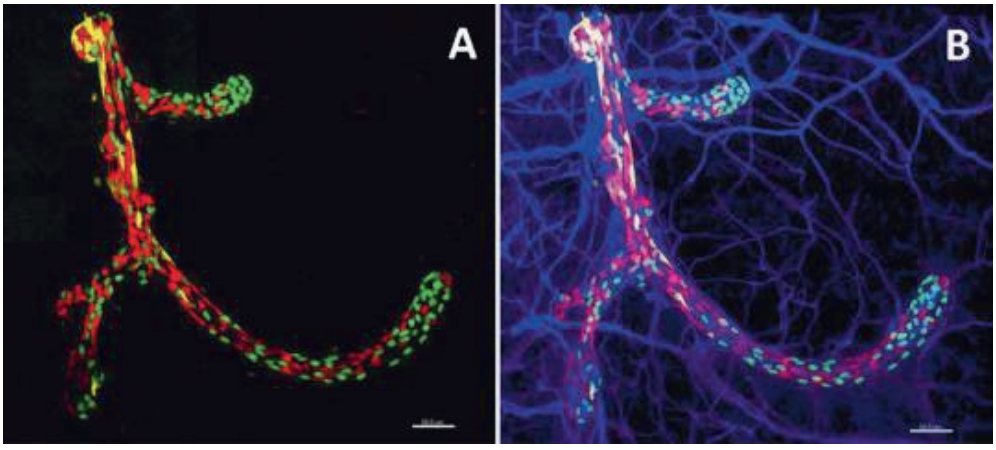

Figure 4: Lineage tracing in the mammary gland. Cells were labelled ("pulse") in puberty and traced for 8 weeks ("chase"). (a) Composite image showing a branched epithelium containing GFP+, RFP+ and YFP+ cells. (b) The same picture as in (a), now including an F-actin staining in blue to help visualise the overall structure of the tissue. Image by Anne Rios. Scale bar is 50µm.

Notes

1. Although alternatives exist (e.g., FLP/FRT technology), Cre/loxP technology remains the most commonly used tool for in vivo lineage tracing experiments. As a result, multiple Cre reporter strains are available to the scientific community (see http://jaxmice.jax.org/list/xprs_creRT1805.html). The reporter lines for lineage tracing in the mammary gland generally express a fluorescent protein gene under control of a strong, ubiquitous promoter once a so-called stop sequence is removed by Cre-mediated recombination (Figure 1). The choice of reporter strain is up to the investigator and depends on the experimental question and/or preferred mode of analysis. For instance, expression of a fluorescent reporter can be visualized directly, but this endogenous signal decreases over time in both whole-mount preparations (as described in this chapter) and tissue sections. In contrast, enzymatic detection of lacZ activity provides a permanent record, but lacZ detection is not easily compatible with fluorescence-activated cell sorting (FACS) or confocal microscopy.

2. Both the Cre^{ERT2}/loxP and the rtTA/tetO-Cre/loxP systems can be used for inducible lineage tracing analyses. The Cre^{ERT2} system involves less breeding as it relies on double-transgenic mice, and a wide variety of *Cre^{ERT2}* driver strains are available, offering flexibility in choosing the experimental setup. However, one needs to be very cautious about the toxicity of tamoxifen, which is required to induce Cre activity in this system. For example, *Bmi1*-expressing stem cells in the intestinal crypt have been shown to undergo apoptosis in response to tamoxifen, resulting in unintentional bias during lineage tracing (Zhu et al., 2013). It remains unclear whether mammary stem cells are also sensitive to tamoxifen, but the hormone sensitivity of this population and their susceptibility to the inhibitor of estrogen biosynthesis letrozole (Asselin-Labat et al., 2010) suggest that they will be. Furthermore, haematopoietic stem cells are highly sensitive to estrogen and hence may be impacted by tamoxifen (Nakada et al., 2014). In the mammary gland, where estrogen signalling plays a critical role, tamoxifen can have adverse effects on development, especially when higher doses are administered (Rios et al., 2014; Shehata et al., 2014). A low dose of tamoxifen should be used for lineage tracing in the mammary gland. In this respect, the doxycycline-inducible rtTA driver is more favourable. This three-component system necessarily incorporates a tetO-Cre allele and thus requires more complex breeding than the two-component Cre^{ERT2}/loxP system.
3. In theory, one could opt to perform lineage-tracing experiments with a non-inducible Cre driver (e.g., *Wap-Cre* (Chang et al., 2014; Wagner et al., 1997)). While offering tissue specificity, this eliminates all experimental control over the time point of Cre-mediated recombination and thus complicates the interpretation of the experimental data.
4. Both the Cre^{ERT2}/loxP and rtTA/tetO-Cre/loxP systems rely on either a knock-in or transgenic mouse strain expressing Cre^{ERT2} or rtTA under a lineage-specific promoter. Regarding tissue specificity, any cell- or tissue-specific promoter is only as good as tested. It might not faithfully recapitulate endogenous gene expression, even in the context of a knock-in construct. Therefore, use a promoter that is well characterized or, better yet, carefully characterize the Cre- or rtTA-driver yourself.
5. The tetO-Cre line obtained from Jax can be leaky in the germline, and thus mice carrying both the tetO-Cre and reporter alleles should be avoided as the breeder.
6. Tamoxifen can function as an estrogen agonist or antagonist, depending on the target tissue. It is classified as carcinogenic and toxic. Always wear gloves when handling this product. Tamoxifen is light sensitive.
7. Alternatively, you can use regular sunflower oil fit for human consumption.

8. Tamoxifen and doxycycline are both administered by intraperitoneal injection in this protocol. Another option is oral gavage, or, in the case of doxycycline, administration via the drinking water and the feed. Total uptake might differ for these options (Cawthorne et al., 2007; Hayashi et al., 1989), so choose one for all traces within one study.
9. Doxycycline is a tetracycline antibiotic. It is classified as harmful and is light sensitive.
10. Always follow your institutional or national guidelines for euthanizing animals.
11. You will need to analyse several time points. The first one is 24–48 h after labelling, depending on how long it takes for the marker gene to be expressed. For example, LacZ can easily be detected at 24 h after injection of tamoxifen. GFP is also detectable from 24 h onwards, but robust levels can often only be detected after 48 h. Checking these early time points will show you what population(s) of cells were initially labelled. Any time point(s) after that will likely show the offspring of these cells as well. To follow the trace really well, consider including some intervening time points in addition to the early (24–28 h) and final analytical time point. This is particularly informative if the gland will dramatically change in appearance (for instance, when the trace is initiated during puberty and the glands are analysed during pregnancy, it could be informative to include experimental animals in which the trace is analysed in adult virgins). Importantly, if you want to provide evidence that you are tracing a stem cell population, the mice will have to complete at least one round (and preferably multiple rounds) of pregnancy, lactation, and involution. This ensures complete turnover of the mammary epithelium, since massive turnover of cells takes place during these processes. Stem cells can self- renew and therefore should survive this turnover. Next to that, they should give rise to differentiated offspring.
12. Depending on the parental genotype (homozygous or heterozygous for each of the alleles), only part of the offspring may carry both alleles. Always check all of your animals by genotyping, even if you are sure what the genotype should be! There is always a chance that animals (or their labels) get mixed up, especially in big animal facilities with large numbers of animals and multiple strains. Make sure that your animals have a unique identifier (for instance an ear clip) when you genotype them. Collect a piece of tissue (ear, toe, or tail can all be used) according to your institutional or national guidelines. It is easiest to do this when the animals are weaned (around postnatal day 21, P21). Lyse the tissue, for example in Viagen tail lysis buffer (approx. 100 µl per sample) supplemented with proteinase K (100 µg/ml) and incubate overnight at 55 °C. Next day, inactivate the proteinase K at 85 °C for 15–45 min. This lysate can now directly be used in a genotyping PCR

(use 1–10 µl). Another option is to use a homemade lysis buffer (Laird et al., 1991). This will take more time, but using homemade buffer is cheaper and will lead to a similar quality of DNA.

13. It is important to think well about your controls. It will usually be difficult to include a proper positive technical control, because this requires an independent Cre driver that is known to work in the mammary gland and therefore, potentially, importing an additional transgenic mouse line. If you do not have access to such a line, you can opt to use a different tissue from the double-transgenic mice in which you initiate the trace as a positive control (provided that there is another tissue in which Cre-mediated recombination should take place very effectively using this driver). For instance, when using the Wnt//β-catenin responsive *Axin2*^{CreERT2} allele in combination with the *Rosa26*^{mTmG} reporter (van Amerongen et al., 2012), we often quickly inspect the intestine (which also contains Wnt/β-catenin responsive stem cells) for successful recombination (i.e., the presence of a GFP signal) using an inverted fluorescence microscope.
14. As a negative control, inject double-transgenic mice with the control solution (i.e., the solution without tamoxifen or doxycycline). Should your Cre driver be "leaky" (i.e., activated by endogenous estrogen or not properly shielded by the ER- moiety), the negative control will reveal this. Of note, leakiness will be far less likely with CreERT2, since it contains three point mutations in the ER moiety (compared to Cre^{ER}; another version, Cre^{ERT}, contains one point mutation compared to Cre^{ER}), which make it less sensitive to estrogen and more sensitive to tamoxifen. Because animal numbers are often limiting, it may be difficult to take along a negative control for every single experiment. As an alternative, analyse a negative control at least once per Cre strain and protocol (i.e., time point of tamoxifen administration). Another negative control is to inject a mouse that carries the reporter allele, but not the CreERT2 driver, with tamoxifen and analyse the mammary glands from this animal alongside the glands from double-transgenic mice to determine the background levels (either following enzymatic lacZ detection or "straight up" for fluorescent reporter alleles). In practice however, most of the mammary gland tissue will serve as a negative control in itself, because you should aim to label only a subset of the cells in order to be able to perform clonal analyses (see Note 20).
15. Biological controls are important as well for the correct interpretation of your experiment. Try to use littermates whenever possible and house animals in the same cage.
16. When designing your experiments, be aware that high doses of tamoxifen can have adverse effects on mammary gland development, as mentioned in Note 2 (Rios et al., 2014; Shehata et al., 2014). Set up pilot experiments to

carefully determine the minimum dose you can use with your particular *CreERT2* driver and labelling time point. We have noticed that the efficiency of labelling cells with tamoxifen in the mammary gland can be much lower than the efficiency in other tissues, such as intestine, using the same *CreERT2* driver.

17. It is advisable to always prepare fresh solutions for each experiment, although it is possible to make the solution the day before and store it at 4 °C overnight for use the next morning.
18. Tamoxifen is notoriously difficult to dissolve. Adding ethanol to the oil solution helps (tamoxifen is soluble in pure ethanol), as will heating to 37 °C, but it will still take some time. Tamoxifen is light sensitive, so wrap the tube in aluminium foil.
19. The solution can be very viscous, so be careful when applying pressure to the syringe.
20. When determining the right concentration of tamoxifen or doxycycline to use, keep in mind that, ideally, lineage tracing analyses are performed at clonal density (see Note 14). This means that only a small part of the population you are interested in is labelled at the time of tamoxifen administration, so as to be able to robustly detect the clonal offspring of the initially labelled cells. To be absolutely sure that a labelled patch of cells is indeed a clone (i.e., comes from one cell), one would want to label only one cell per mammary gland. However, this is practically unfeasible, as it would require large numbers of animals to reach sufficient experimental coverage. Therefore, it is advisable to optimize the concentration of tamoxifen such that each labelled cell (clone) is separated by large pieces of non-labelled tissue.
21. The absolute amount of tamoxifen (and thus the injection volume) is dependent on the age and weight of the mice, as well as on the aim of your experiment. When injecting a low dose of tamoxifen (i.e., no more than 0.5 mg), it is helpful to prepare a solution that has a low concentration (2–5 mg/ml) so you have good control about the volume you are injecting.
22. Not much is known about the half-life of tamoxifen in the mammary gland of mice and, related to this, about the duration of Cre activity following a single pulse of tamoxifen. It may take several hours for tamoxifen to be converted into its active metabolite (4-hydroxytamoxifen). Generally speaking, the half-life of tamoxifen will differ depending on the tissue, the age of the mice, the administered dose, and the route of administration (Nakamura et al., 2006; Reid et al., 2014; Reinert et al., 2012).
23. Find out on beforehand which hand to use for injecting and which one for fixing the mouse. This is a personal preference.
24. IP injections can either be administered in the lower right or the lower left quadrant of the mouse abdomen, depending on which hand you are using to

hold the syringe. If you hold the syringe in your right hand, it is easiest to inject the left quadrant of the mouse and vice versa. However, injecting on the right side will ensure not puncturing the caecum and is the safest option.

25. Insert the needle at an angle of approximately 30° to penetrate the skin, while preventing damage to any internal organs such as liver, bladder, small intestine or caecum. Insertion of about 0.5 cm of the needle should be enough. To check if your needle penetrated any organs, you can aspirate a small volume before injecting. The aspirate should appear clear and not coloured red (blood), green/brown (intestine), or yellow (bladder).
26. When injecting multiple animals housed in the same cage, it is possible to re-use the same needle, provided that you check the sharpness of the needle between injections. A blunt needle causes discomfort for the animal. If you want to be absolutely safe and minimize the chance of infections, use a clean needle (i.e., fill a new syringe) for each animal.
27. Never pool injected and non-injected mice. Similarly, do not put mice injected with different drugs in the same cage.
28. Under normal circumstances, no tamoxifen should be spilt when following this procedure.
29. This will prevent hair from sticking to your instruments as well as the mammary tissue.
30. At this point, the incisions should look like an inverted Y.
31. The mammary glands should now be facing up. Usually, the fourth mammary gland is isolated for further analysis. The fifth mammary gland may still be attached to the fourth mammary gland, but the two are readily distinguishable: the fourth mammary gland is the largest gland and has a distinct boomerang shape with a clearly visible lymph node. The third mammary gland is also relatively easy to excise and can be taken along for analysis as well. However, you should take care to prevent isolating muscle in the process (the muscle is a bit more brownish, whereas the mammary gland is more pinkish in colour).
32. The distal tip is the part closest to the peritoneum and may even extend below the peritoneum towards the back of the animal. Push back the peritoneum to find the end of the fat pad.
33. There are multiple ways to analyse a tracing experiment. First, fluorescence-activated cell sorting (FACS) using combinatorial cell surface markers allows the overall contribution of the labelled cell lineage to the basal and luminal cell populations to be assessed (van Amerongen et al., 2012; Rios et al., 2014). These analyses are suitable for fluorescent Cre reporter alleles, but less so for lacZ reporter alleles. In addition, FACS offers a population-based analysis and individual cell clones cannot be analysed. Second, individual cell clones can be analysed by whole mount confocal microscopy (van

Amerongen et al., 2012; Rios et al., 2014), provided that the labelling was performed at clonal density such that individual cell clones can be readily distinguished. Finally, mammary glands can be processed for paraffin embedding and analysed by immunohistochemical or immunofluorescence staining. While this allows co-staining of labelled cells with structural markers, it again complicates the analysis of entire cell clones, although this can be performed using serial sections.

34. In Subheading 3.4 we present one of the strategies used to further investigate the kinetics of stem and progenitor cells in the mammary gland by performing a clonal analysis using the multi-colour Cre-reporter Confetti. Two challenges need to be tackled to perform a non-biased analysis using this Cre- reporter: strong detection of all four fluorescent proteins, and covering a large area of tissue in order to view many clones. The R26R-confetti allele consists of the strong CAGGS promoter, a LoxP-flanked NeoR cassette serving as a transcriptional roadblock, and the original Brainbow-2.1 cassette (Livet et al., 2007; Snippert et al., 2010a). After Cre-mediated recombination, the roadblock is removed and one of the four fluorescent marker genes (membrane- bound CFP, nuclear GFP, YFP, or RFP) is stochastically placed under control of the CAGGS promoter, allowing one to trace the contribution of individual cells in the same population in a given tissue. Detection of signals from the native fluorescent proteins requires fresh tissue as there are no antibodies avail- able to distinguish CFP, GFP, and YFP.

    An additional obstacle to performing a non-biased clonal analysis is the mode of imaging used. Sectioning (a few micrometres of thickness) has been used for many decades but has some limitations as it does not precisely account for cell morphology and their positioning within the ductal mammary epithelium. Indeed, myoepithelial cells are highly elongated (average of 100 μm) while the cuboidal luminal cells are organised in an inner layer that lies perpendicular to the myoepithelial cell layer. This organization and the differing morphologies of the two cell types imposes challenges for scoring cell clones in 2D sections. Different strategies for whole mount fluorescence analysis have been described (Van Amerongen, 2015; van Amerongen et al., 2012). The Visvader lab recently developed 3D confocal imaging to view entire regions of the mammary ductal tree down to the single cell level (Rios et al., 2014). Here, we provide a brief protocol that allows strong detection of the native fluorescent proteins in regions up to 1 cm of tissue at cellular resolution, thus allowing analysis of both clonal localization and composition.
35. Paraformaldehyde is classified as toxic and carcinogenic. Work in the fume hood while heating and dissolving it.
36. Placing the tissues on ice is necessary to preserve fluorescence.

37. Phalloidin is used to label and visualize F-actin containing structures. It is the easiest way to visualize multiple different cell types in a complex tissue. The staining reveals structural information and provides a global overview of the tissue architecture during image acquisition and analysis.
38. A clearing step is required for ex vivo optical imaging to reduce background of surrounding tissue.
39. If the tissue is too large (for example from a pregnant female) you might need to chop it in several pieces before it fits on a slide.
40. Confocal imaging (Figure 4) can be performed using a Leica SP5 confocal microscope equipped with a 40× oil lens NA 1.2. Tile scans with Z-stacks must be acquired to cover a large ductal tree area, 1024 × 1024 pixels and 12 bits. Use sequential scanning for XFP excitations, to prevent spectral bleed-through. Nuclear GFP can be excited using an argon laser 488 nm line; for EYFP 514 nm line; for RFP a red diode laser emitting at 561 nm, and blue membrane-bound CFP can be excited using a laser line at 458 nm. In general, GFP fluorescence can be collected between ~498 and 510 nm; YFP fluorescence collected between ~521 and 560 nm; RFP fluorescence collected between ~590 and 620 nm; CFP fluorescence collected between ~466 and 495 nm. Phalloidin-647 is excited with the laser 633 and the fluorescence collected after 650 nm.

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