Who watches the watchmen?

WNT responsive stem cells and the regulation of their niche ligands

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‘I hope in this year to come you make mistakes.

Because if you are making mistakes, then you are making new things, trying new things, learning, living, pushing yourself, changing your world. You’re doing things you’ve never done before, and more importantly, you’re Doing something.’

Neil Gaiman
Chapter 3

PRIME: A novel multicolour reporter for lineage tracing, cellular visualisation and cell segmentation


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Abstract

Cre/Lox fluorescent reporter mouse strains are an invaluable tool for lineage tracing and transplantation studies in vivo. Here we report the generation and characterization of a new knock-in mouse strain that is designed for multicolour lineage tracing that allows independent and simultaneous visualisation of multiple (stem) cells and their progeny in vivo. Post Cre/Lox mediated recombination, the knock-in allele permanently expresses one of three bright membrane-tethered fluorescent proteins: mScarlet-I, mTurquoise2 or mVenus. We show that the Rosa26\textsuperscript{PRIME} strain can label and trace WNT/CTNNB1 responsive cells during embryonic development and postnatal development. By design, the fluorescent membrane signal of the PRIME reporter robustly visualizes cell shape across experimental setups and is ideal for further downstream analysis that includes cell segmentation and quantification. We therefore expect this mouse strain to be a useful resource for biologists who want to visualize cell morphology and cell-cell interactions as well as for those who want to track and trace the location and fate of stem cells and their progeny, both during development and in postnatal tissues.
Introduction

Stem cells are essential for the development of the mammalian embryo \(^1, ^2\), tissue homeostasis, regeneration and wound healing \(^3\). These cells are defined by their self-renewal potential and ability to generate progeny that encompasses all cell types of a given lineage \(^4, ^5\). Over the years, many different genetically engineered mouse strains have been generated to identify novel stem cells and capture stem cell dynamics in vivo \(^6-11\). Such lineage tracing is highly informative to investigate both the developmental fate and cell division dynamics of stem cells in vivo over time \(^12-17\).

To better address outstanding questions revolving around stem cell dynamics such as ‘stem cell competition’ and symmetric versus asymmetric stem cell division’ several multicolour lineage tracing reporters have been developed that allow independent and simultaneous visualisation of multiple stem cells and their progeny \textit{in vivo} \(^13-17\) (Fig1A-B). All existing available mouse models suffer from drawbacks, however. Specifically, none of the available strains combine universal reporter expression with a visualisation of cell shape. Moreover, these models often contain fluorophores with overlapping emission spectra suboptimal for multicolour imaging in wholemount tissues (Fig1A-B).

Here we describe the design, construction and characterisation of a novel Cre/Lox inducible fluorescent multicolour reporter allele. It combines aspects of existing multicolour mouse models, incorporates novel bright fluorescent proteins and is designed to allow optimal imaging of wholemount tissues, and further downstream analysis that includes cell segmentation (FigC-F). We call it \textit{Rosa26PRIME}, for PRimary colours In the MEembrane.
## Chapter 3

### Table A)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Nr of colours</th>
<th>Expression</th>
<th>Integration</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAGG-mTmG</td>
<td>2</td>
<td>Ubiquitous</td>
<td>Rosa26</td>
<td>13</td>
</tr>
<tr>
<td>Thy1 Brainbow3.2</td>
<td>4 or more</td>
<td>Neuronal</td>
<td>Transgenic</td>
<td>14</td>
</tr>
<tr>
<td>CAGG-Rainbow</td>
<td>4</td>
<td>Ubiquitous</td>
<td>Rosa26</td>
<td>17</td>
</tr>
<tr>
<td>CAGG-Confetti</td>
<td>4</td>
<td>Ubiquitous</td>
<td>Rosa26</td>
<td>16</td>
</tr>
</tbody>
</table>

### Diagram B)

- **Rosa26-mTRM**: M-EGFP
- **Thy1-Brainbow3.2**: M-EGFP
- **Rosa26-Confetti**: mScarlet
- **Rosa26-Rainbow**: mTq2

### Diagram C)

- Normalized absorbance
- Wavelength

### Diagram D)

- 1
- 2

### Diagram E)

- 5’ homology
- 3’ homology

### Diagram F)

- Colony 1: IRFP670
- Colony 2: mScarlet
- Colony 3: mTq2
- Colony 4: mVenus

- 1
- 2
- 3
Figure 1: Design and generation of the Rosa26\textsuperscript{PRIME} knock-in allele. (A) Overview of multicolour lineage tracing strains that are available from public repositories. Information was retrieved from the International Mouse Strain Resource (IMSR) at http://www.findmice.org. Strains are subdivided by three criteria; whether it is a random transgenic insertion (Tg) or a targeted insertion (Rosa26); whether the expression is ubiquitous or tissue specific; the variation in fluorescent proteins that can be expressed post Cre mediated recombination. (B) Cartoons depicting the outcomes of Cre mediated recombination for the strains from A and our new Rosa26\textsuperscript{PRIME}. (C) Excitation (left) and emission (right) spectra of the four fluorescent proteins of the Rosa26\textsuperscript{PRIME} reporter. Spectral data from FPbase.org Spectra Viewer, accessed on 03-01-2020. (D) Schematic representation of the Rosa26 locus. Most Rosa26 knock-in strains insert reporters in the intron between the 1\textsuperscript{st} and 2\textsuperscript{nd} exon. (E) Cartoon depicting the Rosa26\textsuperscript{PRIME} targeting construct. Three pair of heterotypic Lox sites are inserted separating four fluorescent proteins: a nuclear near-infrared fluorescent protein (3xNLS-iRFP670), membrane-bound red fluorescent protein (mScarlet-I), membrane-bound cyan fluorescent protein (mTq2), and membrane-bound yellow fluorescent protein (mVenus). This construct was cloned downstream of a strong ubiquitous CAGG promoter and inserted in a Rosa26 targeting vector with 5’ and 3’ homology arms. The PGK-Neo-polyA cassette, flanked by FRT sites (indicated by white triangles), was used for selection of embryonic stem cells and was removed prior to establishing the colony. (F) Cartoon depicting stochastic Cre mediated recombination of Rosa26\textsuperscript{PRIME}. Prior to Cre activation, nuclear iRFP670 is expressed. Upon Cre activation, either LoxP, LoxN or Lox2272 is excised in a random fashion leading to three possible outcomes, membrane-associated mScarlet-I, mTq2 or mVenus. The corresponding confocal images depict the outcome in mouse ES cells either prior (top) or after (1, 2, 3) switching.

Results and discussion

We designed our new PRIME multicolour lineage tracing allele to meet specific criteria. First, we chose next-generation fluorescent proteins that score high in terms of brightness, quantum yield and photostability, namely mScarlet-I\textsuperscript{18}, mTurquoise2\textsuperscript{18, 19} and mVenus\textsuperscript{20} (Fig1F, supplementary Fig 4). Second, we wanted to include a universal nuclear marker that would be expressed in all cells prior to switching, thus the stop cassette in the PRIME reporter consists of a nuclear-localised far-red nuclear iRFP670\textsuperscript{21} whose emission spectra is compatible with the other PRIME fluorescent proteins (Fig1C,F). Third, our goal was to generate a multicolour lineage tracing reporter that better visualises cell shape and cell-cell interactions within traced tissues and clones, reminiscent of the mTmG reporter\textsuperscript{13}. Therefore, we added a MARCKS membrane tag to the fluorescent proteins\textsuperscript{13, 22, 23}. GFP or any derivatives with similar emission spectra were explicitly excluded from our design to allow the PRIME reporter to be combined with other reporter strains for dual reporter imaging if desired.

To ensure universal expression of the PRIME reporter allele we targeted our reporter to the Rosa26 locus, a safer harbour locus that allows for robust expression across many tissues\textsuperscript{12} (Fig1D-E). A CAGG promoter was chosen to drive the PRIME allele for its ability to promote strong gene expression in a wide variety of tissues when inserted in the Rosa26 locus\textsuperscript{24, 25}. We chose to achieve recombination by excision via three unique and incompatible Lox site variations, LoxP, LoxN and Lox2272, similar to the design of the Brainbow 3.2 construct\textsuperscript{14}. 
This prevents repeated recombination due to prolonged Cre activity as observed in reporter alleles that use LoxP-mediated inversion and/or excision \(^{15,16}\) and should result in stochastic randomisation of membrane-bound mScarlet-I, mTurquoise2 and mVenus expression following Cre-mediated recombination \(^{14-16}\) (Fig1F).

For functional validation of the allele, we tested Cre-mediated recombination in targeted mouse embryonic stem cells (mESCs) that were heterozygous for the Rosa26\(^{PRIME}\) allele by transiently transfecting these cells with a Cre expression plasmid \(^{26}\). As expected, unrecombined cells expressed nuclear iRFP670 (Fig1F, supplementary Fig 1A-C), whereas upon Cre mediated recombination nuclear iRFP670 was lost, and membrane restricted expression of either mScarlet-I, mTurquoise2 or mVenus was robustly induced (Fig1F, supplementary Fig 1A-C). Following blastocyst injections and germline transmission, wildtype, heterozygous and homozygous Rosa26\(^{PRIME}\) mice were born at the expected mendelian ratios from heterozygous intercrosses on a C57BL/6 and FVB/N mixed background (data not shown). We did not detect any phenotype (including differences in weight, fertility and lifespan up to 12 months) associated with the Rosa26\(^{PRIME}\) knock-in allele in hetero- or, homozygotes (data not shown).

To test the functionality of the PRIME allele during embryonic development, we generated compound Axin2\(^{CreERT2;}\)Rosa26\(^{PRIME}\) mice and labelled Axin2\(^{+}\) cells in E7.5 embryos by a single intraperitoneal injection of Tamoxifen into primed pregnant females \(^{8,27}\) (Fig2A). At E9.5, double-heterozygous embryos showed prominent recombination of the Rosa26\(^{PRIME}\) reporter in the posterior half of the embryo (Fig2B-C), consistent with posterior expression of Axin2 at the time of induction at E7.5 \(^{28}\). Using wholemount confocal microscopy, individual clones and cells within these clones that express mScarlet-I, mTurquoise2 and mVenus could be distinguished in these embryos at the indicated sites (Fig2C-E). Moreover, once recombined, the cell shape of individual cells in the somites could be be visualised, revealing protrusions in polarized cells (Fig2F-G). The imaging resolution provided by the membrane-localized fluorescent proteins was sufficient to segment individual cells in recombined clones in 3D for further quantitative analysis of cell characteristics (e.g. size, shape, circularity) or other parameters (Fig2H). Thus, Rosa26\(^{PRIME}\) allows efficient multicolour lineage tracing, direct visualisation of the cell shape of individual traced cells, and further segmentation and quantification of traced clones in the developing embryo. Of note, iRFP670 expression was unexpectedly but notably absent in vivo (Fig2C-E, Supplementary Fig 2A-E).
PRIME: A novel multicolour reporter or lineage tracing, cellular visualisation and cell segmentation

A) Axin2 CreERT2+/; R26 PRIME/+
E0.5  E7.5  E9.5
Plug  TAM  Analyze

B) Transmission

C) mScarlet
mTq2
mVenus

D)

E) mScarlet
mTq2
mVenus

F) 10 μm

G) 25 μm

H) Segmentation
Figure 2: Visualisation and multicolour lineage tracing during embryonic development. (A) Timeline experiments depicted in (B-G). Lineage tracing in Axin2+ cells was induced in utero at E7.5 by administering a single intraperitoneal injection of Tamoxifen to pregnant females. Embryos were isolated and imaged at E9.5 (n=5). (B) Wholemount confocal microscopy image (transmission) of an E9.5 embryo traced from E7.5. (C) Maximum intensity projection of a wholemount confocal microscopy Z-stack of the E9.5 embryo depicted in (B) showing the fluorescent detection of the recombined Rosa26PRIME allele. Clones expressing mScarlet-I, mTq2 and mVenus can be readily detected. Scalebar is 100um. (D and E) Maximum intensity projection of wholemount confocal microscopy images Z-stack of the somites from E9.5 embryos traced from E7.5, showing multiple traced clones expressing mScarlet-I, mTq2 or mVenus. Nuclear iRFP670 signal cannot be detected. Scalebar is 100um. (F and G) Close ups of the areas highlighted in (G). Membrane-associated PRIME fluorescent proteins clearly outline cell shape in embryonic tissue. Arrowheads in (F and G) indicate cell protrusions that are visualised by membrane-bound mTq2. Scalebar in (F) is 10um and in (G) 25um (h) Automated cell segmentation using CellPose and Napari of clone depicted in (G) based on membrane-associated mTq2 signal. Membrane-bound fluorescent signal from the Rosa26PRIME reporter can be used to readily segment individual cells and clones in 3D using wholemount confocal microscopy for further analysis. The embryonic timed matings and tracing experiments were performed in two independent litters.

Postnatally, we validated the functionality of the Rosa26PRIME allele when combined with a universal Cre driver (Rosa26CreERT2) and a lineage-specific Cre driver (Axin2CreERT2) in the small intestine. Rosa26CreERT2 is universally expressed in intestinal cells (Fig3A), whereas Axin2CreERT2 is a WNT/CTNNB1 responsive driver, and thus the strongest expression was found at the crypt bottom in the so-called crypt base-columnar cells (CBCs) (Fig3E).

Others have previously reported unbiased multicolour lineage tracing with a Rosa26CreERT2 driver to unravel clonal dynamics of stem cells in the absence of a defined stem cell marker. Hence, to validate use of the PRIME allele in combination with a universal Cre driver, we generated compound Rosa26CreERT2;Rosa26PRIME mice and labelled cells in adult double-heterozygous mice by a single intraperitoneal injection of Tamoxifen (Fig3A). Recombined cells were chased for 96 hours post injection, after which efficient labelling across the entire crypt-villus compartment could be detected in the small intestine (Fig3B). Using wholemount confocal microscopy, we could clearly distinguish individual cell shape and cell-cell interactions of mScarlet-I, mTurquoise2 or mVenus labelled cells (Fig3C). Individual cells can be readily segmented based on their fluorescent signal for further downstream analysis (Fig3D).

In double heterozygous adult Axin2CreERT2;Rosa26PRIME mice, Cre/lox mediated recombination of the Rosa26PRIME reporter allele in Axin2+ intestinal stem cells was efficiently induced by a single intraperitoneal injection of Tamoxifen (Fig3E). Recombined cells were traced for >12 months post Tamoxifen injection, after which the progeny of fast dividing, long-lived Axin2+ stem cells could be seen to populate the crypt and villus compartments (Fig3F). Prolonged tracing in Axin2CreERT2;Rosa26PRIME mice resulted in sustained labelling of mScarlet-I, mTurquoise2 and mVenus positive...
clones that encompass large sections of the crypt villus compartment (Fig3F) in formalin fixed paraffin embedded (FFPE) tissue sections (see methods for an optimized protocol to preserve endogenous fluorescence in this context), confirming that the PRIME allele can efficiently recombine, label intestinal stem cells and subsequently trace their progeny with a lineage specific Cre driver.

To validate the efficacy of the PRIME reporter allele in other postnatal tissues, we performed confocal microscopy on cryosections of other organs derived from traced Rosa26\textsuperscript{CreERT2};Rosa26\textsuperscript{PRIME} mice. We could not detect any signal in sections from brain (not wholly unexpected given that Rosa26\textsuperscript{CreERT2} has previously been described not to recombine in this tissue \textsuperscript{29}) and heart (Supplementary Fig 3). Recombined cells that express mScarlet-I, mTurquoise2 or mVenus, however, were readily visualised in sections derived from the liver, pancreas and spleen (Fig4A-E). Interestingly, in polyploid cells (present in the liver and pancreas) that express more than one copy of the PRIME allele more than one fluorescent protein can be detected resulting in a dual-colour signal (Fig4B,D).

Figure 3: Visualisation and multicolour lineage tracing in the small intestine. (A) Cartoon illustrating the localisation of Rosa26\textsuperscript{CreERT2} expression along the crypt-villus axis in the intestine. Timeline of experiments depicted in Fig3B-D and Fig4A. (B) Wholemount confocal microscopy image of the small intestine derived from Rosa26\textsuperscript{CreERT2};Rosa26\textsuperscript{PRIME} compound mice traced for 96h, showing endogenous fluorescent detection of the recombined Rosa26\textsuperscript{PRIME} allele in individual cells. Representative image is shown. Scalebar is 50um. (C) Close-up of the area highlighted in (B). Membrane-associated PRIME fluorescent proteins clearly outline cell shape and cell-cell interactions of individual cells in postnatal tissues. Scalebar is 50um. (D) Automated cell segmentation based on membrane-associated fluorescent signal in (C). Membrane-bound fluorescent signal from the recombined Rosa26\textsuperscript{PRIME} allele can be used to readily segment individual cells using wholemount confocal microscopy for further analysis. (E) Cartoon illustrating the localisation of Axin2\textsuperscript{CreERT2} expression along the crypt-villus axis in the intestine. Timeline of experiments depicted in (F). (F) Confocal microscopy image of FFPE small intestine section from >1 year traced Axin2\textsuperscript{CreERT2};Rosa26\textsuperscript{PRIME} compound mice, showing the endogenous fluorescent detection of the recombined Rosa26\textsuperscript{PRIME} allele. Multiple clones expressing mScarlet-I, mTq2 and mVenus are detected along the crypt-villus axis. Nuclear iRFP670 cannot be detected. Nuclei are counterstained with TOPRO3 (grey). Scalebar is 50um. N=2 Rosa26\textsuperscript{CreERT2};Rosa26\textsuperscript{PRIME} mice were traced and imaged, representative images are shown. N=1 Axin2\textsuperscript{CreERT2};Rosa26\textsuperscript{PRIME} mice were traced and imaged, representative images are shown.
Previously published multicolour lineage tracing reporters have been shown to not recombine in near-equal ratios under all conditions \(^{31}\) (published and personal experience). To assess the recombination frequencies of the \(Rosa26^{\text{PRIME}}\) reporter \textit{in vivo}, we quantified bottom-sections of the intestinal crypt from \(Rosa26^{\text{CreERT2}};Rosa26^{\text{PRIME}}\) mice that were traced for 96 hours (Fig4F-J). We chose the universal \(Rosa26^{\text{CreERT2}}\) driver to approach near equimolar Cre concentrations in all cells irrespective of cell type. Notably, cells expressing mScarlet-I and mTurquoise2 were detected in a near-equal ratio, 47% and 43% of all recombined cells respectively (Fig4G-H,J). mVenus was expressed in significantly fewer cells, 10% of all recombined cells (Fig4I-J). Similar results were observed in switched mESC\(^{\text{PRIME}}\) cells analyzed by FACS (Supplementary Figure 1D). Thus, the \(Rosa26^{\text{PRIME}}\) reporter can efficiently undergo stochastic Cre/lox recombination, however, this does not occur in near-equal ratios.

Finally, we established primary 3D mammary gland organoid cultures from \(Rosa26^{\text{CreERT2}};Rosa26^{\text{PRIME}}\) animals to test the functionality of the PRIME allele in and \textit{in vitro} 3D organoid model. Mammary epithelial fragments were plated in Matrigel and supplemented with FGF to induce branching \(^{32,33}\) (Fig5A). 4-OHT was added to the medium to induce Cre/lox mediated recombination and mammary gland organoids were subsequently traced for 3 or 6 days (Fig5A-B). In this setting, Cre/lox recombination was highly efficient, generating multiple clones per organoid (Fig5C). Similar to what we observed in embryos and adult tissues \textit{in vivo}, mScarlet-I, mTurquoise2 and mVenus positive cells were not detected in equal ratios, with fewer mVenus positive cells visibly present per mammary organoid (Fig5D-I). Again, the imaging resolution obtained by confocal microscopy of unfixed samples was sufficient to segment individual cells in 3D, generating a map of all progeny of recombined cells per organoid that can be used for further quantitative analysis of cell characteristics and cell tracking (Fig5E,G,I).
Figure 4: Visualisation and multicolour lineage tracing in postnatal tissues. (A-E) Confocal microscopy images of cryosections from Rosa26\textsuperscript{CreERT2};Rosa26\textsuperscript{PRIME} mice traced for 96h. (A) Confocal microscopy image of a pancreas cryosection, showing the endogenous fluorescent detection of mScarlet-I, mTq2 and mVenus from the recombinant PRIME allele in sporadic cells. Nuclei are counterstained with TOPRO3 (grey). Scalebar is 50um. (B) Close ups of the boxed areas in (A) showing recombinant polyplloid cells in the pancreas that express more than one fluorescent protein. Scalebar is 10um. (C) Confocal microscopy image of a liver cryosection, showing fluorescent detection of mScarlet-I, mTq2 and mVenus from the recombinant PRIME allele in sporadic cells. Nuclei are counterstained with TOPRO3 (grey). Scalebar is 50um. (D) Close ups of the boxed areas in (C) showing recombinant polyplloid cells in the liver that express more than one fluorescent protein. Scalebar is 10um. (E) Confocal microscopy image of a spleen cryosection showing the fluorescent detection of mScarlet-I, mTq2 and mVenus from the recombinant PRIME allele in sporadic cells. Nuclei are counterstained with TOPRO3 (grey). Scalebar is 10um. (F) Cartoon depicting experimental setup to analyse recombination ratio of Rosa26\textsuperscript{PRIME}, transmission wholmount confocal image of bottom intestinal crypt used to detect start of intestinal crypts, and a confocal fluorescent image of mScarlet-I, mTq2 and mVenus in the intestinal crypts 96h post tamoxifen induction. (G-I) Representative wholmount confocal microscopy images of Rosa26\textsuperscript{CreERT2};Rosa26\textsuperscript{PRIME} from the bottom section of the small intestine. Mice were traced for 96h and recombined cells were scored (n=3353 cells). Scalebar is 50um. (j) Percentage of cells expressing of mScarlet-I, mTq2 and mVenus after Cre mediated recombination. Recombination of the PRIME allele does not occur in near-equal ratios. 47.3% (n=1587) of recombintent cells express mScarlet-I, 42.8% (n=1436) express mTq2 and 9.8% (n=330) express mVenus. See methods for details on quantification.

In conclusion, Rosa26\textsuperscript{PRIME} allows for efficient multicolour lineage tracing, and direct visualisation of traced cells and their progeny in vivo and in vitro. The selected bright and photostable proteins should also make it excellently suitable for live imaging for extended periods in organoid models as lower laser powers can be used, thus limiting phototoxicity. Our Rosa26\textsuperscript{PRIME} mouse offers multiple advantages over existing models (Fig1A-B) and as such we expect it to be useful for investigations into (stem) cell dynamics during embryonic development and in adult tissues. The PRIME reporter contains next generation fluorescent proteins with optimal brightness, photostability and spectral compatibility. By adding a membrane tag to each fluorescent protein, we can detect the cell outline of the progeny of traced cells. This offers the additional advantage of visualising both cell shape, including the presence of fine protrusions, and cell-cell interactions. Moreover, the membrane signal generated by the PRIME reporter is sufficient to readily segment individual cells in 2D and 3D or further quantitative analysis.

We demonstrate that, when paired with either a lineage-specific or a universal Cre\textsuperscript{ERT2} driver, the PRIME reporter efficiently undergoes Cre/lox recombination in multiple tissues, allowing multicolour lineage tracing of cell clones in vivo at embryonic and postnatal stages of development (Fig2-4), as well as in vitro in organoid models (Fig5). At the same time, as previously reported for other multicolour lineage tracing reporters, recombination does not occur as expected (1:1:1), resulting in non-equal ratios of mScarlet-I, mTurquoise2 and mVenus.
expression (Fig4J, Supplementary Fig1D). This suggests that recombination efficiency differs depending on either the type of lox site, the distance between these sites, or a combination of the two. Of note, while we readily detected recombination in mouse mammary organoids in vitro, we unexpectedly only achieved very sparse mScarlet-I labelling of the mammary gland in vivo, either following tamoxifen injection in double-heterozygous Rosa26\textsuperscript{CreERT2};Rosa26\textsuperscript{PRIME} mice or upon intraductal Cre injection in homozygous Rosa26\textsuperscript{PRIME} mice (data not shown). For this we have no explanation, but we hypothesise that unique Cre/lox dose response curves exist for each lox site variant, and the final recombination result will thus likely be an interplay between Cre availability and Cre/lox affinity.

Our PRIME reporter incorporates two fluorescent proteins (mScarlet-I and iRFP670) that, at the time of generating the strain, had not been used in in vivo mouse models before. While mScarlet-I performed very well, we could unfortunately not detect iRFP670 positive nuclei in vivo or in mammary gland organoids, although we did detect weak signal in the targeted mESCs by both confocal microscopy and FACS (Fig1F, Supplementary Figure 1B-C). iRFP670 requires the co-factor biliverdin as a chromophore for its fluorescent signal, and while previous publications suggest that endogenous biliverdin is sufficient in cell lines for iRFP670 to fluoresce, this may not be the case in vivo. Biliverdin is a precursor molecule to Bilirubin and part of the Haemoglobin degradation pathway. Biliverdin concentrations in vivo are likely tightly regulated and perhaps too low to allow iRFP670 to fluoresce. We postulate that Biliverdin can be substituted to culture media in vitro before imaging, or incorporated in wholemount processing protocols to increase iRFP670 signal, although we did not explore this yet. We recommend that next generation mouse models incorporating a far-red nuclear protein use emIRFP670 instead and also incorporate a longer linker sequence.

Summarizing, our PRIME reporter offers straightforward multicolour lineage tracing and is expected to be compatible with live cell imaging, visualising cell shape and cell-cell interactions, cell segmentation in 2D and 3D and thus automated cell tracking in most (embryonic) tissues and organoids. As such, our model can be a useful tool for the scientific community to simultaneously visualise and trace multiple cells and their progeny both in vitro and in vivo.
PRIME: A novel multicolour reporter or lineage tracing, cellular visualisation and cell segmentation
Figure 5: Visualisation and multicolour lineage tracing in mammary gland organoids. (A) Cartoon depicting the experimental setup. Mammary gland fragments from uninduced Rosa26CreERT2;Rosa26PRIME mice were isolated and plated in Matrigel droplets and cultured in basal media with FGF. Organoids were induced with 4-OHT directly at plating or at day 3 and imaged at day 6. (B) Confocal microscopy image (transmission) of mammary gland organoid induced at day 3. Scalebar is 50um. (c) Maximum intensity projection of a confocal microscopy Z-stack of the organoid depicted in (B) highlighting mScarlet-I, mTq2 and mVenus fluorescent signal. Scalebar is 50um. (D) Maximum intensity projection of a confocal microscopy Z-stack of the mScarlet-I fluorescent signal from a mammary gland organoid induced at plating. Scalebar is 50um. (E) Automated 3D cell segmentation based on mScarlet-I membrane signal from (D) superimposed on the confocal microscopy transmission image of the mammary organoid from (D). (F) Maximum intensity projection of a confocal microscopy Z-stack of the mTq2 fluorescent signal from the mammary organoid from (D) Scalebar is 50um. (G) Automated 3D cell segmentation based on mTq2 membrane signal from (F) superimposed on the confocal microscopy transmission image. (H) Maximum intensity projection of a confocal microscopy Z-stack of the mVenus fluorescent signal from the mammary organoid from (D) Scalebar is 50um. (I) Automated 3D cell segmentation based on mVenus membrane signal from (H) superimposed on the confocal microscopy transmission image. For panels (B-I) a total of 15 organoids were imaged derived from 2 female mice.

Methods

Generation of PRIME targeting construct

The PRIME construct was assembled using standard cloning methods. The mTmG construct vector 13 (pCA-mTmG; Addgene plasmid #26123) was used as a building vector. The mTmG cassette was excised by BamHI digestion, leaving the CAG promoter and one LoxP site. LoxN and Lox2272 were ordered as single strand oligos and ligated into the vector. First, BglII-Lox2272-BamHI was inserted at a unique BamHI restriction site. Second, the backbone as opened via BamHI/SwaI digestion and an oligo carrying BamHI-LoxN-SwaI was inserted. The fluorescent proteins were inserted sequentially, together with a nuclear or membrane marker sequence and flanking Lox site. The sequences were ordered as IDT gBlock with flanking restriction sites unique to both vector and insert. Briefly, the pCAG vector was digested with BamHI/AflII and Lox2272-m-mTq2-polyA was inserted to generate pCAG-mTq2. Next, pCAG-mTq2 was digested with EcoRI/AflII to insert LoxN-m-mVenus-polyA thereby generating pCAG-mTq2-mVenus. pCAG-mTq2-mVenus was digested with Nhel/Agel and m-mScarlet-I-polyA was inserted to create pCAG-mScarlet-I-mTq2-mVenus. Lastly, pCAG-mScarlet-i-mTq2-mVenus was digested with BamHI/Xbal to insert 3xNLS-iRFP670-polyA-LoxP to finalize the PRIME construct (pCAG-iRFP670-mScarlet-I-mTq2-mVenus). In between cloning steps, the construct sequence was checked by Sanger sequencing and tested for functional Lox recombination by co-transfecting the vector and a Cre plasmid in HeLa cells.
To generate the Rosa26\textsuperscript{PRIME} targeting construct, pCAG-iRFP670-mScarlet-I-mTq2-mVenus was digested with AflII/SwaI to insert an FRT-PGK-Neo-FRT cassette thereby creating pCAG-iRFP670-mScarlet-I-mTq2-mVenus-Neo. Cloned constructs were transformed in chemically competent DH5a cells and grown at 37°C. Constructs larger than 10kb were transformed and grown in Stbl3 chemically competent cells (Fisher) and grown at 30°C.

To generate the Rosa26\textsuperscript{PRIME} targeting construct, the PRIME construct was digested with PacI/SwaI and blunt ligated into linearized pROSA26-1 (Addgene plasmid #21714) digested with XbaI. Single stranded overhangs after digestion were filled with T4 Polymerase before ligation. pROSA26-1 is the original targeting vector from the Soriano lab containing 1kb 5’ and 4kb 3’ homology arms \textsuperscript{12} (Supplementary Figure 4).

**Generation of Rosa26\textsuperscript{PRIME} mice**

Mouse embryonic stem cell targeting was performed at the NKI transgenic and knockout core facility. The targeting vector was linearized with KpnI and electroporated into B6\_colA\_RMCE mESCs. Briefly, individual colonies were grown on selective medium with neomycin and DT. Surviving colonies were picked and checked for correct integration of the construct by long range PCR and by Southern Blot for Rosa26 5’, and qPCR copy number analysis for the Rosa26 3’ side (Neo cassette). Correctly targeted ES\textsuperscript{Rosa26-PRIME/+} clones were expanded and injected into Go germline blastocysts. These were scored by palpation and crossed to generate heterozygous offspring. Mice were crossed with a FLP deleter strain to remove the FRT-Neo-FRT cassette to establish heterozygous offspring in the F1 and F2 generation.

**Mice**

All mice used for this study were maintained under standard housing conditions. Animals were housed in open or IVC cages on a 12h light/dark cycle and received food and water ad libitum. All experiments were performed in accordance with institutional and national guidelines and regulations approved by the Animal Welfare Committee of either the University of Amsterdam or the Netherlands Cancer Institute.

Rosa26\textsuperscript{PRIME} mice were backcrossed to C57BL/6JRccHsd (Envigo) or FVB/NHan®Hsd (Envigo). These mice have been deposited with Jackson Labs (RRID:IMSR_JAX:037018). Most experiments were performed on a mixed C57BL/6 and FVB background, unless noted otherwise.
Other strains used: Rosa26\textsuperscript{CreERT2} [mixed background, MGI:3764519] \textsuperscript{29} and Axin2\textsuperscript{CreERT2} [mixed background, RRID:IMSR_JAX:018867] \textsuperscript{7}

For timed matings, female mice were screened for the presence of a vaginal plug. When a plug was found, this timepoint was recorded as E0.5. For embryo isolation, pregnant dams were euthanized at the indicated timepoints and embryos were isolated for further processing. Yolk sacs were used for genotyping all embryos.

For lineage tracing, mice received a single intraperitoneal injection of 10-20mg/ml stock solution of tamoxifen in corn oil/10% ethanol. For embryonic lineage tracing a single injection was given to pregnant mothers at E7.5, totalling 0.5mg/25g body weight. For postnatal lineage tracing in Axin2\textsuperscript{CreERT2};Rosa26\textsuperscript{PRIME} mice a dose corresponding to 4mg/25g tamoxifen was given, and in Rosa26\textsuperscript{CreERT2};Rosa26\textsuperscript{PRIME} a dose of 1-5mg/25g tamoxifen. We verified that no leakiness (i.e., recombination of the Rosa26\textsuperscript{PRIME} reporter in the absence of tamoxifen) was detected in uninjected double heterozygous animals. As a negative control for imaging experiments, we used uninjected littermates, thus not showing recombination of the Rosa26\textsuperscript{PRIME} reporter.

\textit{PCR genotyping}

Ear clips or yolk sacs were lysed either overnight (ear clips) or for 2hr (yolk sacs) at 55°C in 200ul Direct PCR tail lysis buffer (Viagen) supplemented with 200ug/ml Proteinase K (20mg/ml stock solution). Proteinase K was inactivated by incubating the samples at 85°C for 45 min. Samples were cooled to room temperature and spun down (2min at 14,000 rpm), after which 1ul of supernatant was used as input for a standard 20ul PCR reaction with 0.4ul PhireII polymerase (ThermoFisher, #F-124S). PCR conditions were as follows: initial denaturation at 98C for 30s, followed by 30-35 cycles of denaturation at 98C for 5s, annealing at the relevant temperature for 5s, extension at 72C for 10s, followed by a final extension step of 72C for 1 min. Samples were cooled to 16C and analyzed on a 1-2% agarose gel in standard TAE buffer. Primer sequences, annealing temperatures, number of cycles and expected band size are detailed below:
To prepare cells for FACS sorting, cells were washed with pre-cooled HBSS containing 2% FBS twice before resuspension in ice-cold HBSS with 2% FBS. Cells were kept on ice until analysis an filtered using a cell strainer. Cells were analysed with a BD FACS AriaIII using endogenous fluorescent signal. Analysis was performed using FlowJo. mTurquoise2 was measured with a 407nm laser and emission was filtered using a bandpass (BP) 450/50nm filter. mVenus was measured using a 488nm laser and BP530/30nm filter. mScarlet-I was measured using a 561nm laser and BP582/15nm filter. iRFP670 was measured using a 633nm laser and BP660/20nm filter.

**Cell culture**

Mouse embryonic stem cells (mESCs) were cultured in N2B27 medium with 2i + LIF at 37°C and 5% CO2. N2B27 medium: one volume DMEM/F121 combined with one volume Neurobasal medium and 10mM Glutamax (Invitrogen), supplemented with 0.5% N2 supplement, 1% B27 supplement, 0.033% BSA 7.5% solution, 50uM B-mercaptoethanol, 2mM Glutamax, 100U/ml penicillin and 100ug/ml streptomycin (all Thermofisher). Medium was supplemented with fresh 1000U/ml LIF (Merck) and 2i: CHIR99021 (ITK Diagnostics) 3uM and PD325901 (Sigma) 1uM. Plates were coated with 0.1% bovine gelatin. Cells were transfected with a Cre expression plasmid (Addgene #13775) using either PEI or X-tremeGENE HP DNA (Roche) in a 1:1 ratio (1ug DNA per 1ul X-tremeGENE). A total of 2 independent transfection experiments were performed (experiment 1: 1 control, 4 Cre-transfections, analysed at 72 hours or 10 days post-transfection; experiment 2: 1 control, 2 transfection conditions, analysed at 11 or 16 days after transfection).
Primary mouse mammary organoid cultures

Mammary organoids (third thoracic and fourth inguinal) were harvested from uninduced Rosa26<sup>CreERT2</sup>;Rosa26<sup>PRIME</sup> virgin female mice. Mammary organoids were established according to published protocols<sup>32, 33</sup>. Briefly, fat pads were minced with scissors and transferred to a tube with 10ml collagenase/trypsin solution consisting of DMEM/F12/Glutamax (Gibco) supplemented with 0.02g collagenase type IV (Sigma-Aldrich C5138), 5ml Fetal Bovine Serum (Gibco), 250ul of 1ug/ml insulin (Sigma-Aldrich I6634) and 50ul of 50ug/ml gentamicin (Sigma-Aldrich G1397), and were incubated for 30min at 37°C shaking at 200rpm. The resulting suspension was centrifuged at 1500rpm for 10min, and then resuspended in 4ml DMEM/F12 + 80ul DNase (1U/ul) (Promega M6101). The DNase solution was gently shaken by hand for 2-5min, followed by centrifugation at 1500rpm for 10min. Four differential centrifugations (pulse to 1500rpm in 10ml DMEM/F12) were performed to separate single cells (including fibroblasts) from mammary gland epithelial fragments. Isolated epithelial fragments were mixed with 50ul Growth Factor Reduced Matrigel (Corning), seeded in an 8 well chamber slide pre-coated with 20ul Matrigel per well, and incubated for 30min at 37°C. After Matrigel polymerization, basic organoid growth media was added (DMEM/F12, 1% v/v insulin, transferrin, selenium (Gibco 41400045) and 1% v/v penicillin/streptomycin (Gibco, 100x stock). Organoids were cultured for 6 days in basic organoid medium supplemented with FGF either at plating of day 3. 1uM 4OHT was added to the medium for 2 days to induce Cre mediated PRIME recombination. Media was refreshed every 2-3 days and cultures were analysed at day 5 and 6.

Detecting endogenous fluorescence signal in FFPE tissue sections signal in FFPE

An FFPE processing protocol to maintain endogenous fluorescent signal was adopted from<sup>40</sup>. Isolated tissues were fixed in 4% PFA at room temperature for 2h on a roller bench. Next, tissues were dehydrated through a Tertbutanol (TBA) graded series. Note that Tertbutanol crystalizes at room temperature and thus should be placed at 55°C until liquified before starting the graded series. PFA was replaced with 35% Tertbutanol dilution in milliQ (Merck, #1096290500) and incubated for 2h at room temperature on a roller bench. 35% TBA was discarded and replaced by 50% TBA for 2h at room temperature on a roller bench. 50% TBA was discarded for 70% TBA overnight at 4°C on a roller bench. 70% TBA was replaced with 100% TBA and incubated for 2h at room temperature on a roller bench. As a last step, the samples were incubated with Histoclear II for 2h at room temperature on a roller bench. The isolated tissues were transferred to a 10ml bottle and filled with paraffin.
to soak the samples overnight at 55°C. Fluorescence is preserved up to at least 2 weeks after embedding (3 weeks post tissue isolation). After embedding, tissue sectioning can proceed as normal. We obtained better results when fresh sections (5um on SuperFrost plus slides, Thermo Scientific) were cut shortly before imaging. Deparaffinized and rehydrated slides were washed three times for 5 minutes with PBS and mounted with a coverslip using in Vectashield mounting medium H-1000 (Brunschiwig Chemie) and imaged within 24 hours.

**Cryosections**

Tissues were embedded in OCT and stored at -80°C. Tissues were sectioned with a cryostat in slices of 15um thick and left to thaw at room temperature for 20 minutes. A post-fixation with 4% PFA was performed for 25 minutes. Tissue sections were washed thrice with PBS for 10 minutes. Membranes were dissolved with 0.2% Triton X-100 in PBS for 2 hours. Post-permeabilization, the sections were washed thrice with PBS for 10 minutes and stained with TOPRO3 (Invitrogen, T3605) (1uM) diluted in PBS for 10 minutes. Tissues were mounted in Vectashield mounting medium H-1000 (Brunschiwig Chemie).

**Microscopy**

For wholemount confocal microscopy of embryos and tissues, samples were fixed in 4% PFA made from 16% PFA (methanol free ampules, Thermo Scientific) diluted in PBS for 1h at room temperature. Tissues were dehydrated through a graded glycerol series. Imaging of wholemount embryos and intestine was performed on a Leica SP8 confocal microscope. Fluorophores were excited as follows: mTq2 at 442nm, mVenus at 514nm, mScarlet-I at 567nm, and iRFP670 or TOPRO3 at 633nm. Emission was detected by Leica PMT or HYD detectors. Samples were imaged sequentially and all images were acquired using a 10x dry or 25x long working distance water objective.

Images of primary mammary gland organoids were captured using confocal microscopy on a Leica SP8 with LasX software. For imaging, the samples were cultured and imaged on a glass chamber slide (Ibidi). Mammary organoids were live-imaged in an Okolab stage-top incubator at 37C and 5% CO2 for extended periods of time. Fluorophores were excited as follows: mTq2 at 442nm, mVenus at 514nm, mScarlet-I at 567nm, and iRFP670 at 633nm. Emission was detected by Leica PMT or HYD detectors. Samples were imaged sequentially and all images were acquired using a 25x long working distance water objective.
For imaging tissue section (either cryo- or FFPE) slides, images were captured using confocal microscopy on a Leica SP8 with LasX software. Fluorophores were excited as follows: mTq2 at 442nm, mVenus at 514nm, mScarlet-I at 567nm, and iRFP670/TOPRO3 at 633nm. Emission was detected by Leica PMT or HYD detectors. Samples were imaged sequentially and all images were acquired using a 25x long working distance and 63x water objective.

**Microscopy image analysis**

Fluorescence microscopy images were processed in Fiji using the Image 5D plugin. Automated 2D and 3D cell segmentation was performed in the multidimensional image viewer software Napari (more specifically devbio-napari) 42. We used Cellpose2.0 43, 44, a generalist algorithm for cell and nucleus segmentation, to segment cells based on membrane PRIME signal. Modeltype Cyto2 was used to segment based on the membrane signal. If necessary, the model thresholding was edited manually for each individual image to achieve optimal segmentation. To achieve 3D cell segmentation we processed Z-stacks as 3D in napari. A stitch threshold of 0.1 was chosen to stitch segmented masks of individual slices within the Z-stack together to form a 3D segmentation mask for each cell in the Z-stack. Note: for 3D segmentation to work segmentation masks need to be detected in each individual slice of the Z-stack. Hence, prior processing in Fiji to manually remove slices in which no signal is detected is required.

Quantification of PRIME recombination efficiency in intestinal crypts was performed on *Rosa26CreERT2;Rosa26PRIME* wholemount tissue samples. Images were on a Leica SP8 with a 25x long working distance water objective for optimal endogenous fluorescence detection in thick tissues. The location of the crypt (bottom) was determined by the transmission signal, by identifying crypt morphology and granular Paneth cells. The intestinal samples were isolated 96h post Tamoxifen injection, allowing sufficient time to recombine and fluoresce mScarlet-I, mTurquoise2 and mVenus, however this timepoint also does allow cells to undergo a round of cell division and should be taken into account as a minor uncertainty in the results. Individual mScarlet-I, mTurquoise2 and mVenus positive cells were scored in Fiji by automatic drawing of circular ROIs to keep track of scored cells and the number of counted cells. The counted cells are plotted as the percentage of mScarlet-I, mTurquoise2 or mVenus positive cells in the total recombined cell population. Data was plotted as a boxplot. Individual datapoints represents the percentage of switched cells in the total population of recombined cells for each scored image (n=13, from 2 injected *Rosa26CreERT2;Rosa26PRIME* mice). Representative images for
each mScarlet-I, mTurquoise2 and mVenus are shown in Figure 4J.

Software, statistics and online databases

The following software was used: Fiji (https://imagej.net/Fiji/Downloads) for image analysis. R (R Development Core Team, 2017) and BoxPlotR to generate the boxplot in Figure 4J. Graphpad Prism (v6.01) for the boxplots in supplementary figure 1C-D, Napari and Cellpose 2.0 for automated cell segmentation in 2D and 3D. Final figures were compiled in Adobe Illustrator. For figure 1A, information was retrieved from http://www.findmice.org on the 8th of July 2022.

Author contributions


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Supplementary Figure 1: PRIME recombination in mESC cells. (A) Cartoon depicting the experimental setup. mESPRIME cells were plated and transfected with a Cre plasmid to induce recombination. At minimum 72 hours post transfection cells were sorted and checked for iRFP670, mScarlet-I, mTurquoise2 and mVenus expression. (B) FACS plot depicting iRFP670 expression in the control group versus Cre transfected cells 16 days after transfection. (C) Boxplot of FACS iRFP670 signal shown in (B) as a % of the total population of cells. (D) Boxplot depicting the recombination ratio in mES cells. The percentage of mScarlet-I, mTurquoise2 and mVenus expressing cells in the population of cell that has recombined as detected by FACS. Individual datapoints for two independent experiments are shown. Figure is directly adapted from A.A.A. van de Moosdijk: ‘Developing an analysing novel tools to study endogenous WNT signalling in mice’.

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Supplementary Figure 2: iRFP670 expression is not detected in embryonic and postnatal tissues. (A) Lineage tracing in Axin2+ cells was induced in utero at E7.5 by administering a single intraperitoneal injection of Tamoxifen to pregnant females. Embryos were isolated and imaged at E9.5 (n=5). Wholemount confocal microscopy image trace of an E9.5 embryo traced from E7.5. Maximum intensity projection of a wholemount confocal microscopy Z-stack showing clones expressing mScarlet-I (red) and mTurquoise2 (blue). iRFP670 cannot be detected in non-recombined cells (grey). (B) Individual iRFP670 channel from (A). (C) mScarlet-I and mTurquoise2 channels from (A). (D) Wholemount confocal microscopy of 4% PFA fixed and glycerol cleared small intestinal samples of Rosa26PRIME WT, HET and HOM mice showing transmission and iRFP670 signal. (E) Wholemount confocal microscopy of freshly isolated small intestinal samples of Rosa26PRIME WT, HET and HOM mice showing transmission and iRFP670 signal. (D-E) Representative images are shown. Images are derived from a single experiment.
PRIME: A novel multicolour reporter or lineage tracing, cellular visualisation and cell segmentation
Supplementary Figure 3: Brain and Heart tissue do not show recombination. Confocal microscopy taken from cryosections of the heart and brain. Magnification is either 25x or 63x. TOPRO3 nuclear staining is shown in grey. Panels depict representative images from 2 Rosa26<sup>CriERT2</sup>;Rosa26<sup>PRIME</sup> mice traced for 96h in a single experiment.
Supplementary Figure 4: PRIME targeting vector. Schematic showing the plasmid map of the PRIME targeting vector used to generate mESC_{PRIME} to make Rosa26_{PRIME} mice. Plasmid map was generated in SnapGene. Figure is directly adapted from A.A.A. van de Moosdijk: ‘Developing an analysing novel tools to study endogenous WNT signalling in mice’ 46.
Chapter 3

References


