The use of lineage tracing to study kidney injury and regeneration

Lineage tracing methods to study kidney injury and regeneration, their limitations and advantages

Paola Romagnani[1,2], Yuval Rinkevich[3], Benjamin Dekel[4,5]

[1]Excellence Centre for Research, Transfer and High Education for the development of DE NOVO Therapies (DENOTHE), University of Florence, Italy

[2]Pediatric Nephrology Unit, Meyer Children's Hospital, Florence, Italy.

[3]Stanford Institute for Stem Cell Biology and Regenerative Medicine, Stanford University School of Medicine, Stanford, CA 94305, USA

[4]Pediatric Stem Cell Research Institute, Sheba Medical Center, Tel Hashomer, Sackler School of Medicine, Tel Aviv University, Tel Aviv 52621, Israel

[5] Division of Pediatric Nephrology, Edmond and Lily Safra Children's Hospital, Sheba Medical Center, Tel Hashomer, Sackler School of Medicine, Tel Aviv University, Tel Aviv 52621, Israel

Corresponding authors:

Paola Romagnani
Department of Biomedical Experimental and Clinical Sciences "Mario Serio", University of Florence
Viale Pieraccini 6, 50139, Firenze, Italy
e-mail: paola.romagnani@unifi.it

Benjamin Dekel
Division of Pediatric Nephrology & Pediatric Stem Cell Research Institute, Edmond and Lily Safra Children's Hospital, Sheba Medical Center, Tel Hashomer, Sackler School of Medicine, Tel Aviv University, Tel Aviv 52621, Israel
e-mail: Binyamin.Dekel@sheba.health.gov

Key words:clonal analysis, lineage tracing,renal progenitors, stem cells, regeneration, podocyte, tubular cell, injury

Running title: ***Lineage tracing strategies for kidney regeneration***

Abstract

Lineage tracing strategies is proving to be a powerful approach for tracking cells *in vivo*, and is transforming our understanding of the cell fate changes that underlie kidney pathophysiology. The technologicaladvances in lineage tracing approaches is permitting novel spatial, temporal and kinetic resolutions into mechanisms responsible for tissue renewal and repair. In particular, lineage tracing has been used to investigate the possible role of stem/progenitor cells in kidney development, homeostasis and regeneration. Recently, the production of novel murine models where individual cells are tagged, assist in clarification of mechanisms of kidney regeneration and new answers to old questions are gradually being unraveled. The complexity of these genetically engineered systems requires careful analysis and interpretation. Caution must be paid particularly on the type of promoter, switch and reporter used, as well as on the induction times that are best to study the pre-specified end points.Here, we focus on the main points that need to be considered to interpret results obtained with lineage tracing, as well as on novel techniques that are becoming available, with a particular emphasis on their use for studying putative renal progenitors and the mechanisms of kidney regeneration.

Keypoints

- The principle of lineage tracing is based on marking a single cell in such a way that the mark is transmitted to the cell's progeny.
- Lineage tracing can provide information about the number of progeny of the founder cell, their location, and their differentiation status.
- The critical points to consider for an appropriate evaluation of the data obtained with lineage tracing should be three: the promoter, the switch and the reporter.
- The choice of the promoter should be optimized based on the cell type that has to be studied.
- The best possible promoter is one that is specifically and selectively expressed by the cell type analysed.
- For any kind of promoter system used, the expression pattern has to be verified prior to the actual cell fate tracing experiments.
- If the cell type marked by the promoter is unknown, negative results never allow definitive conclusions.
- The switch is usually a drug-regulated form of the bacterial enzyme Cre recombinase, which activates the transgene to allow turning on and off transcription of the reporter in a time-dependent manner.

- The use of lines that express Creconstitutively is problematic for lineage tracing, because transient or later expression of the gene in other cellsalso likely induces*de novo* activation of Crerecombinase, potentially altering results interpretation.

- Critical points to evaluate the results are also represented by the induction times used to study the pre-specified end points.

- Fluorescent reporters allow not only qualitative evaluation of phenomena but also quantitation and live imaging through dual or multiphoton microscopy.

- Use of multicolor reporters allows evaluation of cell division and clonal analysis and provides the most accurate genealogical descriptions of stem/progenitor behaviors.

Until recently, the behavior of cells *in vivo* could only be inferred indirectly, and consequently the basis of tissue biology in the kidney was ill-defined. However, lineage tracing in transgenic mice is proving to be a powerful approach for tracking cell fate *in vivo*, and it is transforming our understanding of the cell fate changes that contribute to kidney injury and regeneration. Traditionally used in developmental biology, lineage tracing has become an essential tool also in stem cell research, because it provides information about how the cell behaves in the context of the intact tissue or organism.[1-6] Tracking cells in their native habitat has provided insight into how the balance of proliferation and differentiation is achieved to an extent never before envisaged and thus, lineage tracing is increasingly applied to solve numerous scientific problems. In particular, the use of lineage tracing has provided unprecedented levels of information about clonal dynamics and the organization of the stem cell compartment in postnatal tissues[2-7] and has recently been used to investigate the possible role of resident progenitor/stem cells in kidney development, homeostasis and regeneration.[8-12]However, the inherent complexity of genetic recombination techniques and the increasing number of possibilities associated with this technology makes the interpretation of the results of these studies accessible only to experts. Several excellent reviews were focused on the technical aspects of lineage tracing.[13-15]In this Review, we focus on the main points that need to be considered to interpret results obtained with lineage tracing, with a particular emphasis on their use for studying putative renal progenitors and the mechanisms of kidney regeneration.

Lineage tracing: how it works The basic concept of lineage tracing is simple, and is based on marking a single cell in such a way that the mark is transmitted to the cell's progeny. To this aim, expression of a reporter gene, such as the enzyme β-galactosidase or a fluorescent protein, is switched on in a subset of cells in a tissue[16,17]. If the labelled cells divide, they pass on the expression of the reporter to their daughter cells, and form clusters of labelled cells, providing information about the number of progeny of the founder cell, their location, and their differentiation status.

To conduct a lineage-tracing experiment a switch that is usually a drug-regulated form of the bacterial enzyme Cre recombinase, must be engineered into the mouse genome. This enzyme recognizes specific sequences, called *LoxP* sites[16,17], that are not naturally found in the mouse genome, and are inserted only in

transgenic constructs used to create the transgenic line (**Fig.1**). Put simply, Crerecombinase excises the DNA between *LoxP* sites and rejoins the ends[16](**Fig.1**). To control Cre activity have been developed modified promoters that are active only in specific cell populations and allow Cre transcription exclusively in a drug-sensitive fashion, thus leading to reporter expression.[13-17]Already from this summary, it is clear that the critical points to consider for an appropriate evaluation of the data should be three: the promoter, the switch and the reporter.

The promoter By enabling the tracking of cells and their progeny at any time point, lineage tracing is nowadays the most reliable technique to determine the stem or progenitor nature of a cell. However, the choice of the promoter is an essential determinant of results. For example, using lineage tracing Humphreys *et al*proved that tubular regeneration after acute kidney injury is totally derived from intrinsic epithelial cells.[10] Indeed, in this elegant study, the authors employed a mouse line in which the Six2 promoter directly derived the expression of the Cre recombinase fused to the GFP.[10] By crossing it with a Rosa26-LacZ or RFP reporter line they obtained the reporter heritable expression.[10] The author were thus able to tag only $Six2^{+}$renal epithelial precursors, which are present in the metanephric mesenchyme (MM) during the developmental period of active nephrogenesis.[11] All the MM-derived nephron epithelial cells including tubular cells, but no extra-tubular cells, were thus marked.[10] The paper demonstrated that, following an ischemic injury, no dilution of reporter-marked cells was present, and that surviving tubular cells were positive for both the reporter and the proliferation marker Ki67, indicating that all reparative epithelial cells originated from within the MM-derived nephron.[10,18] The use of this promoter for tracing, however, does not discriminate between terminally differentiated epithelial cells and putative intra-tubular progenitors, since cells from which tubular structures of the nephron are derived are marked at the time of embryogenesis. In addition, the use of a constitutive system cannot completely exclude upregulation of the label within the tubules after development (as discussed in detail below). Thus, to finally dissect the contribution of potential intratubular progenitors to tubular regeneration, lineage tracing strategies based on other promoters were proposed. Indeed, Kusaba et al.[19] used the sodium dependent inorganic phosphate transporter, SLC34a1, as a marker to trace terminally differentiated proximal tubular cells. Based on the fact that there is no dilution of

fate marker after injury and repair, the authors concluded that progenitors do not participate in tubular regeneration.[19] However, this work which relies on lineage tracing of a cell population unified by a cell marker, does not take into consideration the possibility that intrinsic renal progenitors may express differentiated tubular markers to some degree. Indeed, expression of differentiation markers by tissue resident progenitors was reported in several organs.[20-24] Thus, the choice of a tubule differentiation marker for the promoter may not allow excluding the existence of tubular-committed progenitors since they would be genetically tagged similarly to differentiated tubular cells.

Interestingly , several studies have proposed the existence in adult human kidney of a putative population of tubular progenitors characterized by co-expression of surface markers CD133 and CD24. These cellscan co-express tubular markers although at lower levels than other differentiated tubular cells and localize within the tubule, scattered among differentiated tubular cells.[25-27]To trace these putative tubular progenitors,Berger *et al.*[28], used a conditional mouse originally designed to reproduce the expression pattern of the endogenous podocalyxin gene within podocytes.[8] In this mouse, transgene expression was unexpectedly detected within parietal epithelial cells of the Bowman's capsule but not within podocytes.[8] This allowed tracing the migration of parietal epithelial cells within the glomerular tuft and demonstrate that these cells can act as podocyte progenitors during kidney development[8].In further studies, the same authors demonstrated that parietal epithelial cells represent a major constituents of sclerotic lesions in focal segmental glomerulosclerosis and,together with podocytes,[29], in crescents[30,31]. In a more recent article, Berger *et al*. proposed that committed podocytes are recruited from Bowman's capsule even after birth, and that this represents a committed intrinsic "podocyte reserve", which can be recruited to allow glomerular growth[32] and may be particularly active in human[33].Interstingly, Sagrinati *et al*. had described a progenitor potential of parietal epithelial cells in human.[34-38]Of note, in human, parietal epithelial cells of the Bowman's capsule appear as undifferentiated at the urinary pole[38] and as podocyte-committed in more proximity to the vascular pole[38] and, like putative tubular progenitors, are characterized by co-expression of CD133 and CD24.[34-38]. In their study, Berger *et al.* assumed that, similarly to CD133, their mouse may tag tubular progenitors in addition to parietal epithelial cells and then described a lack of amplification of tagged tubular cells following acute kidney injury.[28]However, at difference with cells expressing CD133 in human kidney, that were previously reported to behave as progenit*ors in vitro* and following their transplantation in models of

acute tubular injury *in vivo*,[26,34-41] the scattered tubular cells in the transgenic mouse described by Berger *et al.*were not functionally studied.[28]In this mouse, multiple different cell types in addition to parietal epithelial cells are tagged, and the progenitor nature of the tagged scattered tubular cells is hypothesized based on expression of four markers that are shared by CD133+ human renal cells as well as by mouse parietal epithelial cells of the Bowman's capsule in addition to other cell types. Of note, the distribution of tagged tubular cells is also different from that reported for the CD133+ human tubular progenitors, that are mostly localized in the S3 segment and distal tubule,[26,34] while tagged cells in this mouse mostly localize in the S1+S2, as well as S3 segment.[28]. Since it is unknown if tubular progenitors in this mouse are really tagged, the absence of their amplification in response to injury does not allow to conclude about their existence and role. Thus, as a general concept, if lineage tracing strategies demonstrate self renewal and differentiation capacity of the tagged cell population, one can conclude positively on the existence of a progenitor population. However, if the cell type marked by the promoter is unknown, negative results never allow definitive conclusions. Thus, the questions if tubular progenitors exist in the mouse and are involved in tubular regeneration remain open. This further underlines how critical is the choice of the promoter used for lineage tracing. For all these reasons, to draw conclusions about putative tubular progenitors, models that exploit physiologically expressed genes at single cell resolutions that are progenitor-specific and which, consequently, may unify human and mouse studies, are mandatory.

In fact, human stem cell studies usually rely on prospective isolation based on specific markers followed by functional analysis including *in vivo* transplantation assays).[34-41]Accordingly, in the human fetal kidney and Wilms tumor, Ncam1 and Aldh1 (especially the Aldh1a2 paralogue),[42-47] CD133 and CD24[48] have been put forward as stem/progenitor cell markers in early mesenchyme ($Six2^{+}$) and derived epithelial precursors ($Six2^{-}$).[42-48]In the human adult kidney, CD133, CD24 and Aldh have been suggested to mark putative*in situ* epithelial progenitor populations while Ncam1 down-regulated after completion of nephrogenesis and reactivated in culture and following injury,[49]delineated aclonogenic cell subset that exhibited de-differentiation, epithelial-to-mesenchymal transition characteristics converting to a stem cell-like state.[49] Lineage tracing based on such markers may define a role in tubular homeostasis and regeneration for progenitors similar to the ones described in human. A prerequisite for this analysis is that a given marker/promoter would share homology in humans and mice, similar mRNA or protein levels and similar

188 expression domain. This could be relevant to Ncam1/Aldh1a2 and hence generation of conditional
189 Ncam1/Aldh1a2-CreERT2 mice would clarify a role in development and in adults. In contrast, markers such
190 as human CD133 cannot be used in the mouse kidney.[50] Indeed, human CD133 was first isolated from
191 hematopoietic stem cells by a monoclonal antibody recognizing a specific epitope designated as AC133.[51,52]
192 Once included in the Cluster of Differentiation nomenclature (CD), it was thus classified based on the name
193 of the epitope (CD133).[51,52] CD133 currently serves as a useful marker for the isolation of many different
194 types of stem and progenitor cells in adult human tissues, even for clinical purpose.[51,52] However, antibodies
195 that recognize portions of the human CD133 protein different from the AC133 epitope do not specifically
196 recognize stem cells, but are rather expressed by many differentiated epithelia.[51,52] Recent results suggest that
197 only antibodies that recognize epitopes localize in the second extracellular loop, like the AC133 and 293C3
198 clones used to identify human renal progenitors,[50] are suitable for stem cell and progenitors recognition.[51,52]
199 Although for these reasons use of CD133 mRNA or gene to detect renal progenitors in the mouse is currently
200 not possible,[34,50]a lineage tracing approach that uses a promoter that is co-expressed with CD133 on renal
201 progenitors may be suitable. Future studies are required to address this possibility. Importantly, there may
202 exist the opposite situation in which a murine marker such as Sca1, previously shown to identify an MSC-
203 like cell population in the adult mouse kidney),[49] has no homologous protein in humans, precluding
204 transplantation assays with counterpart human cells. Therefore, for any kind of promoter system used, the
205 expression pattern has to be accurately chosen and verified prior to the actual cell fate tracing experiments.

206

207 ***The switch*** The power and specificity of lineage tracing is in the variety of 'switches' that are now available
208 to allow a temporal control over the activity of the Crerecombinase. Indeed, it must be underlined that the
209 simple use of a cell-specific promoter that directly drives Cre expression without the use of a switch that
210 allows turning on and off of Crerecombinasetranscription over time, can alter the results obtained with
211 lineage tracing. Indeed, transient expression of the gene in another cell population, or later expression in cells
212 of the tissue under study can induce *de novo* activation of Cre recombinase in a way that a part of the newly
213 generated cells won't be derived from initially tagged ones. This *in vivo* genetic fate mapping is frequently
214 confused with lineage tracing analysis and cannot give conclusive results, and often leads to over

interpretation of the data. For example, Sakamoto et al.[53] concluded, in a model of FSGS, that podocytes transit into a parietal epithelial cell phenotype[53]. However, in the constitutive mouse model used in this study, upregulation of podocyte markers may have occurred in whatever moment in parietal epithelial cells leading to Crerecombinase activation and cell tagging, and thus the labelled cells may also represent parietal epithelial cells differentiating into podocytes.[53] For this reason, lineage tracing needs to be based on temporal regulation of marker gene expression. To this aim,inducible models have been created, also termed conditional models. Cell-specific promoters have been modified with regulatory elements that control Cre expression only when activated by specific exogenous molecules. As a consequence, the Cre recombinase will recombine the DNA in the desired cells that express the marker gene, only during the administration of the inductor molecule, thus opening a transient window in which cells may be labeled. Upon withdrawal of the molecule, no other cell that has not been labeled during the administration window can undergo genetic recombination, neither if it express the cell-specific promoter. Conditional assays principally rely on Tamoxifen-regulated systems ($CreER^T$) (**Fig. 1A**) or on Tetracycline-regulated systems (**Fig. 1B**). Tamoxifen-based systems are built to constitutively express an inactivated form of Crerecombinase fused to a modified Estrogen Receptor (ER^T) (**Fig. 1A**). When tamoxifen binds to the ER the protein can modify its structure and enter the nucleus, gaining access to DNA, where activated Cre may recombine (**Fig. 1A**). In Tetracycline-controlled systems the cell-specific promoter drives the expression of a reverse tetracycline-controlled transactivator (rtTA) (**Fig. 1B**). When this protein is ligated by administered Doxycycline, it will bind DNA sequences of Tetracycline-controlled transcriptional activation-elements (Tet), which in turn drives Cre expression (**Fig. 1B**). Cre will than act on the reporter transgene by ligating loxP elements (**Fig. 1B**). An interesting comparison of how different the result can be when obtained with constitutive versus conditional mice can be observed in two recent studies by Pippin *et al*., who used the renin gene as a cell-specific promoter in order to label and trace cells of the juxtaglomerular apparatus.[54,55] The authors demonstrated that renin expressing cells have regenerative capacity and are able to replace podocytes as well as parietal epithelial cells in a mouse model of FSGS, suggesting that they may serve as upstream mesenchymal progenitors for both parietal epithelial cells and podocytes.[54] In both of these studies, the authors used a constitutive model in which renin drives the recombination that leads to fluorophoreZsGreen expression, within cells.[54,55]Using this fate mapping approach, the authors reported an increase in cells of

renin lineage in the intraglomerular compartment at 14 days after injury, with a subset that coexpressed the podocyte markers nephrin, podocin and synaptopodin, indicative of a podocyte-like marker phenotype.[54]Since any cell population that had expressed renin, in any moment of animal life, would result as genetically tagged, the use of a constitutive model here, limites our interpretations into whether the renin-positive population contributes to podocyte replenishment. Moreover podocytes can express renin under some circumstances, which could be an alternative explanation for for the co-expressions of podocyte markers and fluorescence signal.[56-58] In one of the two studies[54] the authors also used a conditional mouse system to perform lineage tracing of renin cells, and similarly concluded that these cells can act as podocyte progenitors.[54]However, when traced with the conditional mouse in the same FSGS model, renin expressing cells that derived from podocytes appeared to be extremely rare, more than ten fold less than with the conditional mouse.[54,55]These studies reveal that these methodological aspects can have strong impact on data interpretation and conceptual conclusions.

Interestingly, a recent study by Starke *et al* [9]further analyzed the hypothesis that cells of renin lineage act as precursors for other renal cells by using an inducible mouse model that labeled renin cells with β-gal. [9] In that paper, the authors demonstrated that two-thirds of the glomerular tufts became β-gal positive following mesangial injury and intraglomerular renin descendant cells colocalized only with mesangial but not with endothelial, podocyte, or parietal epithelial cell markers, suggesting that renin cells can act as precursors for mesangial cells, but not for podocytes.[9] Thus, further studies are required to effectively address the contribution of renin cells to podocyte replenishments.

Although the use of a time dependent switch is an essential pre-requisite for a correct lineage tracing experiment, further important caveats should be considered when designing such an experiment and interpreting results. First of all, it has to be considered that the doses of the drug given to the animal will affect the analysis, and thus must be chosen depending on the experimental purpose. Indeed, low or sub-optimal administration will label the population of interest at clonal density, and it is therefore used to study origins of particular cell population, as demonstrated by Rios *et al*.[3] High doses will instead maximize the labeling of the entire stem/progenitor pool, enabling the visualization of their cumulative contributions within the entire compartment.[13,14] Second, an important limitation for conditional experiments can be leakiness of the system: where there is residual recombination even in the absence of induction. Because of

this limitation, multiple control groups must be estimated in all experimental conditions, and various tissues must be evaluated. Additional limitations include, transgene constructs that may spontaneously silence and lead to under representation of data.Cell fusion events may also occur *in vivo* in a number of tissues[59]leading to spreading of fluorophore expression, and to false interpretations of multi-lineage contributions.[13,14,60]Third, it must be considered that drugs like tamoxifen or doxycycline can be given through different routes of administration, like intraperitoneally, by chow or drinking water, introducing a variability in results, depending on timing, health conditions, age, weight, and more. Finally, mosaic activation of cre recombination can occur. Indeed, ideally, the reporter gene is driven by a ubiquitously active promoter or gene locus. Unfortunately, no such promoter has been identified so far. Viral promoter fragments usually are transcriptionally active in a mosaic fashion, and the ROSA26 locus is transcriptionally active at a low level in most cells but it may be transiently down-regulated in diseased tissue."

All these considerations, highlight how challenging lineage tracing experiments can be, and how an accurate choice of the switch and of its timing, are critical determinants of the results and of their interpretations.

The reporter The third critical ingredient for a lineage tracing experiment is the choice of the reporter. Traditionally, the *Escherichia coli* lacZ gene, β-galactosidase, was one of the first reporters used for lineage tracing and has been used extensively.[13,14]β-galactosidase produces an intense blue color when incubated with the substrate analog X-gal, but is technically difficult and can be variable. Alternatively, beta-gal can be visualized using immunofluorescent stainings, although with such methods podocytes may show low-level background staining.[13-15] Fluorescent reporters have become the new standard, and in this case detection by epifluorescence is far superior to antibody-enhanced methods, which are subject to nonspecific binding of antibodies and also raises the possibility of imaging clones of cells in living tissue through the use of multicolor reporters. Such dynamic analysis of cell-fate decisions is facilitated by advances in imaging, such as two- or multiphoton fluorescence microscopes, which enable in-depth scanning and optimal fluorophore separation of the multicolor fluorescence, as recently illustrated by an elegant study that used this approach to visualize podocyte response to injury.[61] In multicolor reporters, attention should be paid to the use of lines that express Cre constitutively because a change in cell color (“flipping”) may occurs even after the initial Cre recombination. Therefore, multicolor reporters are optimal when used in combination with inducible Cre

models. The high choice of different reporters that are becoming available is now one of the major strengths of lineage tracing approaches, but can also limit comparisons of results and their interpretations. For instance, the sensitivity to Cre-mediated recombination of different reporter alleles targeted to the *Rosa26* locus can vary substantially. Some *Rosa26*-targeted reporters will respond efficiently to Cre but can show leaky expression (background expression) in the absence of the Cre-inducing drug.[13-15] Others are less efficient, and so have less background.[13-15]These issues are crucial when comparing studies in which Cre is being used to report gene expression or signaling activity, as the proportion of cells labeled may reflect the reporter that is used as much as the level of Cre activity.[13-15] However, a careful choice of the reporter may even allow sophisticated quantitation of regeneration after injury processes. To this aim, one of the most used reporter genes is the *mT*/*mG*(*membraneTomato*/*membraneGreen*) transgene, which is characterized by a constitutive expression of the membrane bound fluorescent Tomato Red protein in all the cells of the animal body (**Fig. 2A**). As the sequence encoding the Tomato Red is floxed by two loxP site, only cells that will undergo recombination events will be able to express the membrane bound GFP which is downstream of Tomato Red, thus turning red into green (**Fig. 2A**). One example of a paper which used this system is Wanner *et al.*that studied podocyte turnover and regeneration in aging, in a unilateral nephrectomy models and following acute podocyte loss.[62] To study severe podocytes depletion the authors employed a quadruple transgenic model in which the podocyte marker Podocin conditionally induced the shift of the *mT*/*mG* transgene present on one *Rosa26* allele, while inducing the expression of the human simian Diphtheria toxin receptor (iDTR) transgene, codified under the other *Rosa26* allele (**Fig.2**).[62] Using this methodology,podocytes were tagged in green and were induced to express the iDTR following doxycycline administration, making them susceptible to a specific ablation as a consequence of the Diphtheria toxin injection.[62] With this approach, newly generated podocytes can only be red colored due to the withdrawal of doxycycline before the onset of the damage.[62]While this approach enables quantification of *de novo* generated podocytes, it does not permit to identify the source of the novel podocytes population, neither does it allow to establish the origins or identities of the progenitor population.[62] By employing this transgenic mouse model the authors reported, at 4 week from Diphtheria toxin (DTA) injection, a significant increase in the percentage of red-positive (newly generated) podocytes after ablation (**Fig. 2B**), corresponding to regeneration of 38% of lost podocytes, while after nephrectomy and in an aging model no increase in

Tomato Red^{+}podocytes was reported.[62]Interstingly, the DTA model is the only one that is characterized by podocyte depletion, while nephrectomy and aging do not necessarily lead to podocyte loss. This suggests that podocyte regeneration may occur only after podocyte detachment or death.

Even if genetically modified mice with inducible recombination under different promoters have been used for *in vivo* fate mapping in the kidney, these analyses were performed on the population level, and not on individual cells. As such, cellular models for kidney development, physiologic tissue maintenance, and regeneration *in vivo* remained open questions. To this aim, multicolour reporters, that enable combinatorial expression of multiple fluorescent proteins in a stochastic manner under the control of a specific promoter,like the 'Brainbow' (**Fig. 3**), 'Rainbow'or 'Confetti' constructs, have opened a new wide range of possibilities.

Rinkevich *and*Dekel.,[12] have recently utilized such a genetic marking strategy for lineage tracing of individual renal precursors in the adult mouse kidney. This strategy entails the use of a multicolor (red, yellow, green, blue) Cre-dependent reporter construct within the ROSA locus (*R26*$^{VT2/GK3}$, termed 'Rainbow'), in addition to a lineage tracing strategy that is independent of candidate markers (using an inducible Cre-ER fusion protein under the ubiquitous Actin promoter). *Actin*^{CreER}; *R26*$^{VT2/GK3}$offspring injected with tamoxifen, induced cytoplasmic fusion protein to enter the nucleus and permanently recombine a random single color-encoding gene in all renal epithelial cells, regardless of their locations, marker gene expressions or developmental potentials.[12] Using this unbiased assay, clonal analysis of individual cells over a 7-month period was performed, revealing the emergences of unipotent clones of three major types (proximal, distal tubule, and collecting tubules) each of which contributed to individual tubule segments over that tracing period[12] (**Fig. 4A**).Because of the cellular density of the kidney tissue, clonal analysis using a 4-color reporter construct may mask cellular contributions from adjacent and similarly colored cells to these clones.[12]To circumvent this possibility, Rinkevich *and* Dekel., have employed a protocol using single and low doses of tamoxifen. In these experiments, the tissue frequency of recombination was low enough (<1%) as to sparsely label single cellswith individual fluorescent colors within large and non-colored kidney domains.[12] These low-dose experiments demonstratedthat large and single colored clones are outputs of individual cells that generate and regenerate the kidney epithelia[12] (**Fig. 4B, white arrows**). Thus, using a ubiquitous, unbiased promoter (Actin), clonal analysis helped identify a distinct mode of renal epithelial cell

355 turnover in maintenance and following ischemia and toxic AKI.[12] Long-term lineage tracing *in vivo* revealed
356 significant tubulogenesis has occurred within the mature kidney epithelia that produce slowly expanding
357 clonal foci with segmental boundaries. To visualize the full sizes and distributions of clones Rinkevich *and*
358 Dekel isolated intact nephron segments from *Actin*^{CreER}; *R26*$^{VT2/GK3}$ mice that were chased for 7-months[12]
359 (**Fig. 4, C, D**). Intact tubules exhibited large epithelial clones within individual segments indicating that cells
360 with lineage-restricted capacities and progenitor characteristics reside within tubule segments of the
361 nephron.[12] Following this 7-month clonal analysis, large clones also appeared within multiple glomeruli (**Fig.**
362 **4, E-G**), indicating that both adult tubules and glomeruli house active clone forming cells.[12]

363 Another system that is employed for lineage tracing and clonal analysis during embryonic development
364 involves the creation of multicolored chimeric mice, termed tetrachimera mice.[63] These mice are generated
365 by injecting mouse embryonic stem cells that express separate fluorescent proteins (GFP-mES, RFP-mES,
366 CFP-mES) into wild-type blastocysts.[63] Kidneys from generated tetrachimera mice revealed that mature
367 nephrons were polyclonal,indicating derivation during development from mixed contributions of clones,
368 each of which contributed to individual tubule segments and to individual regions within nephrons[63] (**Fig.**
369 **4H**).

370 Clonal analysis can further be used in the context of generating renal organoids. Following our previous
371 success in establishing long-term kidney sphere cultures from single cell suspensions of human patients,
372 recapitulating renal features and *in vivo* renal capacity ,[42,64]Rinkevich et al.[12]recently used the Rainbow
373 system to investigate the *in vitro* fates from individual renal precursors, by establishing a culture system of
374 growing murine renal epithelial organoids in suspension.[12] In this assay, kidneys were harvested from
375 *Actin*^{CreER}; *R26*$^{VT2/GK3}$ mice immediately after a tamoxifen administration regimen, dissociated into single
376 cells, and plated with matrigel. Within several days of culturing, monoclonal renal organoids developed from
377 individual cells that gradually enlarged, and then opened into hollow spheres resembling renal tubes *in*
378 *vivo*.[12] Similar to in vivo observations, clonal progeny developing within renal organoids maintained segment
379 identity. When combined with isolation of renal cell fractions using flow cytometry, This type of approach
380 allows the identifications of *in vitro* clonal efficiencies as well as progenitor frequencies within isolated
381 FACS-sorted renal fractions[12] (**Fig. 4, I-I''**).

The same study also examined the clonal capacities of Wnt-responsive cells by using a Wnt reporter *Axin*^{CreER}that expresses the Cre-ER fusion protein under the promoter of the *Axin* gene; which provides negative feedback in the Wnt-β-catenin signaling pathway.Single cell analysis was performed on the population of renal cells that where Wnt responsive by crossing *Axin2*^{CreER} mice to 'Rainbow' reporter mice.[12] *Axin2*^{CreER}; *R26*$^{VT2/GK3}$ that were lineage traced from e17.5 up to the 5th postnatal months showed single colored and large clones within the adult tubules, indicating that they derived from individual Wnt-responsive precursors[12] (**Fig. 4J**). The authors performed a comparison of clone size between the unbiased clonal analysis (*Actin*^{CreER} promoter) and clonal analysis using Wnt-responsive cell fractions (*Axin*^{CreER}promoter).[12] Their analysis suggests that Wnt-responsive cells (WRCs) are cells with significant proliferative capacity, behaving *de facto*as long-lived unipotent progenitors when an appropriate stimuli to clonally expand/self-renew is received.[12]

Consistent with this, Barker *et al*[7]has identified cells within developing kidney (following epithelial induction) that express LGR5^{+}(a Wnt co-receptor).The authors crossed *LGR5*^{CreER} with a similar multicolor (red, yellow, green, blue) Cre-dependent reporter construct within the ROSA locus (the Confetti reporter) and found individual LGR5^{+}cells (most likely Wnt responsive cells) as the immediate progenitors that generate the thick ascending limb of Henle's loop and distal convoluted tubule.[7]Despite the fact that LGR5 is silenced at postnatal stagesof development and fails to trace Wnt-responsive cells in the adult, both reports demonstrates that constant tubulogenesis is occurring within the mammalian kidney via a similar mechanism involving Wnt-responsive precursors giving rise to other cells.

Taken altogether, the *in vivo*clonal analysis demonstrates that lineage-committed cells with progenitor characteristics continuously maintain and self-preserve the mouse kidney throughout life and after AKI.

An outlook on emergingmethodologies In the most recent years, novel techniques emerged that can potentially solve many of the ongoing questions and debates about kidney regeneration. The recent observation that adult progenitors expressing low levels of differentiation markers may be involved in regeneration of adult tissues was for example recently addressed in the brain by using Split-cre. Such a strategy, which can allow lineage tracing of a specific population in response to a combination of two

reporters, can be achieved by expressing the two fragments of a split Cre recombinase from two different tissue-specific promoters.[65,66] This lead to the characterization of different types of progenitors in adult mouse brain based on co-expression of CD133 and neural-specific markers.[22-24,67]Fate mapping experiments based on the overlapping activity of two promoters can also be performed by using two independent recombinases (Cre and Flp) driven by two promoters combined with a special reporter allele.[67] This system has been used to identify and analyse neural progenitors in several brain regions.[23,24,68] These strategies may be applied also to address the existence and potential role of renal progenitors in the mouse kidney.

Although lineage tracing techniques hold the potential tochange our knowledge of kidney biology, they add to their complexity being time consuming and highly costly. Indeed, genetic manipulation in mammalian species are usually made in the germline of an organism, which can then be used to create a stable transgenic strain for experimentation.[69] Recently, howver, the RNA-guided endonuclease Cas9 from microbial type II CRISPR (clustered regularly interspaced short palindromic repeat) systems (previously referred to as Cas5 or Csn1)[70-78] has been harnessed to facilitate genetic manipulations in a variety of cell types and organisms (reviewed in Hsu et al., 2014).[77] A major advantage in CRISPR/Cas gene editing is that reactions can be multiplexed to introduce multiple genome modifications in a single step.[77,78]Cas9 can be easily reprogrammed using RNA guides to generate targeted DNA double-strand breaks (DSBs), which can stimulate genome editing via one of the two DNA damage repair pathways: nonhomologous end-joining (NHEJ), resulting in insertions and deletions (indels) or homology-directed repair (HDR), resulting in precise sequence substitution in the presence of a repair template.[77,78] Unlike other programmable nuclease systems used for genome editing, a unique advantage of the Cas9 system is that Cas9 can be combined with multiple single-guide RNAs (sgRNA). However, commonly used delivery systems based on lentiviral and adeno-associated viral (AAV) vectors have limited packaging capacity which renders it challenging for incorporation of Cas9 along with sgRNA expression cassettes and necessary genetic elements (i.e., promoters, fluorescent proteins, and polyadenlyation sequences). Thus, to facilitate broader applications of CRISPR-Cas9, Platt et al. generated a Cre-dependent Rosa26 Cas9 knockin mouse to overcome the delivery challenges associated with Cas9 (**Fig. 5**).[79]Cas9-expressing cells derived from the constitutive Cas9-expressing mice facilitate genomeediting because it requires only the introduction of sgRNAs, which can be efficiently delivered using viral and nonviralsgRNA delivery methods.[79]This mouse can be used in

conjunction with a variety of guide RNA delivery reagents, including to facilitate genome editing in multiple tissues in vivo (**Fig. 5**). [79]In vivo as well as ex vivo genome editing using adeno-associated virus (AAV)-, lentivirus-, or particle-mediated delivery of guide RNA in neurons, immune cells, and endothelial cells was reported.[79] More importantly, Platt et al. delivered a single AAV vector in the Cre-dependent LSL-Cas9 model to generate loss-of-function mutations in the tumor suppressors *Trp53* (*p53*) and *Lkb1*, and homology-directed repair modification of *Kras* to oncogenic *KrasG12D* simultaneously.[79] Intra-tracheal delivery of this vector, which also carried Cre and a luciferase reporter sequence in addition to the sgRNA and *KrasG12D* homology repair templates, resulted in adenocarcinomas in 100% of the infected animals.[79] These data suggest that the conditional LSL-Cas9 mice provide exciting new tools to perform targeted genomic manipulation in traditionally challenging cell types *in vivo* and *ex vivo*, and to generate novel and fast strategies for lineage tracing which hold great promises for regenerative studies.

Conclusion

Lineage tracing strategies are powerful tools that are now growingly applied to understand the physio-pathological mechanisms that govern kidney regeneration and disease. Novel options to enhance the system and its possible applications are continuously becoming available, further increasing its possible applications. Caution must be paid particularly to the type of promoter, switch and reporter used, as well as on the induction times that are best to study the pre-specified end points. Even with these caveats, lineage tracing strategies are making accessible to our knowledge a wide range of processes, including kidney regeneration, which can now be directly visualized for the first time. Lineage tracing methods at single cell resolutions rather than cell populations would provide the most accurate genealogical descriptions of stem/progenitor behaviors. Consequently, novel types of murine models where renal progenitors are specifically tagged are being produced, and novel techniques to reduce the complexity and costs of lineage tracing experiments are appearing.Gradually,mechanisms of kidney regeneration are being clarified and new answers to old questions are gradually being found. These approaches and their use will allow the identifications and complete characterizing of all progenitor populations within the kidney and their contribution to kidney regeneration with unprecedented resolution and accuracy.

Acknowledgements

This manuscript is supported by the European Community under the European Community's Seventh Framework Programme (FP7/2012-2016), grant number 305436 (PR), The Israel Scientific Foundation Grant Award 910/11 and the NephroTools Marie Curie Program FP7 (BD).

Competing interests

Paola Romagnani is author of two patents on human renal progenitors (patent numbers: PCT/EP2007/054132 and F12013A000303) that are property of the University of Florence as well as of the public paediatric Meyer Children's Hospital.

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LEGEND FOR FIGURES

Figure 1 Cre-recombinase-based lineage tracing system. Schematic representation of two of the most common strategies used for lineage tracing. The genetic background of one animal is altered such that a specific promoter is used to induce Cre recombination in specific cells. These mice are crossed to a reporter animal harbouring a stop codon flanked by Cre-recombinogenic*loxP* sites upstream of a reporter gene, such as *lacZ* or *GFP*, under the control of the *Rosa26* promoter. In mice expressing both genetic elements, Cre recombinase excises the stop codon, such that *Rosa26* drives expression of the reporter in stem cells. Once marked in this way, all descendants propagate the expression of the reporter under *Rosa26*promoter control. In Cre recombinase expression can be activated at defined time-points through treatment with specific drugs. (A) Temporal restriction can be achieved by fusing the Cre recombinase gene to the tamoxifen-responsive hormone-binding domain of the estrogen receptor (Cre-ER^{TAM}). The Cre enzyme is in an inactive state in the absence of the ligand tamoxifen. Once tamoxifen is added, the Cre is active and can translocate to the nucleus. In the absence of tamoxifen, no expression of the reporter gene is observed because of the presence of the stop signal upstream of the reporter gene. When tamoxifen is administered, the Cre is activated and mediates recombination between the loxP sites in cells. As a consequence, the STOP codon is excised and the cells are permanently marked by the reporter gene. ER, estrogen receptor; GFP, green fluorescent protein. (B) Tet-on strategy for inducible and temporal control of transgene expression is based on the tetracycline (tet) bacterial resistance gene operon. Three transgenic lines are required. The first transgenic line contains the tTA under the control of a cell-specific promoter: this line provides the selectivity to the system. The second transgenic line contains the transgene of interest under the control of the tet operon (TetO) DNA-binding element. The TetO promoter element can be activated upon binding of the tetracycline transactivator (tTA) to drive ubiquitous expression. The third transgenic line contains the reporter system. When a tetracycline derivative, such as doxycycline, is given to the trigenic mice (obtained from breeding the tTA line with the TetO line, and then with the reporter line) in the drinking water, doxycycline binds to tTA to induce its interaction with the TetO minimal promoter and thereby turns on transgene expression selectively in the desired cell type.

Figure 2 *mT/mG* Cre-recombinase-mediated reporter for lineage tracing in the kidney.

(A) mT/mG is a double-fluorescent Cre reporter construct that expresses membrane-targeted tandem dimer Tomato (mT) prior to Cre-mediated excision and membrane-targeted green fluorescent protein (mG) after excision. Tomato reporter expression is nearly ubiquitous, allowing visualization of fluorescent markers in live and fixed samples of all tissues examine, while mG labeling is Cre-dependent, complementary to mT at single cell resolution, and distinguishable by fluorescence-activated cell sorting. Both membrane-targeted markers outline cell morphology, highlight membrane structures, and permit visualization of fine cellular processes, and thus are ideal to study podocytes. (B) mT/mG image of an *hNPHS2.rtTA;TetO.Cre;mT/mG;iDTR* kidney previously induced with doxycycline, treated with DT as previously reported[49]and compared with healthy controls. After staining with synaptopodin (blue), this model allows visualization of resident podocytes (green and blue), and of *de novo* generated podocytes (red and blue) after podocyte injury. Left: A healthy glomerulus, where all resident podocytes appear as green and blue after doxycycline induction of Cre recombinase activation. Right: After doxycycline washout, induction of podocyte injury with iDTR treatment is followed after one month by generation of novel podocytes that are not derived from resident podocytes, as demonstrated by the lack of green signal. Newly generated podocytes appear as synaptopodin positive cells (blue) that are red labelled, showing they were generated from an external progenitor.

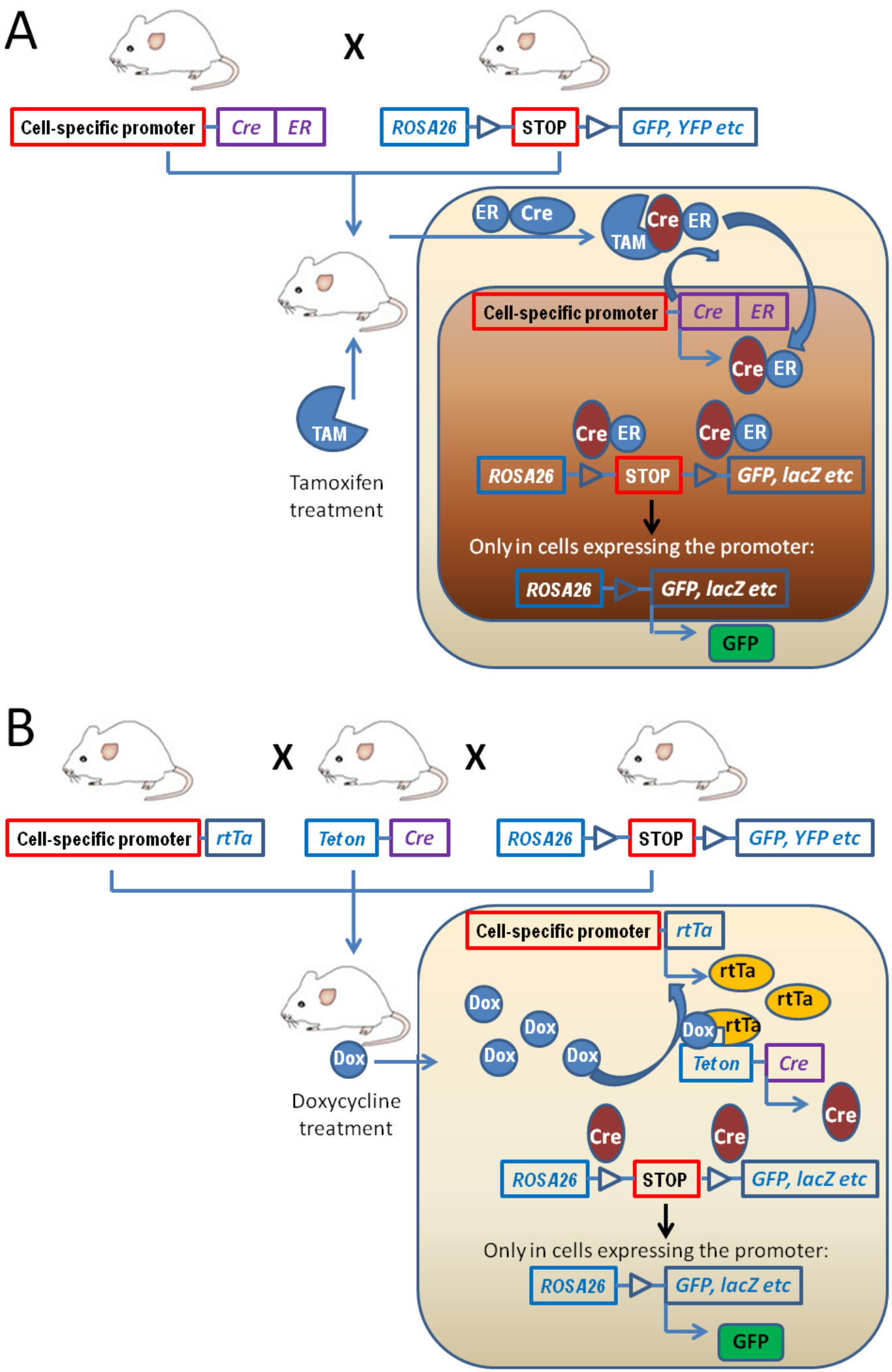
Figure 3: MulticolourCre-recombinase-mediated reporter for lineage tracing and clonal analysis.

(A) Schematic representation of the genetic strategy to mark cells with multiple fluorescent proteins. One animal harbours a transgene encoding a cell-specific promoter driving Cre recombinase expression. These mice are crossed to a reporter animal that, under the control of the ubiquitous *Rosa26* promoter, harbour a neomycin resistance gene flanked by Cre-recombinogenic*loxP* sites, and multiple genes, in sense and antisense orientations, encoding the fluorescent proteins, GFP, RFP, YFP and CFP (green, red, yellow and cyan fluorescent protein, respectively) that are flanked by Cre-recombinogenic*loxP* and inversion sites. Cre recombinase stochastically excises and inverts at the *loxP* sites to generate the possible transgenes shown, and allow *Rosa26* to drive expression of multiple combinations of fluorescent proteins in cells and their progeny.

Figure 4. Clonal analysis of the renal epithelia.

(**A**) Merged Rainbow image showing single colored clones within the adult kidney following a 7-month chase in *Actin*^{CreER}; *R26*$^{VT2/GK3}$ mice. (**B**) Merged DAPI and Rainbow image showing outcomes of clonal analysis following low dose tamoxifen administration. Large and separate single colored clones are visable within tubule segments. (**C, D**) Intact nephrons isolated fromkidneys of *Actin*^{CreER}; *R26*$^{VT2/GK3}$ mice indicate that significant tubulogenesis has occured within adult nephrons. (**E-G**) Merged Rainbow images of glumeruli showing sinle colored clones. (**H**) Merged tetrachimera image of a developing kidney. A large red clone contributing to a nephron segment. (**I-I''**) Merged images of monoclonal renal organoids following clonal analysis in-vitro. (**J**) A large single colored clone emerge from a Wnt-responsive precursor, as observed by clonal analysis in *Axin*^{CreER}; *R26*$^{VT2/GK3}$ mice.

Figure 5 CRISP-based systems for lineage tracing. Generation of a CRISP-based mouse was recently achieved bsed on Rosa26-LSL-Cas9 knockin mice have a floxed-STOP cassette preventing expression of the downstream bicistronic sequences (Cas9 and EGFP). Although under control of a CAG promoter, widespread expression cas9 and EGFP is prevented by the STOP cassette. After exposure to Cre recombinase, expression of cas9 and EGFP is observed. Cas9 expression is tightly controlled in a Cre-dependent manner, whereas gene editing via viral delivery of cas9 is burdened by packaging size limits.These Rosa26-LSL-Cas9 mice only require one to select a Cre recombinase driven by the promoter of their choosing and a specific single guide RNA (sgRNA) for generating single or multiple simultaneous mutationsediting because it requires only the introduction of sgRNAs, which can be efficiently delivered using lentiviral vectors.


A
x
Cell-specific promoter
Cre
ER
ROSA26
STOP
GFP, YFP etc
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Cre
ER
Cre
ER
ROSA26
STOP
GFP, lacZ etc
TAM
Tamoxifen
treatment
Only in cells expressing the promoter:
ROSA26
GFP, lacZ etc
GFP
B
x
x
Cell-specific promoter
rtTa
Teton
Cre
ROSA26
STOP
GFP, YFP etc
Cell-specific promoter
rtTa
rtTa
rtTa
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Dox
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treatment
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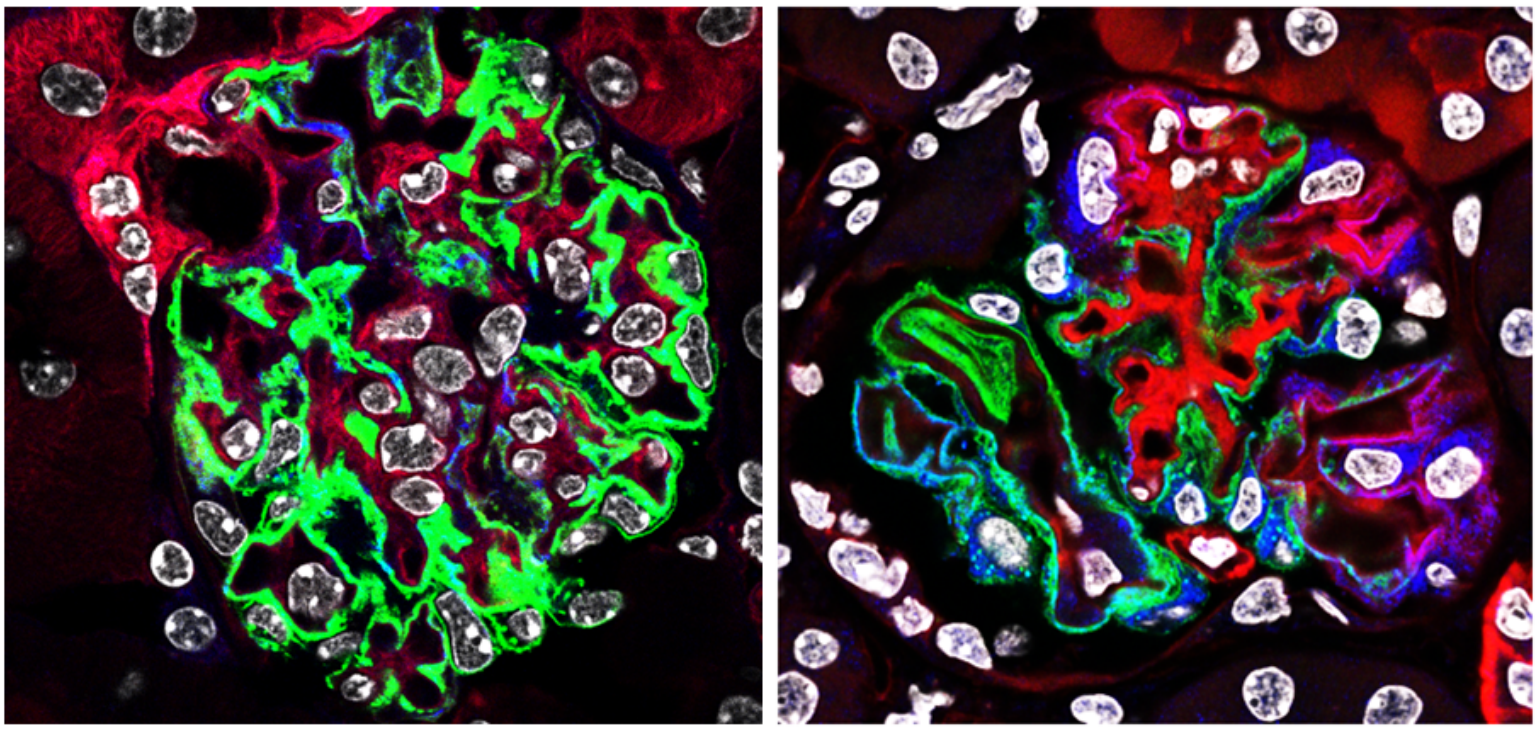
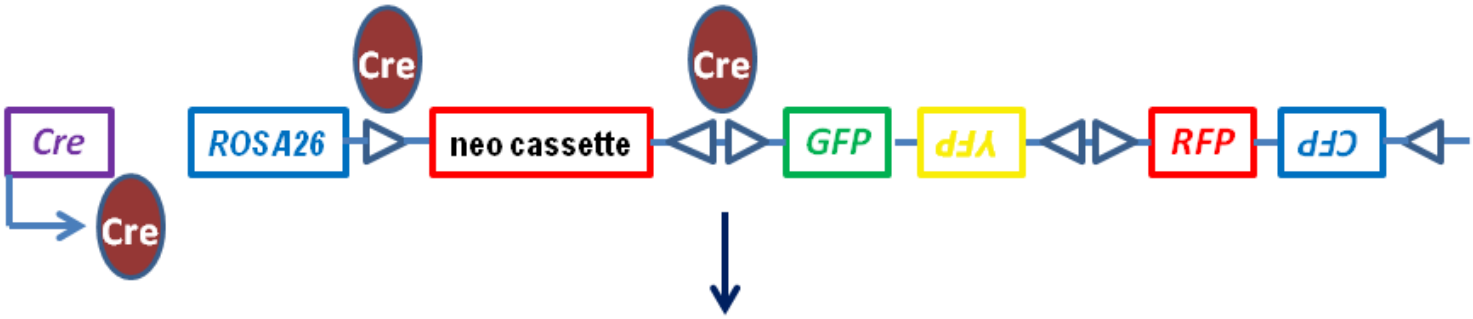
STOP
GFP, lacZ etc
Only in cells expressing the promoter:
ROSA26
GFP, lacZ etc
GFP

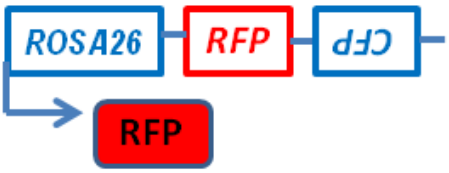
A

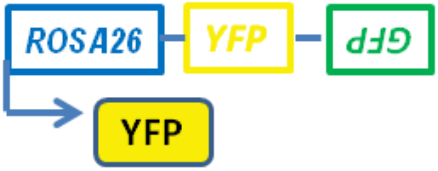
B

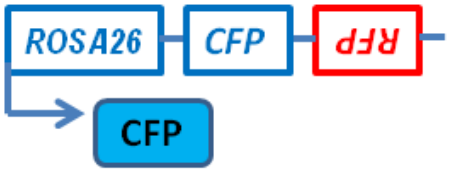


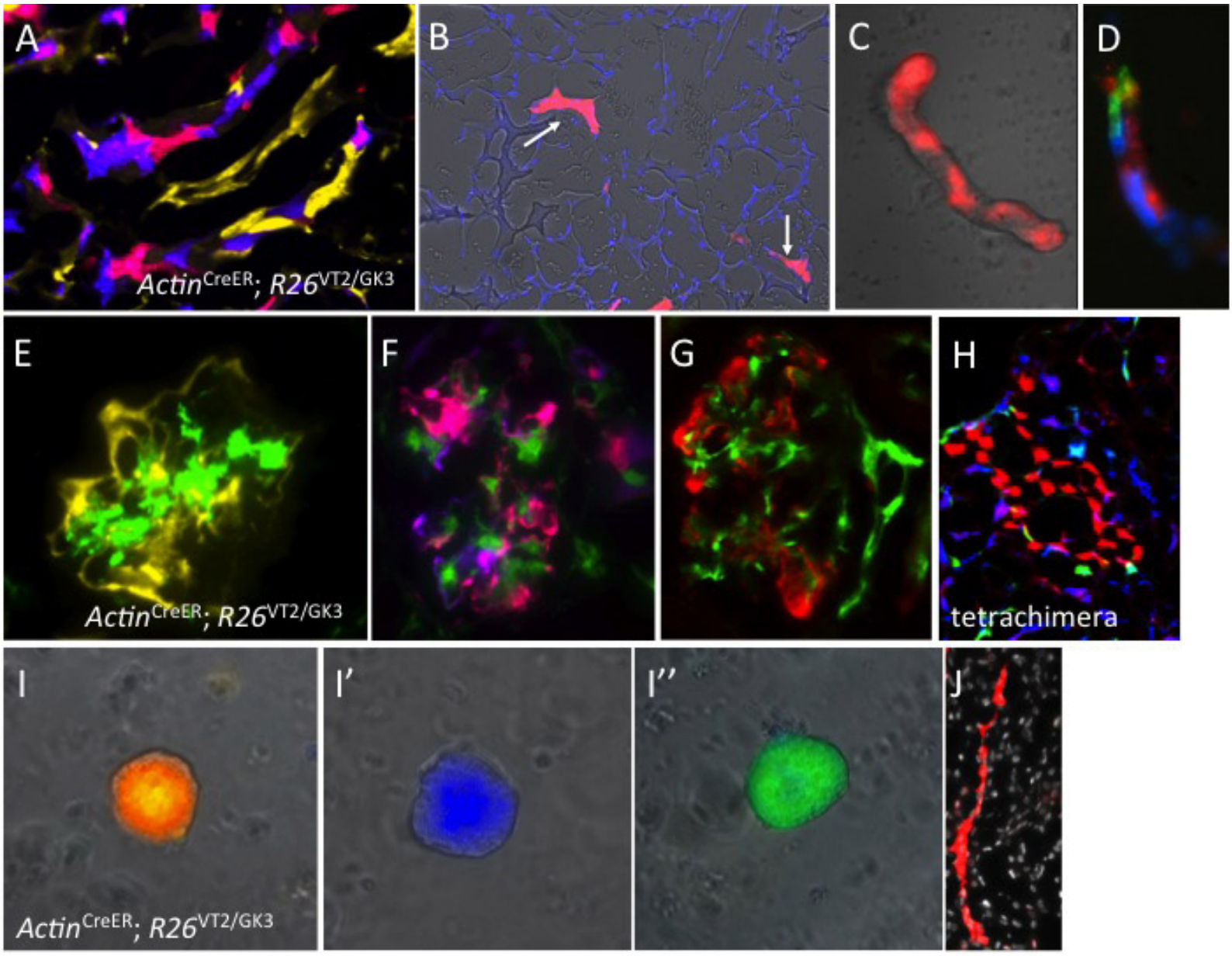


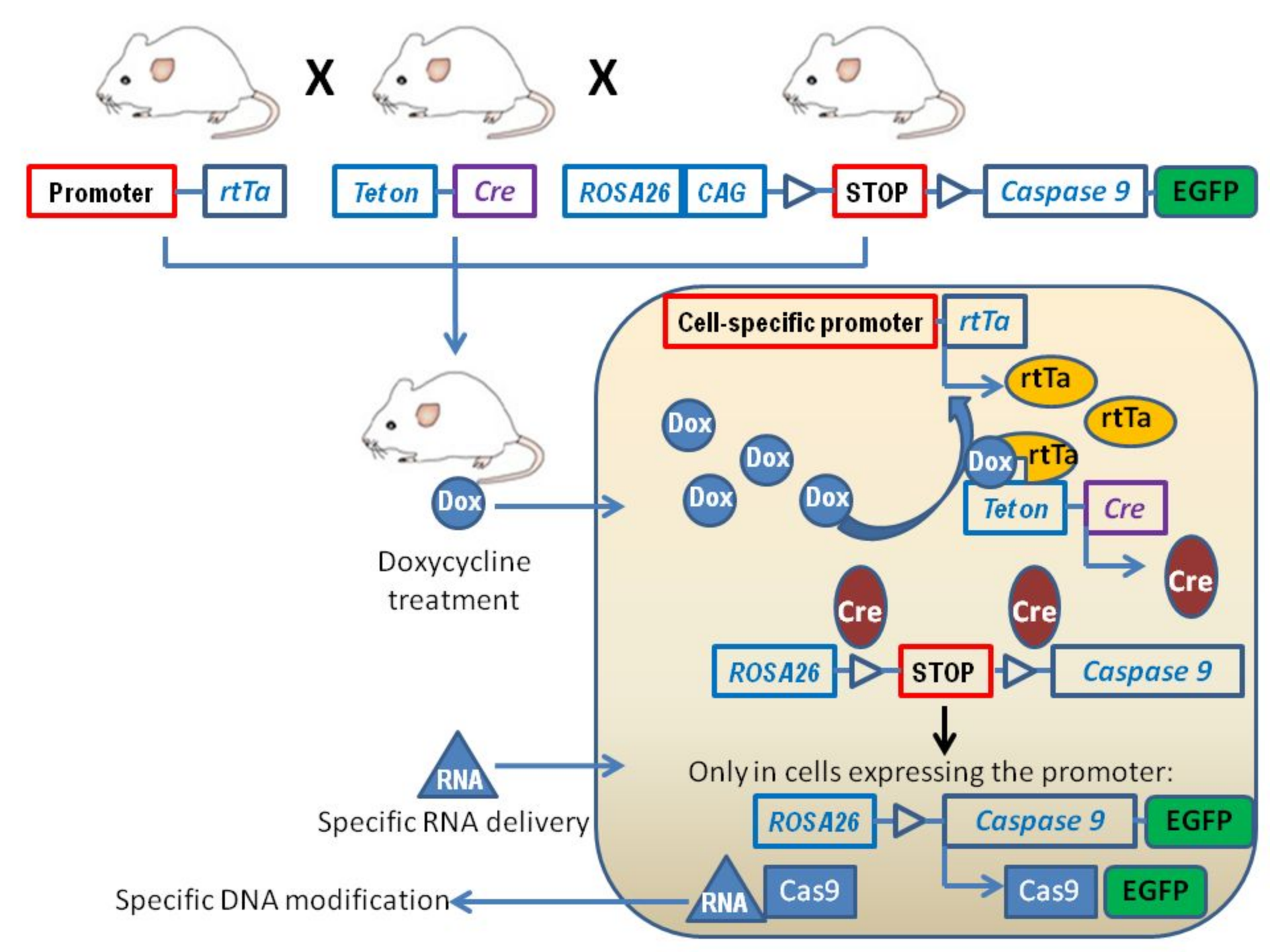
Stochastic possibilities after Cre recombination:








A
Actin^CreER; R26^VT2/GK3
B
C
D
E
Actin^CreER; R26^VT2/GK3
F
G
H
tetrachimera
I
Actin^CreER; R26^VT2/GK3
I'
I''
J


X
X
Promoter
rtTa
Teton
Cre
ROSA26
CAG
STOP
Caspase 9
EGFP
Dox
Doxycycline
treatment
Cell-specific promoter
rtTa
rtTa
rtTa
rtTa
Dox
Dox
Dox
Dox
Dox
Teton
Cre
Cre
Cre
Cre
ROSA26
STOP
Caspase 9
RNA
Specific RNA delivery
Only in cells expressing the promoter:
ROSA26
Caspase 9
EGFP
RNA
Cas9
Cas9
EGFP
Specific DNA modification