Nuclear double-fluorescent reporter for *in vivo* and *ex vivo* analyses of biological transitions in mouse nuclei

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Abstract

Cre-responsive dual-fluorescent alleles allow *in situ* marking of cell lineages or genetically modified cells. Here we report a dual-fluorescent allele, *ROSA^{nT-nG}*, that directs nuclear accumulation of tdTomato in Cre-naïve lineages. Cre converts the allele to *ROSA^{nG}*, which drives nuclear EGFP accumulation. Conditions were established for analyzing marked nuclei by flow cytometry on the basis of red-green fluorescence and ploidy, with a particular focus on liver nuclei. Hydrodynamic delivery of a Cre-expression plasmid was used to timestamp arbitrary hepatocytes for lineage-tracing. The distinct green fluorescence of nuclei from Cre-exposed lineages facilitated analyses of ploidy transitions within clones. To assess developmental transitions in liver nuclei, *ROSA^{nT-nG}* was combined with the hepatocyte-specific *AlbCre* transgene, facilitating discrimination between hepatocyte- and non-hepatocyte-nuclei. Nuclei extracted from postnatal day 2 (P2) livers were 41% green and 59% red, and reached a stable level of 84% green by P22. Until P20, green nuclei were >98% diploid (2N); at P40 they were ~56% 2N, 43% 4N, and <1% 8N; and by P70 they reached a stable distribution of ~46% 2N, 45% 4N, and 9% 8N. In conclusion, *ROSA^{nT-nG}* will facilitate *in vivo* and *ex vivo* studies on liver and will likely be valuable for studies on tissues like muscle, kidney, or brain, in which cells are refractory to whole-cell flow cytometry, or like trophectoderm derivatives or cancers, in which cells undergo ploidy transitions.

Introduction

Cre-responsive marker alleles in mice have become important tools for cell-lineage or -fate tracing and as surrogate markers to identify cells in which a Cre-responsive conditional allele has undergone conversion (Akagi et al. 1997). Fluorescent markers have proven advantageous over β -gal-based markers in that they allow live-cell real-time visualization (Kisseberth et al. 1999; Mao et al. 2001; Muzumdar et al. 2007). Single-color fluorescent reporters (e.g., “floxed-stop” reporters) (Srinivas et al. 2001) are useful for visualizing or quantifying minority Cre-exposed populations, but less useful for assessing the purity of majority Cre-exposed populations. For example, it is easy to see a single fluorescent cell

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(Cre-exposed) in a non-fluorescent (Cre-naïve) background (Srinivas et al. 2001), but it can be challenging to see a single non-fluorescent Cre-naïve cell in an otherwise fluorescent milieu (e.g., ref. (Malato et al. 2011)).

The non-essential *ROSA26* gene on chromosome (chr) 6 is weakly ubiquitously expressed (Soriano 1999), so transgenes knocked into this locus are typically ubiquitously expressed and non-disruptive. The original *ROSA26*-targeted marker containing a floxed-stop β -gal was useful for analyzing domains of Cre expression (Soriano 1999). However, early fluorescent protein insertions in this locus were problematic, as the weak expression of the locus generally yielded insufficient protein accumulation for direct visualization. Rather, detection often required antibody staining of fixed tissues (Srinivas et al. 2001; Guo et al. 2009; Malato et al. 2011).

More recently, a strongly expressed dual-fluorescent reporter allele was developed, here abbreviated *ROSA^{mT-mG}* (for *ROSA26*-membrane-tdTomato-membrane-EGFP) (Muzumdar et al. 2007). This allele solves the “one-color” problem by having a floxed-tdTomato (tdT, red-fluorescent) cistron upstream of an EGFP (green-fluorescent) cassette. Two-color fluorescent reporters allow real-time visualization of both minority and majority cell populations (Muzumdar et al. 2007). Thus, it is easy to see either one green (Cre-exposed) cell in a red (Cre-naïve) milieu (e.g., (Suvorova et al. 2009)) or to see that an otherwise purely Cre-exposed sample contains a few red cells (e.g., (Iverson et al. 2011)). *ROSA^{mT-mG}* also solved the weak expression problem of *ROSA26* by incorporating transcriptional enhancers that make both the red and green fluorescence directly visible in most live or cryosectioned tissues (Muzumdar et al. 2007). Finally, *ROSA^{mT-mG}* incorporated signals that direct the fluorescent proteins to the outer membrane (Muzumdar et al. 2007). This last attribute provides outstanding histological recognition on sectioned material and, being at the cell periphery, is favorable for flow cytometry of explanted whole cells (Muzumdar et al. 2007).

In most situations, *ROSA^{mT-mG}* is an excellent Cre-responsive marker allele, and our group has used it for such applications since it became available (Suvorova et al. 2009; Weisend et al. 2009; Rollins et al. 2010; Iverson et al. 2011). However, for some applications including many concerning liver biology, a version of this allele directing nucleus-targeted fluorescent protein accumulation would also be desirable. Thus, hepatocytes are challenging to explant or analyze by flow cytometry. Also, the hepatocytes, themselves, exhibit dramatic nuclear transitions, including changes in ploidy or transitions between mono- and bi-nuclear states (e.g., ref. (Duncan et al. 2010)), the study of which could be aided by nuclear markers. Therefore, we modified *ROSA^{mT-mG}* to generate a nucleus-targeted two-color reporter allele, entitled *ROSA^{nT-nG}* (nuclear tdT-nuclear EGFP). From mice bearing this allele, we also generated *ROSA^{nG}*, which directs ubiquitous accumulation of nuclear EGFP. The description and characterization of these alleles, and some analyses using these alleles, are presented here.

Materials and methods

Mice, care conditions, and animal procedures

C57Bl/6J, B6.Cg-Tg(Alb-cre)21Mgn/J (Postic et al. 1999) (bearing the transgene here abbreviated *AlbCre*) and STOCK *Gt(ROSA)26Sor^{tm4}(ACTB-tdTomato,-EGFP)Luo/J* (Muzumdar et al. 2007) (abbreviated *ROSA^{mT-mG}*) were obtained from Jackson Labs (Bar Harbor, ME, stock #s 000664, 003574, and 007576, respectively). The *ROSA^{nT-nG}* allele was targeted into the *ROSA26* locus on chr 6 in a C57Bl/6 x 129XI hybrid embryonic stem (ES) cell line. Chimeras were produced by blastocyst injection and founders were established by crossing

male chimeras to C57Bl/6J. This is the first report of these mice, and with this publication, the *ROSA^{nT-nG}* mice are being made available for unrestricted non-profit research.

By convention, defined loci are italicized and allelic quality follows as a superscript with each allele separated by a slash (/). When noted, wild-type alleles are designated by a superscript +. Unmapped transgenes, when applicable, are italicized and given a superscript designation of 0, 1, or 2, to indicate whether the transgene occurs in 0 (absent), 1 (hemizygous), or 2 (homozygous) copies. Loci are separated by a semicolon. Thus, *ROSA^{nT-nG/+};AlbCre¹* would have one wild-type and one nT-nG allele at the *ROSA26* locus, and would be hemizygous for the *AlbCre* transgene.

Animals received humane care according to the criteria outlined in the “Guide for the Care and Use of Laboratory Animals” and all animal procedures were reviewed and approved by the Montana State University Institutional Animal Care and Use Committee. Animals were maintained on a 14:10 light:dark cycle in HEPA-filtered forced-air conditions (Tecniplast, Buguggiati, Italy) on sterilized bedding with unrestricted access to sterilized water and sterilized feed (PicoLab 5058, LabDiet, St. Louis, MO). Administration of the AdCre vector (Suvorova et al. 2009) was performed by intravascular inoculation via a retroorbital route (Rollins et al. 2010). Hepatocyte-targeted delivery of a Cre-expression plasmid (Mansour et al. 1988) was performed by hydrodynamic tail vein (HDTV) delivery as described previously (Zhu et al. 2006; Keng et al. 2011), using 1/10th the body mass of lactated Ringer’s solution containing the amounts of plasmid DNA indicated in text or legends. Hepatic oxidative stress was induced by chronic inclusion of 0.7% (w/v) of phenobarbital (Siegfried Chemical) in drinking water, as indicated in figure legends and text. Partial (2/3) hepatectomies were performed as described previously (Mitchell and Willenbring 2008; Rollins et al. 2010).

Allelic design, construction, and validation

The targeting vector for the *ROSA^{mT-mG}* allele (Muzumdar et al. 2007) was kindly made available to us by Dr. L. Luo, Stanford University, through Addgene (Addgene Plasmid #17787, Cambridge, MA). This was modified to remove the membrane-targeting domain from the N-termini of tdT and EGFP, replace these with strong start-codons, and append the 50 amino acid nuclear assembly domain from the human SRm₁₆₀ protein to the C-terminus of each protein (Wagner et al. 2003). Details of allelic design are presented in Supplemental Figure S1. The SRm₁₆₀ nuclear assembly domain was reported to direct stable integration of proteins, including EGFP, into the nuclear matrix (Wagner et al. 2003), which we expected would both prevent loss from isolated nuclei and be unlikely to disrupt nuclear functions. The domain proved effective for nuclear localization; however we did detect slow diffusion of the proteins from isolated nuclei, which was noticeable in less than one hour at room temperature (data not shown). Cold temperatures, viscous buffers, or mild fixation allowed longer or permanent nuclear retention (see text and legends of specific experiments), and will be important considerations for using this allele on isolated nuclei.

Liver nuclei preparation and nuclei flow cytometry

Nuclei preparation methods were modified from classical protocols for isolation of native rat liver nuclei (Gorski et al. 1986; Tian and Schibler 1991; Schmidt and Schibler 1995). Briefly, mice were sacrificed and livers were harvested into petri dishes on ice. Liver was minced and 1.4 g was transferred to a Teflon-glass homogenizer (loose pestle). To this was added 18 ml of ice-cold 1X nuclei homogenization buffer (2.0 M sucrose, 10% glycerol, 15 mM KCl, 10 mM HEPES, pH 7.6, 0.5 mM spermidine, 0.15 mM spermine, 0.5 mM EDTA). Homogenization was performed at < 0°C by having the homogenizer in an ethanol-ice bath in a cold room using a motorized pestle drive. If the nuclei were to be used for flow

cytometry, 0.6 ml of ice-cold 10% formalin was added during homogenization (0.3% final formalin concentration in homogenate); this was replaced with 1X PBS for other applications. The homogenate was layered onto 2.5 ml cushions of 1X nuclei homogenization buffer in SW41Ti tubes and centrifuged for 1 h at 24,000 rpm, 0°C. Pelleted nuclei were recovered and resuspended in nuclei freezing buffer (50% glycerol, 10 mM HEPES, pH 7.6, 15 mM KCl, 0.5 mM spermidine, 0.15 mM spermine, 0.5 mM EDTA), and were either snap-frozen in liquid nitrogen and stored at -80°C or were used fresh.

For flow cytometry, sheath fluid was supplemented to 0.15 mM spermine and 0.5 mM spermidine ("polyamine-supplemented sheath", or PSS) to retain chromatin and nuclei integrity. Nuclei were diluted 10-fold with PSS and strained through fine mesh. Where indicated, nuclear DNA was quantitatively stained with DRAQ5 by the manufacturer's recommendations (AbCam, Cambridge, MA). Flow cytometry was performed on a FACSaria II (BD Biosciences, Franklin Lakes, NJ) at 70 p.s.i. and a 70 μ m nozzle, using excitation lasers of wavelengths 488 nmeter for EGFP, 561 nmeter for tdT, and 633 nmeter for DRAQ5. Emission was measured at 510 nmeters (EGFP), 582 nmeters (tdT), or 780 nmeters (DRAQ5).

Histology, fluorescence microscopy, and morphometry

Fluorescent dissecting microscope images were taken using a Nikon SMZ-800 binocular dissecting microscope outfitted with an Exfo X-Cite 120Q UV light source, red (Nikon C3394 R/Dil 31002a) and green (Nikon C127265 EN/GFP 83457) filter sets, and a Nikon DS-Fi1 digital camera. For higher magnification images, cryoblocks of tissue were prepared in O.C.T. (Tissue-Tek, Sakura Finetek, Torrance, CA) and 5 μ m sections were fixed 7 min in 10% neutral-buffered formalin, rinsed in PBS, and mounted in Fluoromount-G with DAPI (Electron Microscopy Sciences, Hatfield, PA). Images were taken on a Nikon Eclipse 80i microscope using a Nikon DS Ri1 digital camera, Nikon NIS Elements BR acquisition software, and standard red (TRITC), green (FITC), and DAPI filter sets.

Morphometric analyses of nuclei size were used as an indication of ploidy. Total nuclei purified as above, or isolated 2N, 4N, or 8N nuclei sorted by flow cytometry, were fixed on slides, stained with DAPI, and photographed with DAPI, tdT, and EGFP filters. The area of nuclei on images was determined by morphometry using NIH Image J software (<http://rsbweb.nih.gov>) and was converted to a volumetric function to approximate ploidy. Measurements on flow-sorted hepatocyte nuclei verified that 2N, 4N, and 8N isolates each differed by 2-fold increments in volume (Supplemental Fig. S2C) and were used to calibrate morphometric analyses on slides of total nuclei harvested over a developmental time-course. In each case, 150–300 random green nuclei were measured from each animal to establish the ploidy distribution at each stage.

Results

Generation and characterization of *ROSA^{nT-nG}* mice

The *ROSA^{mT-mG}* targeting vector was used as the backbone for producing the *ROSA^{nT-nG}* targeting vector (Supplemental Fig. S1). Conversion from red to green is strictly Cre-dependent. Thus, the allele marks cell lineages and can be used to follow descendants of a single cell (Fig. 1A). The targeting vector was linearized, electroporated into male C57Bl/6 x 129XI ES cells, and one clone was selected to generate chimeric mice; male chimeras were bred to C57Bl/6 dams. Pups were screened for the allele (founders) and these were bred to C57Bl/6 mice to establish the line.

Expression of *ROSA^{nT-nG}* and *ROSA^{nG}*

Postnatal day-10 (P10) tail-biopsies from heterozygous founders showed strong red fluorescence (Fig. 1B). Founders were bred with C57Bl/6 mates and an embryonic day 13.5 (E13.5) litter was harvested. Observation under the epifluorescent dissecting microscope showed that half of the pups were non-fluorescent and half were strongly ubiquitously red fluorescent (Fig. 1C & D). Other litters were raised to term to establish the line. In subsequent husbandry the allele showed Mendelian distributions; mice heterozygous or homozygous for the allele showed no overt defects (data not shown), consistent with the allele being benign. Tissues showed strong ubiquitous nuclear red fluorescence (Fig 1E–J).

ROSA^{nT-nG} mice were bred with a germline-expressing Cre-transgenic mouse (Gu et al. 1993) to generate a sub-line, *ROSA^{nG}*, that ubiquitously expressed nuclear EGFP. *ROSA^{nG/nG}* mice are also healthy and they exhibit a pattern of green nuclei reflecting the distribution of red nuclei in *ROSA^{nT-nG/nT-nG}*. This pattern included more intense nuclear fluorescence in hepatocytes near portal triads (PT) as compared to hepatocytes near central veins (CV; Figs 1E, K, N); linear arrangements of fluorescent nuclei along myofibrils in skeletal muscle (Figs 1I, L, O); brighter fluorescence in kidney cortex, in particular in glomeruli (G; Fig. 1M & P); and weakly fluorescent nuclei in brain, with the exception of interspersed tracts of small brightly fluorescent nuclei (Fig. 1H).

Exogenous delivery of Cre activity to *ROSA^{nT-nG}* hepatocytes

Delivery of Cre activity from exogenous sources to hepatocytes having a two-color fluorescent marker can allow one to synchronously convert few to most hepatocytes *in situ* (Iverson et al. 2011). Here we used delivery of a naked Cre-expressing plasmid (Mansour et al. 1988) by HDTV (Sebestyen et al. 2006; Keng et al. 2011) to *ROSA^{nT-nG/nT-nG}* mice to show the utility of *ROSA^{nT-nG}* for hepatocyte lineage tracing. HDTV was performed in P21 mice with 5 µg of pMC1-Cre plasmid. Eleven days later, mice were 2/3-hepatectomized to induce a burst of replication (Mitchell and Willenbring 2008). One week later, mice were put under chronic hepatic stress by supplementing their drinking water with phenobarbital. The liver shown in Fig. 2A–C was harvested 110 days after the partial hepatectomy. Imaging the surface of the liver with an epifluorescence dissecting microscope at harvest revealed the lobular structure of the liver (Fig. 3A, representative lobule outlined in black dotted line) and the few time-stamped green nuclei in fields of red Cre-naïve nuclei. Clusters of green nuclei were interpreted as clones derived from a single time-stamped hepatocyte (Fig. 2B, C; a representative clone is encircled in white dotted line). Use of *ROSA^{nT-nG}* for this allows one to assess clone sizes and lobular positions. Moreover, based on the relative size and proximities of nuclei, one can roughly discriminate between mono-nucleate diploid (2N) hepatocytes (Fig. 2C, e.g., orange arrow), mono-nucleate 4N hepatocytes (pink arrow), bi-nucleate 4N hepatocytes (white arrow), mono-nucleate 8N hepatocytes (yellow arrow), and bi-nucleate 8N hepatocytes (blue arrow). This will be useful for studying hepatocyte lineages in terms of proliferation, lobular position characteristics, and ploidy transitions over time.

For other applications, more widespread conversion by exogenous Cre might be useful. Using a single inoculation with 10⁸ PFU of AdCre, ~95% of hepatocyte nuclei in *ROSA^{nT-nG/nT-nG}* mice converted from red to green (Fig. 2D–F).

Flow cytometry of liver nuclei

One reason for developing *ROSA^{nT-nG}* was to allow flow-analysis of fluorescently marked hepatocyte nuclei. Although fixed nuclei tolerate flow (Tamura et al. 1992), standard flow conditions disrupt the integrity of native nuclei. By using PSS buffer, we found we could analyze native nuclei by flow (Supplemental Fig. S2). To calibrate and validate flow

parameters for $ROSA^{nT-nG}$ liver nuclei, we used identically prepared liver nuclei from (i) wild-type (non-fluorescent) mice, (ii) $ROSA^{nT-nG/nT-nG}$ mice (red-fluorescent), (iii) $ROSA^{nG/nG}$ mice (green-fluorescent), (iv) $ROSA^{nG/nT-nG}$ mice (both red- and green-fluorescent alleles), as well as (v) mixtures of i, ii, and iii nuclei (Fig. 3). In analyzing the red- and green-fluorescence of nuclei of type i, ii, iii, or iv, all events were restricted to the lower-left, upper-left, lower-right, or upper right quadrants, respectively (Fig. 3A). Nuclei type v (a mixture of type i, ii, and iii) segregated to the lower-left, upper-left, and lower-right quadrants, but the upper-right quadrant (“double-positive” quadrant) remained empty (Fig. 3B, upper panel). Importantly, there was not fluorescence-overlap between genotypes, although increased tdT expression was accompanied by a subtle increased green fluorescence in $ROSA^{nT-nG/nT-nG}$ nuclei, as indicated by the diagonal pattern of nuclei (Fig. 3A, upper left).

By flow cytometry, all types of fluorescent nuclei (types ii, iii, and iv) showed three distinct clusters of fluorescence intensities, labeled in red as “a”, “b”, and “c” in Fig 3. These clusters were most pronounced in the $ROSA^{nG/nG}$ nuclei (Fig. 3A, lower right panel) and were retained in mixed nuclei populations (Fig. 3B top). These proved not to be simply differential accumulation of fluorescence in 2N, 4N, or 8N nuclei, as type v nuclei (the mixed population) stained with DRAQ5 showed that green 2N nuclei contained classes “a”, “b”, and “c”, green 4N nuclei contained predominantly class “c” with a smaller number of class “b”, and green 8N nuclei were almost exclusively class “c” (Fig. 3B, lower panel). Interestingly, in livers of genotype $ROSA^{nT-nG/+}; AlbCre^l$ mice, in which hepatocyte nuclei will be green and other nuclei in the liver will be red, 2N green nuclei were all of classes “b” and “c”, and the class “a” nuclei were red (Supplemental Fig. S3 and data not shown), indicating that these were from cells that do not express *AlbCre*. Our interpretation is that class “a” nuclei are from non-hepatocyte cells of the liver, such as endothelial and Kupffer cells, whereas classes “b” and “c” represent distinct dim- and bright-fluorescent hepatocyte nuclei subpopulations (see Discussion).

Postnatal age-dependent transitions in liver nuclei populations

Nuclei extracted from $ROSA^{nT-nG/nT-nG}; AlbCre^l$ mice over a developmental time-course from P2 to P180 were evaluated for their proportion of red (Cre-naïve lineages) versus green (Cre-exposed lineages) nuclei (Fig. 4A). Within the green subpopulation, the proportions of 2N, 4N, and 8N nuclei were assessed by morphometric analyses on micrographs of unsorted nuclei. Fetal livers have a predominant hematopoietic function, which migrates to the bone marrow around birth as the liver takes on its hepatic character (Weisend et al. 2009). Consistent with this, nuclei extracted from P2 livers were 41% green and 59% red, and the proportion of green nuclei rapidly increase thereafter until about P22, when isolated liver nuclei reached a stable level of ~84% green (Fig. 4A).

As previously established (Shima and Sugahara 1976; Ohtsubo and Nomaguchi 1986), postnatal increases in hepatocyte nuclei ploidy showed a delay as compared to the increase in liver hepatocyte content and an incremental developmental transition. Thus, whereas by P20, $ROSA^{nT-nG/nT-nG}; AlbCre^l$ livers were approaching their stable adult ratio of red-to-green nuclei (Fig. 4A, region highlighted in green), their green nuclei remained >97% 2N (Fig. 4B). At this time, 4N nuclei began appearing, and these increased until ~P40 (Fig. 4B, region highlighted in yellow). It was not until ~P40 that the first 8N nuclei were counted, and these increased until ~P70 (region highlighted in magenta), by which age the hepatocytes had reached a stable distribution of ~46% 2N, 45% 4N, and 9% 8N nuclei (region highlighted in peach).

Discussion

The liver undergoes dramatic postnatal transitions, both developmentally and in response to hepatic damage, that can be challenging to quantify (Styles 1993; Celton-Morizur and Desdouets 2010). Hepatocytes are one of very few differentiated cell types in mammals that can proliferate (Overturf et al. 1997; Grompe et al. 1999; Shafritz and Oertel 2011). Moreover, hepatocytes undergo atypical replication cycles leading to alterations in nuclei content and in the ploidy of individual nuclei (Duncan et al. 2010). Here we report a Cre-responsive dual-fluorescent marker allele that specifically labels nuclei. This allele will facilitate *in situ* and *ex vivo* analyses on mouse nuclei.

Flow analyses on time-stamped nuclei

We report a system in which Cre-marked fluorescent hepatocyte nuclei could be analyzed by flow cytometry. In preparing liver nuclei, the tissue is chilled on ice immediately at harvest, which will better preserve some *in vivo* attributes than will the extended 37°C digestions used for explanting whole hepatocytes (Severin et al. 1984a; Ohtsubo and Nomaguchi 1986; Tamura et al. 1992; Schmidt and Schibler 1995; Lu et al. 2007). Also, although whole explanted hepatocytes can be analyzed by flow (Ohtsubo and Nomaguchi 1986; Tamura et al. 1992; Overturf et al. 1999; Duncan et al. 2010), their large size makes them susceptible to damage by shear forces, and this will be greater for larger hepatocytes (Fung 2000). By contrast, purified hepatocyte nuclei are rigid, dense, and smaller than whole hepatocytes, approximating the sizes of the immune cells that flow cytometry has been optimized for analyzing. Thus, diploid hepatocyte nuclei are roughly the size of whole resting lymphocytes (7–8 μ meters) and 8N hepatocyte nuclei are the size of stimulated lymphocytes (12–16 μ meters) (Ponder and Ponder 1959).

In the nuclei purification protocols we based our approach on (Gorski et al. 1986; Tian and Schibler 1991; Schmidt and Schibler 1995), buffers contain multivalent cations to maintain the integrity of native chromatin and nuclei during purification. Here we supplemented the sheath fluid to these conditions, which sustained nuclear integrity during flow. Our adult *ROSA^{nT-nG}; AlbCre^L* liver nuclei preparations were ~84% green and 16% red (Fig. 4A). Since other studies have reported that ~30% of adult mouse hepatocytes are binucleate (Celton-Morizur and Desdouets 2010), our data suggests that adult liver contains ~76% hepatocytes and 24% non-hepatocytes. This value approximates histology-based estimates (Blouin et al. 1977; Weisend et al. 2009; Celton-Morizur and Desdouets 2010). The ability to analyze *ROSA^{nT-nG}*-labeled liver nuclei is expected to provide a useful tool for analyses of liver and hepatocyte biology.

In addition to 4N and 8N nuclei in hepatocytes, other forms of atypical nuclear ploidy, including uniparental chromosome representation, have been shown (Duncan et al. 2010). In the current study, flow analyses of nuclei from young adult *ROSA^{nG/nT-nG}* livers did not show detectable levels of single-color nuclei, suggesting that all detected nuclei were from cells that contained both maternal and paternal copies of chr 6 (Fig. 3A). Further studies will be required to understand whether this resulted from sharing of gene products between nuclei in binucleate cells or the age or conditions of the mice. Stresses like regeneration impact ploidy (Tamura et al. 1992; Melchiorri et al. 1993; Styles 1993; Celton-Morizur and Desdouets 2010), and the late onset of appearance of 8N nuclei (Fig. 4B) suggests that some normal ploidy transitions do not occur until advanced ages (Shima and Sugahara 1976; Severin et al. 1984b). We anticipate that *ROSA^{nG/nT-nG}* mice will be useful for future studies of chr 6 mis-representation.

Unanticipated in our development of *ROSA^{nT-nG}* was that it could segregate hepatocyte nuclei into bright- and dim-subpopulations (Fig. 3B). On histological analyses of

ROSA^{mT-mG} mice, we previously noted that green fluorescence was stronger in membranes of periportal as compared to centrilobular hepatocytes (Rollins et al. 2010; Iverson et al. 2011), like we report for nuclear fluorescence in *ROSA^{nT-nG}* mice here (Fig. 1E, K, N). Ongoing studies aim to isolate populations of bright and dim hepatocyte nuclei by flow, test whether these, indeed, reliably reflect identifiable subtypes of hepatocytes, and use these to assess differences between the two populations.

It should be emphasized that the ploidy-based analyses on isolated nuclei shown here do not represent cellular ploidy distributions in liver. Thus, a portion of the isolated 2N nuclei will be from 4N cells having two 2N nuclei. It has been reported that these appear earlier in development than do 4N nuclei (Bohm and Noltemeyer 1981; Styles 1993; Celton-Morizur and Desdouets 2010). Similarly, whereas 8N nuclei constitute only ~9% of hepatocyte nuclei in mature adults, 8N hepatocytes are likely a substantially higher proportion of the liver, as this will include hepatocytes with two 4N nuclei.

Hepatocyte lineage tracing and lineage life-histories

In fetal stages, hepatocyte lineages are born from 2N progenitor cells (Grompe 2005). Each differentiation event gives rise to a new 2N hepatocyte lineage in which cells can proliferate, accumulate damage, undergo changes in ploidy, and die. Different hepatocytes on a lineage might occupy different sub-lobular zones, have different metabolic functions, be exposed to different stresses, and have different replicative potentials. Entire lineages might go extinct. We define the hepatocyte lineage life history as the sum of all of these events, starting with birth of a lineage from a progenitor cell to death of the last hepatocyte on that lineage.

For tissues in which cells are not migratory, synchronous marking of a few cells can allow one to infer clonal relationships between adjacent marked cells at later times (Gehring 1967). Using HDTV of Cre plasmid to mark small numbers of hepatocytes in *ROSA^{nT-nG}* livers followed by a period of hepatic stress (Fig. 2A–C), we identified clones of hepatocytes that had, during the period between time-stamping and harvest, undergone expansion and ploidy diversification. The variance in clonal expansion was remarkable, such that within a liver, some marked hepatocytes had given rise to large clones whereas other clones were represented by one or few marked cells (Fig. 2 and see Cover Image submission). Additional information, such as bromodeoxyuridine- and TUNEL-labeling indexes, will be needed to discern the contributions of proliferative rates versus death rates in causing such large differences in clone sizes, and it will be important to investigate whether this difference in clonal size is arbitrary or relates in a predictable way to the life history of that lineage. Interestingly, within clones we frequently found cells having different nuclear or genomic content, consistent with these parameters being rapidly dynamic within hepatocyte lineages (Duncan et al. 2010).

Recently, we and others have shown that new hepatocytes can differentiate at low frequencies from progenitor cells in postnatal liver (Iverson et al. 2011; Malato et al. 2011; Espanol-Suner et al. 2012). It is debated whether this is a physiologically important contribution to the liver. To resolve the debate, it will be useful to trace the fates of individual hepatocytes and map hepatocyte lineage life histories. If, as we have proposed (Iverson et al. 2011), these “newborn hepatocytes” can each give rise to a large lineage, then a low birth-rate could feed a substantial turnover in hepatocyte lineages over the course of a lifetime. On the other hand, if newborn hepatocytes do not give rise to expanded lineages, then this contribution might be of little importance (Malato et al. 2011; Espanol-Suner et al. 2012). The *ROSA^{nT-nG}* system we report here will be useful for many studies in liver biology and likely in other organs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used

2N	diploid
4N	tetraploid
8N	octaploid
alb	albumin
chr	chromosome
E	embryonic day
EDTA	ethylenediaminetetraacetic acid
EGFP	enhanced green fluorescent protein
floxed	flanked by <i>loxP</i>
HDTV	hydrodynamic tail vein injection
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
P	postnatal day
PSS	polyamine-supplemented sheath buffer
tdT	tandem-dimer tomato

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Fig. 1. *ROSA^{nT-nG}* and *ROSA^{nG}* alleles. (A) Schematic of the alleles (left) and representative hepatocytes (right), showing nuclear tdT expression in Cre-naïve lineages, allelic conversion by Cre, and nuclear EGFP expression in all members of Cre-exposed lineages. “enh”, enhancer; N, SRM₁₆₀ nuclear assembly domain; bent arrow, transcription initiation site; yellow triangles, loxP sites; “STOP” signs, transcription termination signals. (B) Tail-snip from a P10 founder under the red channel of an epifluorescence dissecting stereomicroscope. (C & D) Identical frames of two live littermates from a mating of a *ROSA^{nT-nG/+}* founder at embryonic day 13.5 (E13.5) photographed under white light (C) or red-fluorescence (D) on the dissecting stereomicroscope. (E – J) Whole organs from a P42 *ROSA^{nT-nG/+}* mouse photographed under red fluorescence on epifluorescence dissecting stereomicroscope. No fluorescence is visible on the green channel (not shown). The boxed area in panel E corresponds to the region expanded in panel F. The tracks of representative central veins (CV) and portal triads (PT) are indicated by yellow and blue dotted lines, respectively. (K – P) Whole (K & L) or bisected (M) organs, or cryosections of organs (N –

P), as indicated, from a P100 *ROSA^{nG/nG}* mouse. G, glomeruli. In panels K & N, representative lobules are circumscribed; in L & O, arrows denote the orientation of myofibrils; in M, dotted line separates kidney cortex from medulla. Scale bars: in panels B – D, 5000 μ meters; in panels E – P, 500 μ meters.

Fig. 2.

Exogenous delivery of Cre activity for time-stamping, lineage-tracing, or global conversion of hepatocytes. (A – C) Clonal analysis of time-stamped hepatocyte nuclei. A mouse was time-stamped with 5 μ g of pMC1-Cre plasmid by HDTV at P21 and was 2/3-hepatectomized at P32. Beginning at P39, the drinking water contained 0.7% phenobarbital. The mouse was harvested at P142 and the liver was photographed with an epifluorescence dissecting stereomicroscope. Panel A is a merge of the red and green channels; black dashed line circumscribes a representative lobule. Because this is an image of the whole organ and nearly all nuclei are red, underlying out-of-focal plane red nuclei blur visualization of in-focus red nuclei. Panel B is the same frame under green fluorescence only; dashed box indicates the region enlarged in panel C. White dashed circle denotes a clone of three hepatocytes (one binucleate and two mononucleate) in which all four nuclei are 4N. Orange arrow: 2N nucleus in a mononucleate hepatocyte; white arrow: 2N nuclei in binucleate hepatocyte; pink arrow: 4N nucleus in mononucleate hepatocyte; blue arrow: 4N nuclei in binucleate hepatocyte; yellow arrow: likely 8N nucleus in mononucleate hepatocyte, although this could be two close vertically juxtaposed 4N nuclei. (D – F) Global marking of hepatocytes using AdCre. A P49 *ROSA^{nT-nG/nT-nG}* mouse was inoculated IV with 10^8 pfu of AdCre and was sacrificed 1 week later. Whole liver was photographed as above. Although red channel (panel D) shows many red nuclei, most are deeper in the tissue (in lower focal planes); merged image (panel F) showed that ~95% of the hepatocytes were green. Scale bars in panels A, B, D–F, 250 μ m; in panel C, 115 μ m.

Fig. 3.

Flow analyses on purified fluorescent liver nuclei. (A) tdTomato- versus EGFP-fluorescent flow analyses on liver nuclei from mice of genotypes *ROSA^{+/+}* (lower left plot, all nuclei non-fluorescent), *ROSA^{nT-nG/nT-nG}* (upper left plot, all nuclei red-fluorescent), *ROSA^{nG/nT-nG}* (upper right plot, all nuclei both red and green fluorescent), and *ROSA^{nG/nG}* (lower right plot, all nuclei green fluorescent), as indicated. The quadrants in each plot are separated by vertical and horizontal lines at intensities that were used uniformly throughout all analyses in this study. For all fluorescent samples, the nuclei segregated into three distinct peaks, labeled in red as “a”, “b”, and “c”. Note that both the red and green fluorescence was lower in the *ROSA^{nG/nT-nG}* nuclei as compared to *ROSA^{nT-nG/nT-nG}* or *ROSA^{nG/nG}*, respectively. This is attributed to the *ROSA^{nG/nT-nG}* hepatocytes having only one allele of each color per 2N genome. (B) Relationship between DNA content and EGFP intensity on liver nuclei. Purified nuclei from *ROSA^{+/+}*, *ROSA^{nT-nG/nT-nG}*, and *ROSA^{nG/nG}* livers were mixed, stained with DRAQ5, and analyzed by flow for red- versus green-fluorescence (upper plot) or DRAQ5 versus green fluorescence (lower plot). The *ROSA^{nG/nG}* nuclei still showed three peaks differing in EGFP intensity in the red versus green plot. When these were analyzed by DRAQ5 versus green, the EGFP fluorescence was found to be independent of ploidy. Thus, 2N nuclei showed all three green peaks, whereas 4N and 8N nuclei were predominantly only in peak “c”. Further analysis revealed that peak “a” was non-hepatocyte 2N nuclei, and peaks “b” and “c” were dim or bright hepatocyte nuclei, respectively (see Supplemental Fig. S3).

Fig. 4. Nuclear transitions during postnatal liver development. Mice of genotype *ROSA^{nT-nG/nT-nG}; AlbCre^f* were sacrificed at the indicated postnatal ages and liver nuclei were purified. Slides of total liver nuclei were analyzed for red-versus-green fluorescence (panel A), and green nuclei were morphometrically analyzed for volume, as an indication of ploidy (panel B; see Supplemental Fig. S2C). The time from P0 to P20 (shaded green) is the period of time when the liver loses its hematopoietic function and becomes specialized for hepatic functions. Only diploid hepatocyte nuclei were detected during this period. From P20 – P40 (yellow), 4N hepatocyte nuclei appeared, but no 8N nuclei were detected. From P40 – P70 (magenta), 8N nuclei appeared, and after P70 (peach) the abundance and ploidy of hepatocyte nuclei appeared to be at steady state.