STEM CELLS®

TISSUE-SPECIFIC STEM CELLS

The Majority of Multipotent Epidermal Stem Cells Do Not Protect Their Genome by Asymmetrical Chromosome Segregation

PANAGIOTA A. SOTIROPOULOU, AURÉLIE CANDI, CÉDRIC BLANPAIN

Université Libre de Bruxelles, Interdisciplinary Research Institute, Brussels, Belgium

Key Words. Stem cells • Cell division • Chromosome segregation • Cancer

ABSTRACT

The maintenance of genome integrity in stem cells (SCs) is critical for preventing cancer formation and cellular senescence. The immortal strand hypothesis postulates that SCs protect their genome by keeping the same DNA strand throughout life by asymmetrical cell divisions, thus avoiding accumulation of mutations that can arise during DNA replication. The in vivo relevance of this model remains to date a matter of intense debate. In this study, we revisited this long-standing hypothesis, by analyzing how multipotent hair follicle (HF) SCs segregate their DNA strands during morphogenesis, skin homeostasis, and SC activation. We used three different in vivo approaches to determine how HF SCs segregate their DNA strand during cell divisions. Double-labeling studies using pulse-chase experiments during morphogenesis and the first adult hair cycle showed that HF SCs incorporate two different nucleotide analogs, contradictory to the immortal strand hypothesis. The co-segregation of DNA and chromatin labeling during pulse-chase experiments demonstrated that label retention in HF SCs is rather a mark of relative quiescence. Moreover, DNA labeling of adult SCs, similar to labeling during morphogenesis, also resulted in label retention in HF SCs, indicating that chromosome segregation occurs randomly in most of these cells. Altogether, our results demonstrate that DNA strand segregation occurs randomly in the majority of HF SCs during development, tissue homeostasis, and following SC activation. STEM CELLS *2008;26:2964–2973*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Cellular homeostasis represents the balance among cell proliferation, differentiation, and apoptosis, maintaining constant the number of cells within adult tissue. Stem cells (SCs) are responsible for tissue replenishment throughout life by providing cells that maintain tissue homeostasis and repair following injuries [1]. In tissues with high cellular turnover such as the skin epidermis, the intestine, and the hematopoietic system, SCs divide many times during the life of the organism, thus being at high risk of accumulating errors during DNA replications that may have deleterious consequences, such as cancer formation and cellular senescence. Consequently, SCs may have acquired during evolution specialized mechanisms to ensure the maintenance of their genome integrity.

Cellular quiescence could be one of the mechanisms that protect SCs from exhaustion, as well as from accumulating DNA mutations. In the skin epidermis, multipotent SCs reside in a specialized location within hair follicles (HF) called the bulge [2]. Bulge SCs are activated during HF regeneration and after wounding to repair the damaged epidermis. Different experimental evidence, such as label retention studies independent of DNA labeling, as well as direct cell cycle analysis together with SC markers, demonstrated unambiguously that bulge SCs are more quiescent than their transit amplifying progenies [3–5].

Another putative mechanism by which SCs may protect their genome from accumulating mutations arising during DNA replication is known as the immortal strand hypothesis [6]. According to this theory, during tissue homeostasis, SCs undergo asymmetric divisions in which they retain the older DNA strand (the "immortal" strand), whereas the daughter cell committed to terminal differentiation inherits the newer DNA strand, which may contain de novo mutations. One common experimental argument to support the existence of the immortal strand hypothesis derives from label retention studies in which a nucleotide analog, such as 5-bromo-2-deoxy-uridine (BrdU), is administered to young animals and supposedly incorporated into the DNA of symmetrically proliferating SCs. Thereafter, the labeled DNA would remain indefinitely present in SC genome, even after multiple rounds of cell division, because they will always retain the older immortal DNA strand [7–11]. On the other hand, similar pulse-chase experiments have been widely used as experimental argument to demonstrate the relative quiescence of SCs in many adult tissues, assuming that chromosome segregation is random and label retention represents only the relative quiescence of these cells [12–14]. Three decades after this theory has been proposed, the immortal strand hypothesis still remains a matter of intense debate [15, 16]. Only one study hitherto investigated the in vivo relevance of the immortal strand theory during homeostasis using double-labeling proto-

Author contributions: P.S.: conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing; A.C.: collection and assembly of data, data analysis and interpretation, manuscript writing; C.B.: conception and design, data analysis and interpretation, manuscript writing, final approval of manuscript; P.A.S. and A.C. contributed equally to this work.

Correspondence: Cedric Blanpain, M.D., Ph.D., Université Libre de Bruxelles, IRIBHM, 808, Route de Lennik, Brussels 1070, Belgium. Telephone: +32-2-5554175; Fax: +32-2-5554655; e-mail: Cedric.Blanpain@ulb.ac.be; web site: http://blanpainlab.ulb.ac.be/index.htm Received July 4, 2008; accepted for publication August 23, 2008; first published online in STEM CELLS *EXPRESS* September 4, 2008. 1066-5099/2008/$30.00/0 doi: 10.1634/stemcells.2008-0634

cols together with well-characterized stem cell markers and suggested that hematopoietic SCs do not retain BrdU label and segregate their chromosomes randomly [17].

The recent progress made in the isolation and functional characterization of the slow cycling multipotent bulge SCs of the skin epidermis [3–5,18] allows testing of the immortal strand hypothesis in a well-characterized model of quiescent epithelial SCs. The skin epidermis is an ideal model to tackle this unresolved question because bulge SCs can be accurately identified by high CD34 expression [3, 18] and they alternate cycles of proliferation and quiescence in a timely defined and synchronized manner [2], which facilitates the design and interpretation of double-labeling studies. In addition, a transgenic mouse model allowing the quantification of cell division independently of DNA labeling has been developed and tailored to skin SC biology [4].

Hair follicle morphogenesis in mice begins during embryonic development and continues during the first week of life. Once HFs reach their final size, the lower two-thirds stop proliferating and ingress rapidly by programmed cell death (catagen), whereas the upper part survives and gives rise to a specialized structure known as the bulge, which represents the cellular niche of the multipotent adult SCs of the skin. After a short resting stage (telogen), HFs undergo a new cycle of regeneration (anagen), during which bulge SCs are activated to proliferate transiently and give rise to transit amplifying cells that will eventually differentiate into the distinct mature cells of the HF [2]. Administration of nucleotide analogs, such as BrdU or tritiated thymidine, during HF morphogenesis results in labeling of most proliferative epidermal cells including the progenitors of the prospective bulge cells. After a month of chase, label-retaining cells (LRCs) are preferentially located in the bulge region of the HF [12]. The presence of LRCs during this experimental procedure is compatible with the greater quiescence of the prospective bulge cells, as suggested by the high expression of NFATc1, a critical regulator of SC proliferation, in the future bulge cells during this developmental stage [19] and as demonstrated directly by label retention studies performed during hair follicle morphogenesis [20], but it is also compatible with the immortal strand hypothesis. Indeed, the expansion of developmental progenitors during morphogenesis may involve symmetric divisions, which would result in the retention of the nucleotide analog that would become the mark of the "immortal" strand, as has been argued for other epithelial tissues, such as the tongue papilla, mammary epithelium, and intestinal crypts [7, 8, 10]. Upon administration of a second nucleotide analog, putative stem cells of these tissues could be double-labeled but progressively lost the second analog while keeping the first, which is thought to represent the mark of an immortal DNA strand.

However, recent studies showed that LRCs obtained from pulse-chase experiments performed during the first week of life become progressively undetectable, as they dilute the BrdU label upon cell division [13, 21, 22]. This important characteristic demonstrates that the nucleotide analog pulse during the first week of life does not result in the labeling of an immortal DNA strand but it does not rule out definitively the immortal strand hypothesis because the oldest DNA template may have been established earlier during development, before BrdU administration.

In the present study, we took advantage of the use of double-labeling pulse-chase experiments utilizing two different uridine analogs, such as 5-chloro-2-deoxy-uridine (CldU) and 5-iodo-2-deoxy-uridine (IdU), which provides the possibility to study label incorporation in sequential cell divisions and subsequently to develop models of the potential outcomes associating cell labeling and strand segregation. As illustrated in Figure 1A and because LRCs generated during the first week of life do not represent the mark of an immortal strand as mentioned above, the asymmetrical strand segregation hypothesis predicts that SCs cannot be double-labeled during the administration of the second analog (Fig. 1A). On the other hand, if CldU LRCs represent only the mark of relative quiescence during the chase period, the random strand segregation model predicts that each chromosome has a 50% chance of being double-labeled after the first cell division, due to the semiconservative nature of DNA replication (Fig. 1B). Because mice do not have 1 but 40 chromosomes, the actual probability for a cell to become double-labeled would be close to 100% (Fig. 1C). These double positive cells would be progressively lost thereafter according to the number of divisions accomplished, as nucleotide analogs cannot be detected after three to four cell divisions [17, 22].

Here, we used different approaches, including double-labeling studies performed during HF morphogenesis and tissue homeostasis, as well as pulse-chase experiments combining DNA and chromatin labeling to investigate precisely how bulge SCs segregate their DNA strands during morphogenesis and tissue homeostasis and following SC activation. Our results clearly demonstrate that the vast majority of bulge SCs segregate their DNA templates randomly and that the immortal strand model does not represent a mechanism responsible for the maintenance of bulge SC genome integrity.

MATERIALS AND METHODS

Mice

CD1 mice were obtained from Charles River (Wilmington, MA, http://www.criver.com). Cytokeratin 5-inducible tetracycline transcriptional repressor (K5Tet^{off}) mice were a kind gift from A. Glick (Laboratory of Cellular Carcinogenesis and Tumor Promotion, National Cancer Institute, Bethesda, MD). Fusion protein between histone-2B (H2B) and the green fluorescent protein (GFP) under the control of a tetracycline response element (TRE), TRE-mCMV-H2B-GFP mice were a kind gift from E. Fuchs (Howard Hughes Medical Institute, The Rockefeller University, New York). TRE-mCMV-H2B-GFP:K5Tet^{off} mice were bred and genotyped as described previously [4]. All animal experiments were performed in accordance with the guidelines of the Free University of Brussels (ULB) and the Ethical Committee for Animal Welfare (reference number 260N).

Nucleotide Analog Administration

BrdU, 5-chloro-2-deoxy-uridine (CldU), and 5-iodo-2-deoxy-uridine (IdU) (Sigma-Aldrich, Schnelldorf, Germany, http://www.sigmaaldrich.com) were administered intraperitonealy twice daily at an equimolar concentration of 65 mM at a quantity of 50 mg/kg, following the experimental settings described in each section. For the chase of H2B-GFP in TRE-mCMV-H2B-GFP:K5Tet^{off} mice, doxycycline diet (Plexx b.v., Elst, The Netherlands, http://www.plexx.eu) was provided for the indicated period.

12-*O*-Tetradecanoylphorbol-13-Acetate Treatment

CD1 mice were topically treated with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (Sigma) daily from PD25 to PD30 (PD, postnatal day). Back skin was shaved and 100 μl of freshly prepared TPA (60 μg/ml) diluted in acetone or 100 μl of acetone alone as a control was applied on each mouse.

Extraction of Keratinocytes

Isolation of keratinocytes was performed as previously described [3]. Briefly, back skin was cleaned from adipose tissue and blood vessels and incubated overnight in trypsin/EDTA at 4°C (Gibco-Invitrogen Inc., Grand Island, NY, http://www.invitrogen.com). The following day epidermis was scraped so that single-cell suspension was obtained and cells were stained for flow cytometry.

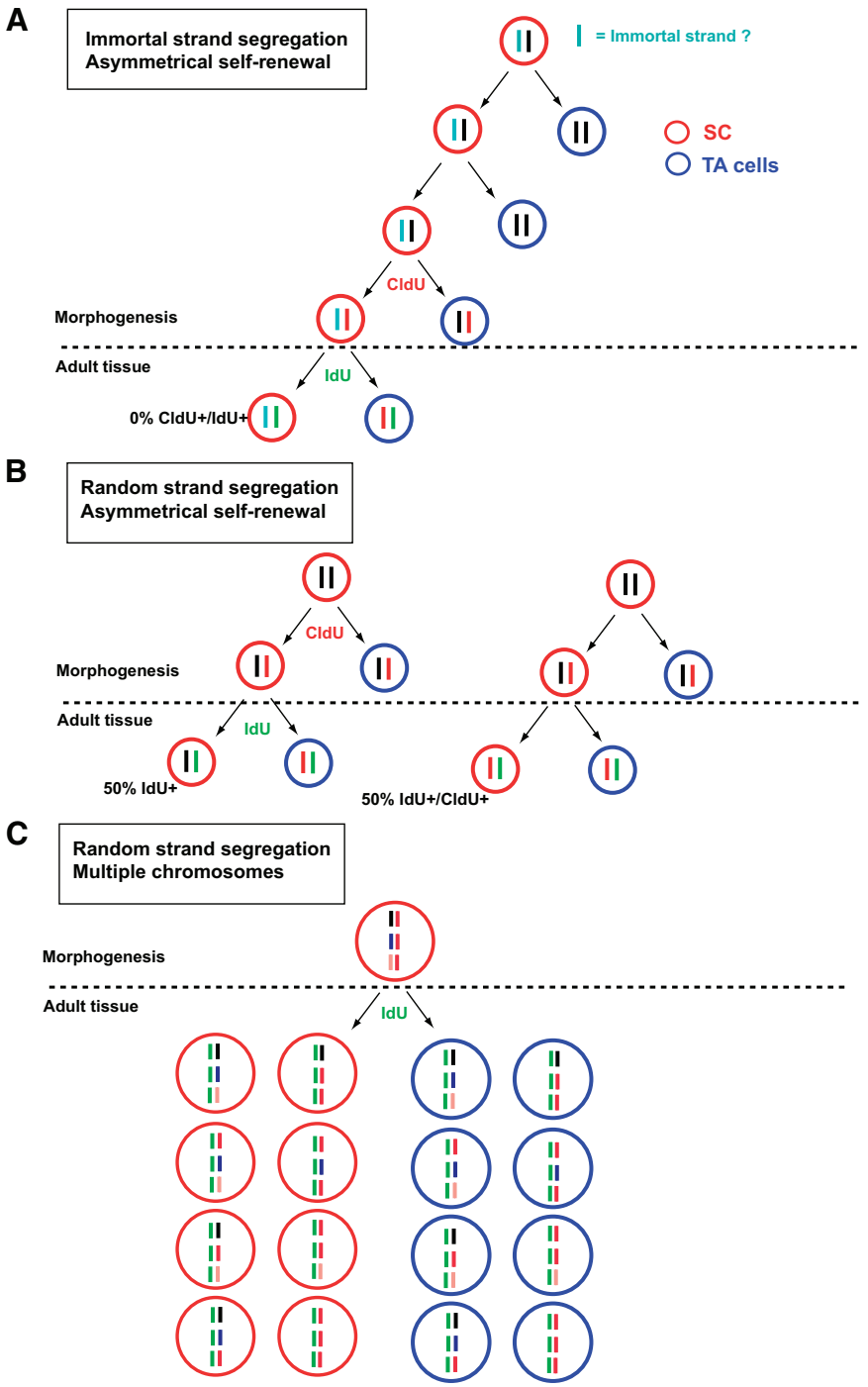

Figure 1. Possible outcome of double-labeling studies according to the asymmetrical and random strand segregation models. **(A):** If stem cells segregate their chromosomes according to the immortal strand hypothesis and the older "immortal" strand is established early during the embryonic development, pulsing with CldU during postnatal life will mark the newly formed strand, which will be transferred to the more differentiated cell in the subsequent division and will be replaced with the novel IdU labeled strand. Therefore, in the immortal strand model, no $CldU^{+}IdU^{+}$ double-labeled cells can be observed. **(B):** If DNA templates are segregated randomly, there is a 50% chance that the daughter SC inherits the previously labeled CldU chromosome and will be double positive. **(C):** If we take into account the existence of multiple-labeled chromosomes (three chromosomes are represented in this example), then after the second analog pulse the vast majority of the cells will be double-labeled. Abbreviations: CldU, 5-chloro-2-deoxy-uridine; IdU, 5-iodo-2-deoxy-uridine; SC, stem cell; TA, transit-amplifying.

Immunofluorescence

The following primary antibodies were used: anti-BrdU clone UBI 1/75 (at 1/800) (Abcam, Cambridge, MA, http://www.abcam.com) and clone B44 (at 1/800) (BD Biosciences, San Diego, http://www.bdbiosciences.com), which are specific for CldU and IdU, respectively, and chicken anti-GFP (1/2000) (Abcam). Secondary antibodies were purchased from Jackson Immunoresearch (Newmarket, Suffolk, U.K., http://www.jireurope.com) and were used as follows: anti-rat Rhodamine RedX (RRX) (1/400), anti-mouse Fluorescein Isothiocyanate (FITC) (1/400), anti-mouse Cyanin5 (1/400), and anti-chicken FITC (1/400). Back skin was fixed in 4% paraformaldehyde for 24 h and embedded in paraffin. Sections of 4 μm were carried out using a microtome Leica RM2245 (Newcastle upon Tyne, U.K., http://www.leica-microsystems.com). Antigen unmasking procedure was performed for 30 minutes at 98°C in a PT Module (Lab Vision, Fremont, CA, http://www.labvision.com) using a PT Module Buffer one (Lab Vision).

Nonspecific antigen blocking was performed using a M.O.M. Basic Kit reagent (Vector Laboratories, Burlingame, CA http://www.vectorlabs.com) for mouse antibodies, or with phosphate-buffered saline supplemented with 5% Normal Donkey serum (Jackson Immunoresearch), 1% bovine serum albumin, and 0.1% Triton X-100 (both from Sigma) for 1 h at room temperature otherwise. Antibodies recognizing CldU and IdU respectively were incubated overnight at 4°C, followed by anti-mouse fluorescein isothiocyanate (FITC), anti-Rat RRX and 4′,6-diamidino-2-phenylindole (DAPI) (1/1000, Molecular Probes, Eugene, OR, http://probes.invitrogen.com) for 1 hour at room temperature. In TRE-

mCMV-H2B-GFP:K5Tet^{off} mice, anti-GFP antibody was detected with anti-chicken-FITC, whereas anti-mouse Cy5 was used as secondary antibody to detect the anti-IdU antibody. Pictures were acquired using a Zeiss Axio Imager.M1 (Thornwood, NY) fluorescence microscope and objectives ×20 Zeiss EC Plan-NEOFLUAR (.5 numerical aperture) and ×10 Zeiss EC Plan-NEOFLUAR (.3 numerical aperture), with a Zeiss Axiocam MRm camera, using Axiovision Rel. 4.6 software.

Flow Cytometry

Surface staining was performed as described previously [3] using biotin-conjugated anti-CD34 (clone RAM34; eBiosciences, San Diego, http://www.ebioscience.com), followed by streptavidin-Allophycocyanin (BD Biosciences, San Diego, http://www.bdbiosciences.com). The IgG fraction of anti-CD34 antibody was blocked using anti-rat-Fab fragment from Jackson Immunoresearch (at 20 μg/ml). CldU and IdU were detected using the BrdU Flow Kit (BD Biosciences) and respectively anti-BrdU clone UBI 1/75 (at 1/40), followed by anti-rat-Phycorythrin (at 1/75) (Jackson Immunoresearch) and anti-BrdU clone B44 FITC-conjugated (at 1/50) (BD Biosciences). When TRE-mCMV-H2B-GFP:K5Tet^{off} mice were used, the secondary antibody for CD34 was substituted by streptavidin-Peridinin-chlorophyll-protein Complex-Cyanin5.5 (at 1/400), whereas IdU was detected using the anti-BrdU clone B44 unlabeled (BD Biosciences), followed by anti-mouse-Cy5 (at 1/500). Fluorescence-activated cell sorting (FACS) analysis was performed using FACSCalibur and CellQuestPro software (BD Biosciences).

Statistical Analysis

Statistically significant differences in the parameters tested were assessed by applying Student *t* test statistics to the experimental data. The number or individuals in each experiment, as well as the number of independent experiments, are indicated in the respective figure legend.

RESULTS

Double-Labeling Studies during Skin Morphogenesis and First Hair Cycle Indicate Random Chromosome Segregation in Hair Follicle Stem Cells

To directly investigate how chromosomes are segregated during bulge SC division, we used a novel and very sensitive double-labeling method consisting of the sequential administration of CldU and IdU that can be recognized by specific antibodies (Fig. 2A). We administered CldU from PD3 to PD5 and chased the mice until next anagen. This developmental window is a part of the HF morphogenesis when the future bulge SCs proliferate actively and has been used in the past for in vivo SC labeling, resulting in almost complete labeling of the skin epidermis including wide labeling of the prospective bulge SCs [22]. Administration of nucleotide analogs during the first 2 weeks of life has also been used successfully to label bulge SCs [12, 13] and gives identical results. However, we have chosen to pulse mice from PD3 to PD5, to label cells in early developmental stage, and to avoid toxicity of prolonged nucleotide analog administration. Subsequently, mice were injected with IdU from PD23 to PD25 (Fig. 2B), to label bulge SCs during their next proliferative phase [23]. Immunofluorescence on back skin sections revealed the presence of double positive CldU$^+$IdU$^+$ cells within the bulge (Fig. 2C), indicating that some CldU LRCs divided during the first anagen and incorporated IdU. Quantification of CldU and IdU positive cells within CD34$^+$ bulge SCs by FACS (Fig. 2D) revealed that 4.92% ± 0.82% of CD34^{high} cells were CldU$^+$, 14.9% ± 1.54% of CD34^{high} bulge cells were IdU$^+$, and 1.88% ± 0.35% of total CD34^{high} cells were CldU$^+$IdU$^+$ double positive (Fig. 2E). Similar results were obtained when IdU was administered from PD3 to PD5 followed by CldU from PD23 to PD25, showing that CldU and IdU are incorporated equally (data not shown). The percentage of double positive cells found after CldU pulse-chase and IdU pulse in the bulge fraction (1.88%) was somehow greater than the percentage of double positive cells (0.75%) that would be expected (% CldU$^+$ cells × % IdU$^+$ cells) if bulge LRCs divide at the same rate as the rest of the bulge cells (15%), suggesting that some bulge SCs might divide more than once during SC activation. Overall, the presence of double CldU$^+$IdU$^+$ positive cells demonstrates directly that chromosome segregation occurs randomly in bulge SCs during HF regeneration.

To directly test whether bulge SCs divide more than once during HF regeneration, we combined the former double-labeling experiment together with a novel transgenic approach, allowing the quantification of cell divisions independently of DNA labeling. To this end, we used transgenic mice co-expressing the tetracycline inducible transcriptional repressor (tTA or Tet^{off} and H2B-GFP) under the control of a tetracycline-responsible element (TRE-mCMV-H2B-GFP), which has been used successfully to isolate and characterize slow cycling cells of the skin epidermis [4]. In the absence of tetracycline (Dox), all epidermal cells express a high level of H2B-GFP. Following Dox administration, the transcription of H2B-GFP is totally repressed and consequently, upon division, cells dilute the H2B-GFP label equally among the two daughter cells [4, 22, 24]. Therefore, the intensity of H2B-GFP expression in a given cell is directly proportional to the number of divisions accomplished during the chase period. As described above, we first administered CldU from PD3 to PD5 and, after chasing took place for 18 days, we administered from PD23 to PD25 IdU together with Dox to chase H2B-GFP and analyzed the co-expression of CldU, IdU, and H2B-GFP in the back skin of mice at PD26 (Fig. 3A).

As illustrated in Figure 3B, bulge LRCs that did not divide during the chase period expressed a higher level of H2B-GFP than LRCs or bulge cells that did divide and incorporate IdU, indicating that the H2B-GFP system faithfully recapitulates the history of cell divisions in the bulge. We used FACS analysis to quantify the number of divisions that the double-labeled cells had undergone during this chase period. During each round of cell division, H2B-GFP fluorescence is divided by two in the daughter cells, so by defining the brightest peak as no SC division, we can infer how many times a cell had divided during the chase period. As illustrated in Figure 3C, the CldU LRCs that did not incorporate IdU during the chase period showed one homogenous peak corresponding to the brightest H2B-GFP fluorescence, demonstrating that these cells did not divide during the chase, whereas all CldU bulge LRCs that incorporate IdU during the pulse presented a lower H2B-GFP fluorescence intensity as expected. All cells that divided and incorporated IdU present a lower level of fluorescence compared to the brightest H2B-GFP cells. Subsequent quantification of the number of divisions that a cell accomplished during the chase period based on H2B-GFP fluorescence clearly demonstrates that the vast majority of LRCs labeled only with CldU have not undergone cell division, whereas the double CldU$^+$IdU$^+$ positive cells divided in average two times during the chase period (Fig. 3D). Cells positive for IdU without having undergone any division most probably represent cells within the S-phase, which have already incorporated the uridine analog but have not divided yet. Our results revealed that

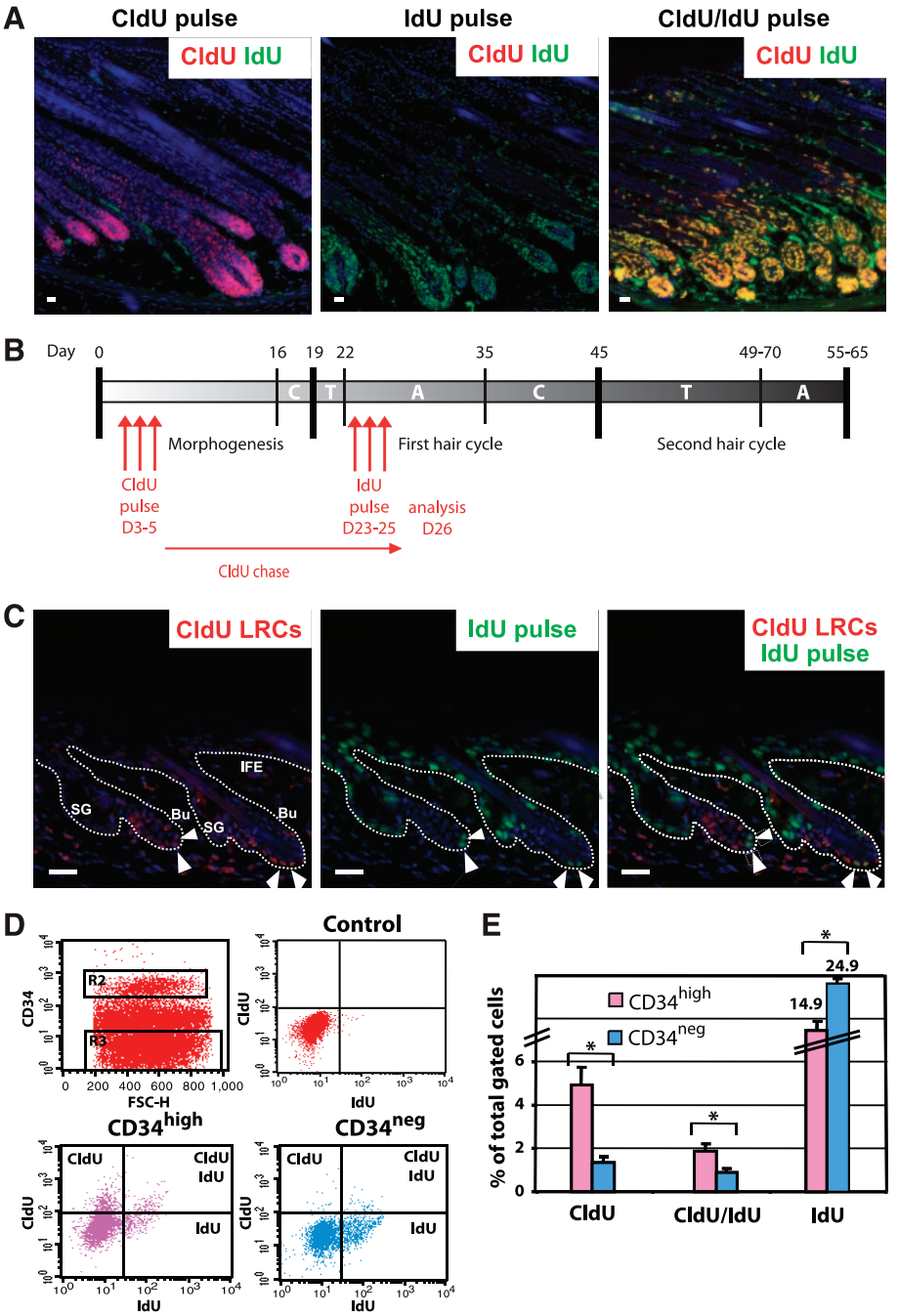

Figure 2. Bulge label-retaining cells incorporate the second uridine analog upon stem cell division. **(A):** Immunostaining of CldU and IdU on skin sections from mice pulsed only with CldU (left panel), only with IdU (middle panel), or with both analogs (right panel). No cross reaction of the antibodies is observed. **(B):** Chart illustrating hair follicle morphogenesis, the two first hair cycles and the double-labeling protocol used in these experiments. CldU was administrated twice daily from PD3 to PD5 and chased thereafter. IdU was injected twice per day from PD23 to PD25 (PD, postnatal day). Mice were sacrificed and analysis was performed at PD26. **(C):** Representative fluorescent images of CldU and IdU incorporation of the back skin. $CldU^+$ LRCs are shown in red in the left panel. In the middle panel, the actively proliferating cells during the IdU pulse from PD23 to PD25 are detected by the IdU immunostaining (green). 6-Diamidino-2-phenylindole (blue) stains the nuclei. The right panel represents a merged image. Arrowheads are pointing to the $CldU^+IdU^+$ double-labeled cells. **(D):** Fluorescence-activated cell sorting (FACS) analysis of epidermal cells from a representative experiment on PD26 stained for CD34, gated on the $CD34^{high}$ and $CD34^{neg}$ (respectively gates R2 and R3 on the left plot), and analyzed for the expression of CldU and IdU. The control plot shows the same staining procedure and analysis on mice that did not receive nucleotide analog. **(E):** Histogram representing the quantification of the CldU and IdU incorporation by FACS, demonstrating that around 40% of the LRCs do incorporate the second nucleotide analog during the next anagen, in contrast to what is predicted by the immortal strand hypothesis. The data are expressed as mean ± SEM of results obtained from 25 mice. Scale bars: 20 μm. Abbreviations: *, $p < .05$; *t* test; A, anagen; Bu, bulge; C, catagen; CldU, 5-chloro-2-deoxy-uridine; HS, hair shaft; IdU, 5-iodo-2-deoxy-uridine; IFE, interfollicular epidermis; LRCs, label-retaining cells; SG, sebaceous gland; T, telogen.

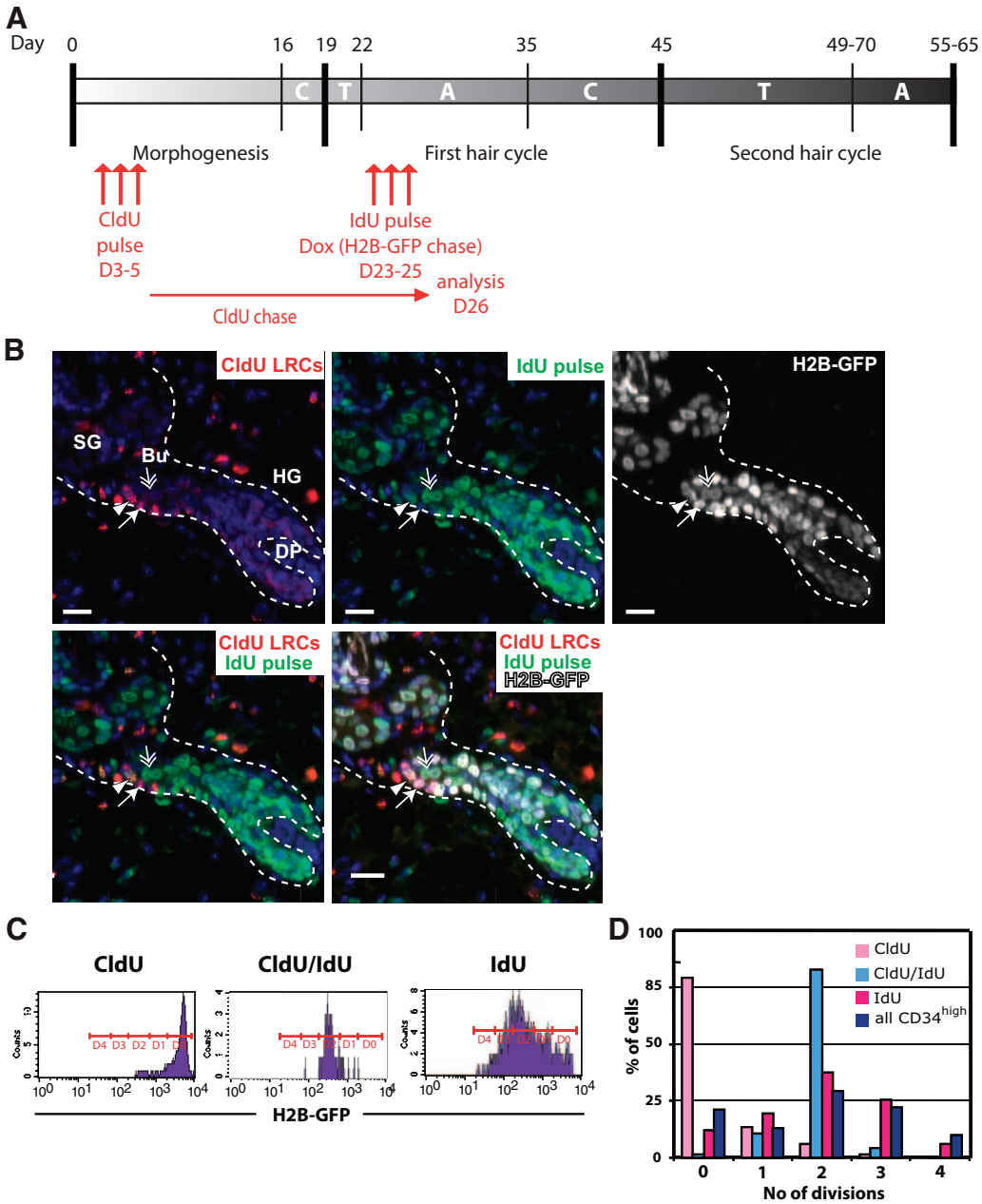

Figure 3. Bulge stem cells retain the newly formed DNA strands after more than one division. **(A):** Chart illustrating the experimental protocol used in these experiments. Mice were pulsed twice daily with CldU from PD3 to PD5 (PD, postnatal day), followed by a chase period until the next anagen. From PD23 to PD25 IdU was administered twice per day, together with doxycycline administration to chase the H2B-GFP. Mice were sacrificed and skin cells were analyzed on PD26. **(B):** Immunostaining of CldU and IdU, and GFP expression of the back skin of PD26 TRE-mCMV-H2B-GFP: K5Tet^{off} mice. Antibodies and epifluorescent markers are color-coded. Arrow points to a CldU^{+}, H2B-GFP^{bright} LRC, indicating that this cell did not divide during the chase period. Double arrow shows two H2B-GFP^{low} IdU+ cells, indicating these two cells divided during the chase. Note the higher expression levels of GFP in the bulge, in contrast to the rest of the hair follicle segments, which exhibit higher proliferation rates. Arrowhead points out a double positive CldU^{+}IdU^{+} cell that is also H2B-GFP^{low}, indicating that this cell divided during IdU administration, incorporated IdU, and diluted the H2B-GFP. **(C):** Representative Fluorescence-activated cell sorting analysis of H2B-GFP expression within the CldU^{+}, CldU^{+}IdU^{+}, and IdU^{+} subpopulations of CD34^{high} cells. **(D):** Quantification of cell divisions, based on H2B-GFP expression **(C)**. The vast majority of cells expressing only CldU did not divide, whereas CldU^{+}IdU^{+} double-labeled cells had undergone on average two cell divisions. Scale bars: 20 μm. Abbreviations: A, anagen; Bu, bulge; C, catagen; CldU, 5-chloro-2-deoxy-uridine; DP, dermal papillae; GFP, green fluorescent protein; HG, hair germ; IdU, 5-iodo-2-deoxy-uridine; LRCs, label-retaining cells; SG, sebaceous gland; T, telogen.

during SC activation that accompanied the initiation of follicle regeneration some bulge SCs proliferated actively and accomplished on average two divisions from PD23 to PD25. The persistence of CldU^{+}IdU^{+} double-labeled cells in SCs that had undergone more than one cell division is totally incompatible with the immortal strand hypothesis.

Double-Labeled Bulge Cells Represent Functional Stem Cells of the Hair Follicle

To determine whether these double CldU^{+}IdU^{+} labeled bulge cells still represent bona fide SCs, which are able to sense their surrounding environment and proliferate upon stimulation, we

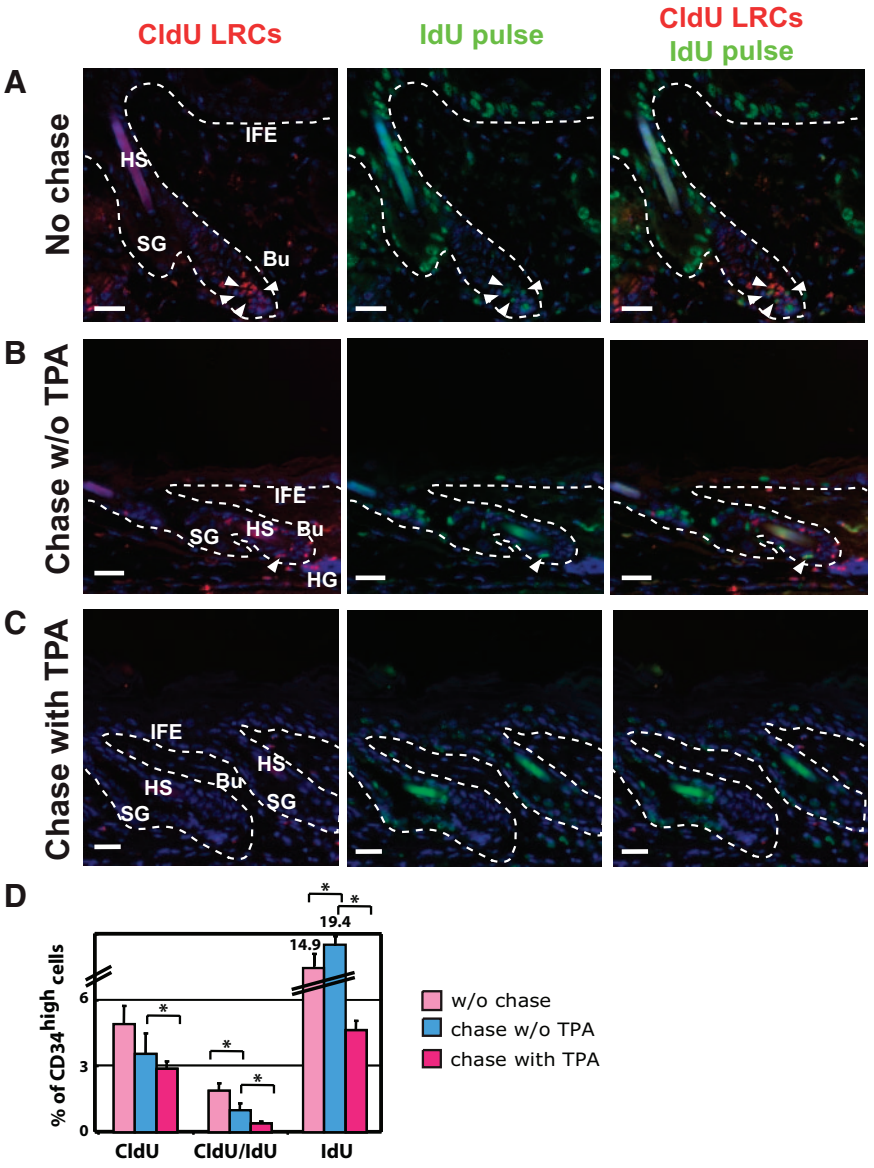

Figure 4. Bulge stem cells (SCs) are able to proliferate upon stimulation and dilute their label during cell division. **(A):** Immunostaining of CldU and IdU of the back skin of a PD25 (PD, postnatal day) animal, after the 3-day IdU pulse. Antibodies are color-coded. Many $CldU^+$ LRCs are observed in the bulge area. Arrowheads point to double positive cells, corresponding to LRCs that have incorporated the second analog upon division. **(B):** Immunostaining of CldU and IdU of the back skin of a PD30 mouse, treated with acetone for 5 days after IdU administration. The fewer LRCs, both $CldU^+$ and double positive $CldU^+IdU^+$ (pointed to by the arrowhead), demonstrate that bulge SCs proliferated during this period. **(C):** Immunostaining of CldU and IdU of the back skin sections from PD30 mouse, treated with TPA for 5 days after IdU administration. The absence of LRCs (both single $CldU^+$ and double $CldU^+IdU^+$ labeled) indicates that bulge SCs, including double-labeled cells, divided actively following proliferative stimuli and dilute their labels. **(D):** Quantification by fluorescence-activated cell sorting analysis of the incorporation of CldU and IdU within the $CD34^{high}$ population after 5 days of chase following IdU administration, with or without TPA treatment. The lower percentage of labeled cells ($CldU^+$, $CldU^+IdU^+$, and IdU^+) demonstrates that all bulge SCs respond to the application of TPA and proliferate, irrespective of their proliferation history. Values represent the means ± SEM of results obtained from four mice. Scale bars: 20 μm. Abbreviations: $*$, $p < .05$; *t* test; Bu, bulge; CldU, 5-chloro-2-deoxy-uridine; HG, hair germ; HS, hair shaft; IdU, 5-iodo-2-deoxy-uridine; IFE, interfollicular epidermis; LRCs, label-retaining cells; SG, sebaceous gland; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

treated mice, after the last administration of IdU, for 5 days with TPA, which stimulates the proliferation of bulge SCs at a rate of about 10% per day [13, 22]. As demonstrated in Figure 4A–4C and quantified in Figure 4D, 5 days of TPA treatment resulted in a stronger decrease of $CldU^+$, IdU^+, and $CldU^+IdU^+$ double positive bulge cells as compared to that of the acetone-treated mice, indicating that the double $CldU^+IdU^+$ labeled bulge cells are still able to proliferate upon stimulation and dilute their labeling following SC division as expected by the random segregation model.

Pulse-Chase and Double-Labeling Studies Performed Exclusively in Adult Animals Demonstrate That Chromosome Segregation Occurs Only Randomly in Hair Follicle Stem Cells

In contrast to pulse-chase experiments performed during morphogenesis, the immortal strand hypothesis predicts that administration of nucleotide analogs during tissue homeostasis will not result in LRCs in adult SCs. Under steady-state conditions, adult SCs undergo on average asymmetrical self-renewal to maintain the SC pool constant. Accordingly, the incorporation of nucleotide analogs in SCs during DNA synthesis should be lost in the subsequent division to keep only immortal strands in SCs. To test this hypothesis, we administered uridine analogs during the proliferation stage of bulge SCs that accompanied the first cycle of hair regeneration in adult mice (Fig. 5A). Administration of uridine analogs from PD23 to PD25 resulted in the incorporation of nucleotide analogs in about 15% of $CD34^+$ bulge SCs as determined by immunofluorescence and FACS analysis (Fig. 5B, 5C). After a month of chase, LRCs are found preferentially within the bulge region, similarly to what is found in pulse-chase experiments during HF morphogenesis (Fig. 5D). Quantification by FACS analysis revealed that 84% of the BrdU LRCs was $CD34^{high}$ cells, corresponding to 1.24% ± 0.41% of all bulge cells, whereas $BrdU^+$ LRCs outside the SC population were very rare (0.24% ± 0.1%) (Fig. 5E). These results reveal that bulge SCs retain DNA labels, not only after pulse-chase experiments performed during morphogenesis but also during tissue homeostasis in adult mice, a characteristic that is not expected from the immortal strand hypothesis but only from their relative quiescence.

As the type of chromosome segregation might be different between developmental progenitors and adult SCs, we

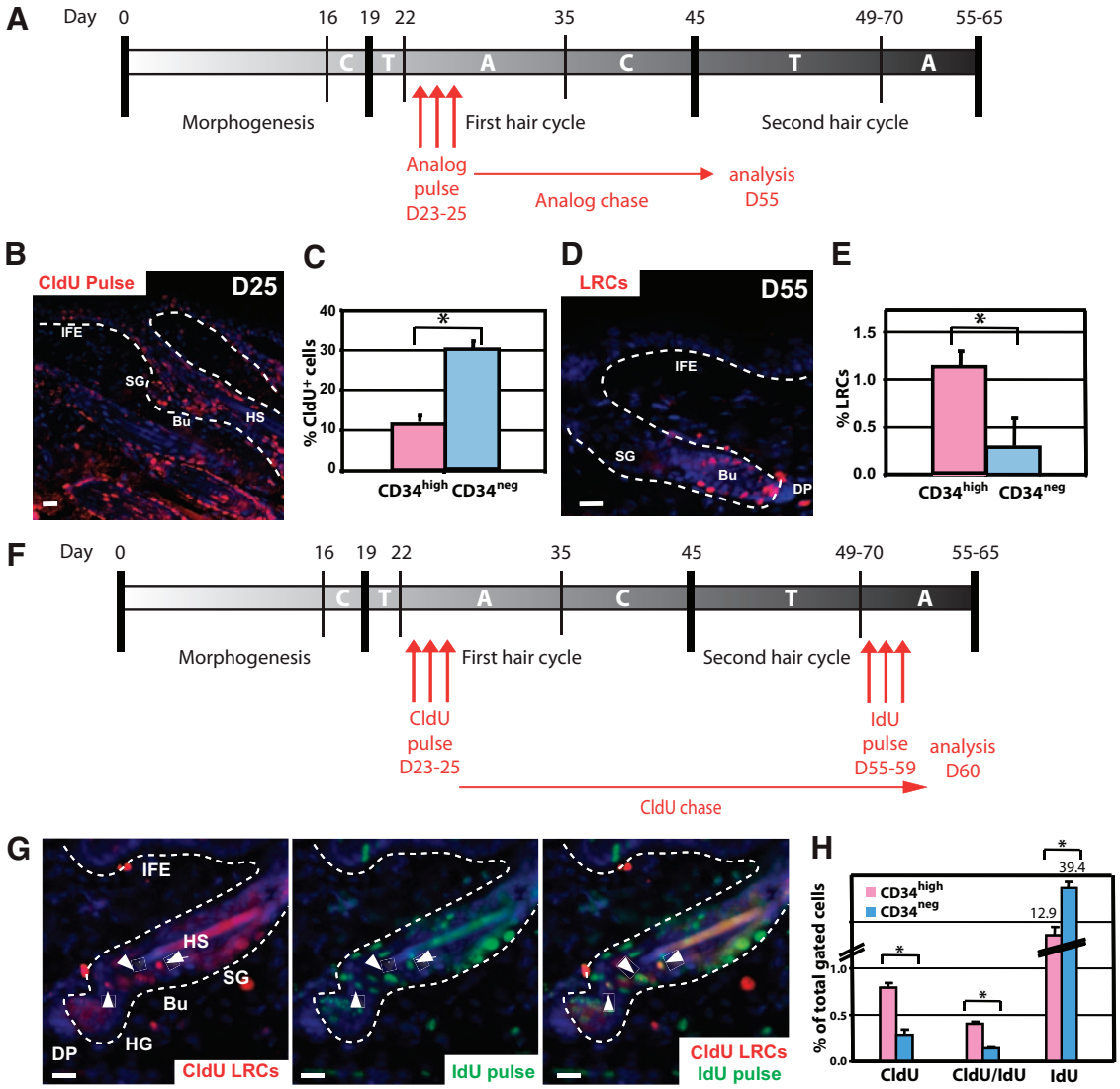

Figure 5. Adult bulge stem cells (SCs) retain DNA labels and segregate their chromosomes randomly. **(A):** Chart illustrating the protocol used in the following experiments. Uridine analog was administered twice daily from PD23 to PD25 (PD, postnatal day) and chased for 1 month thereafter. Mice were sacrificed and analysis was done on PD55. **(B):** Immunostaining of back skin sections of PD25 mice pulsed with CldU from PD23 to PD25. CldU positive cells, shown in red, are observed throughout the epidermis, including the bulge SCs. **(C):** Quantification by fluorescence-activated cell sorting (FACS) of $CldU^+$ cells within the $CD34^{high}$ population in PD25 mice. Bulge SCs incorporated CldU analog, although to a lesser extent than the rest of the epidermal cells, as expected by their greater quiescence. **(D):** Immunostaining of CldU in back skin sections of PD55 mice pulsed with CldU from PD23 to PD25 and chased for a month. $CldU^+$ LRCs are detected specifically in the bulge. **(E):** Quantification by FACS of $CldU^+$ cells within the $CD34^{high}$ population in PD55 mice, showing that the vast majority of LRCs are located within the bulge region, in contradiction to the immortal strand hypothesis prediction that label retention cannot be a characteristic of adult SCs. **(F):** Chart demonstrating the double-labeling protocol used in these experiments. CldU was administered twice a day from PD23 to PD25; IdU was injected twice per day from PD55 to PD59 and mice were analyzed at PD60. **(G):** Immunofluorescence of back skin sections from PD60 mice. $CldU^+$ LRCs are shown in red (left panel) and IdU^+ proliferating cells in green (middle panel). CldU and IdU incorporation are merged in the right panel. Arrowheads are pointing to double positive $CldU^+IdU^+$ cells in the bulge. **(H):** Histograms representing the quantification of the CldU and IdU labeling by FACS analysis, showing that half of the LRCs do incorporate the second nucleotide analog during the next anagen, as expected by random DNA strand segregation. Bars represent the mean values ± SEM from the results obtained from 3, 6, and 10 mice respectively for **(C)**, **(E)**, and **(H)**. Scale bars: 20 μm. Abbreviations: *, $p < .05$; *t* test; A, anagen; Bu, bulge; C, catagen; CldU, 5-chloro-2-deoxy-uridine; DP, dermal papillae; HG, hair germ; HS, hair shaft; IdU, 5-iodo-2-deoxy-uridine; IFE, interfollicular epidermis; LRCs, label-retaining cells; SG, sebaceous gland; T, telogen.

investigated how chromosomes are segregated specifically in the adult bulge SCs, using double-labeling experiments performed solely during adulthood. We first administered a pulse of CldU in adult mice from PD23 to PD25 during the first postnatal hair cycle and then chased until the subsequent hair cycle. During PD55–PD59, when a fraction of HFs enters the new anagen, mice received a pulse of IdU and were analyzed thereafter (Fig. 5F, 5G). These pulse-chase experiments in adult mice resulted in the presence of $CldU^+$ LRCs in the bulge as demonstrated above, whereas SC proliferation during early anagen could be detected by the presence of IdU in some bulge cells, including the $CldU^+$ LRCs (Fig. 5G). FACS quantification (Fig. 5H) revealed that nearly half of the total $CldU^+CD34^+$ bulge LRCs did incorporate IdU during the pulse period, which again is contradictory to what is expected from the immortal strand model.

DISCUSSION

Altogether, our analysis of chromosome segregation in multipotent bulge SCs using various in vivo approaches are not consistent with the predictions based on the immortal strand hypothesis. In contrary, our results rather support the belief that label retention reflects mainly the relative quiescence of bulge SCs during the hair cycle. The persistence of $CldU^+IdU^+$ double-labeled cells in SCs during HF morphogenesis after accomplishment of more than one cell division is totally incompatible with the immortal strand hypothesis and demonstrated unambiguously that the majority of bulge SCs segregate their DNA templates randomly. Our results are consistent with the recent demonstration that H2B-GFP and BrdU are co-segregated following cell division during pulse-chase experiments and the level of H2B-GFP fluorescence is proportional to the intensity of BrdU immunostaining, which also indicated that bulge cells segregate their chromosomes randomly [22].

Like most epithelial SCs, HSCs are also more quiescent than their transit amplifying progenies [17, 25–27]. However, in the hematopoietic tissue, label retention does not seem to be a specific marker of HSCs because HSCs are very rare cells ($<0.01\%$) and due to the presence of more frequent slow cycling cells in the bone marrow that are not HSCs [17]. In the skin epidermis, about a month of chase is required to specifically mark bulge SCs [12], whereas the optimal chase period to specifically mark SCs may differ greatly between different tissues and depend on the activation state of SCs during the chase period.

The relative quiescence of bulge SCs is not constant throughout the hair cycle. During the resting stage bulge SCs are very quiescent. However, during the early stage of hair follicle regeneration, bulge SCs proliferate actively to provide the cells that will form the new hair [23, 28, 29]. Our double-labeling experiments coupled with H2B-GFP label retention showed that during the early stage of hair follicle regeneration (e.g., D23–25), about 15% of bulge cells accomplished more than one cell division. Our data are consistent with the recently published bulge proliferation kinetics observed over the hair cycle, which demonstrated that the majority of bulge cells divide between two and five times during the course of one hair cycle [22]. It is generally assumed that bulge cells consisted of a homogenous SC population. Interestingly, our double-labeling studies demonstrated that the proportion of double-labeled cells is more frequent than the one theoretically expected if all bulge cells divide at the same rate, suggesting that bulge LRCs might be preferentially recruited and activated during early stage of hair follicle regeneration.

It has been suggested that the frequency of asymmetrical inheritance of template strands increase considerably during muscle regeneration, which involved both symmetric and asymmetric cell divisions [30]. However, the similar results we obtained from double-labeling experiments performed during morphogenesis and adult homeostasis, as well as those following TPA treatment, which stimulate SC division similarly to tissue repair, demonstrated that bulge SCs segregate their DNA templates randomly irrespective of their activation state.

The mechanism underlying asymmetrical strand segregation reported in other types of SCs such as muscle, intestinal, neuronal, or mammary SCs remains unclear [8–11]. Clearly, further in vivo studies using CldU/IdU double labeling together with specific SC markers [31–33] will be needed to understand the significance of these findings and whether these results could be the consequences of sister chromatin exchange, in vitro culture of SCs, lack of SC specific markers, poor sensitivity of the previously used methods for DNA labeling studies, and lack of quantitative data in some of these studies [16].

Nevertheless, we cannot rule out the presence within the $CD34^+$ population of some very rare cells, below the detection limits of the experimental methods used here, that segregate their DNA strands asymmetrically and may explain the long-term persistence of very rare LRCs within the skin epidermis [34]. Similarly, we cannot exclude the possibility that only a small fraction of the chromosomes presented a biased mode of DNA strand segregation, like what has been suggested to occur during embryonic stem cell differentiation into endodermal cells [35].

CONCLUSION

Our data demonstrate that most developmental progenitors and adult bulge SCs segregate their DNA templates randomly during tissue morphogenesis and homeostasis and that immortal strand segregation does not represent a genome protection mechanism in adult bulge SCs. Our results demonstrate that label retention in skin epidermis is rather a sign of the relative quiescence of bulge SCs and not a mark of the asymmetrical strand segregation. The absence of asymmetrical strand segregation observed in bulge SCs is similar to what has been recently observed in hematopoietic SCs, suggesting that random DNA strand segregation may be a general mechanism in adult SCs rather than an exception.

ACKNOWLEDGMENTS

We thank the members of Blanpain and Vanderhaeghen laboratories for their constructive comments during the realization of this study. We thank C. Govaerts and P. Vanderhaeghen for their critical comments on the manuscript. We also thank E. Fuchs and A. Glick for providing the mouse strains. This work was supported by a "mandat d'impulsion scientifique" of the FNRS, a career development award of the Human Frontier Science Program Organization (HFSPO), from a research grant of the Schlumberger Foundation, from the program CIBLES of the Wallonia Region, and from a starting grant of the European Research Council (ERC). C.B. is a chercheur qualifie of the FRS/FNRS. A.C. is a research fellow of the FRS/FRIA.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

REFERENCES

1 Morrison SJ, Spradling AC. Stem cells and niches: mechanisms that promote stem cell maintenance throughout life. Cell 2008;132:598–611.
2 Blanpain C, Fuchs E. Epidermal stem cells of the skin. Annu Rev Cell Dev Biol 2006;22:339–373.
3 Blanpain C, Lowry WE, Geoghegan A et al. Self-renewal, multipotency, and the existence of two cell populations within an epithelial stem cell niche. Cell 2004;118:635–648.
4 Tumbar T, Guash G, Greco V et al. Defining the epithelial stem cell niche in skin. Science 2004;303:359–363.
5 Morris RJ, Liu Y, Marles L et al. Capturing and profiling adult hair follicle stem cells. Nat Biotechnol 2004;22:411–417.

6 Cairns J. Mutation selection and the natural history of cancer. Nature 1975;255:197–200.
7 Potten CS, Hume WJ, Cairns J. The segregation of DNA in epithelial stem cells. Cell 1978;15:899–906.
8 Potten CS, Owen G, Booth D. Intestinal stem cells protect their genome by selective segregation of template DNA strands. J Cell Sci 2002;115: 2381–2388.
9 Karpowicz P, Morshead C, Kam A et al. Support for the immortal strand hypothesis: neural stem cells partition DNA asymmetrically in vitro. J Cell Biol 2005;170:721–732.
10 Smith GH. Label-retaining epithelial cells in mouse mammary gland divide asymmetrically and retain their template DNA strands. Development 2005;132:681–687.
11 Shinin V, Gayraud-Morel B, Gomes D et al. Asymmetric division and cosegregation of template DNA strands in adult muscle satellite cells. Nat Cell Biol 2006;8:677–687.
12 Cotsarelis G, Sun TT, Lavker RM. Label-retaining cells reside in the bulge area of pilosebaceous unit: implications for follicular stem cells, hair cycle, and skin carcinogenesis. Cell 1990;61:1329–1337.
13 Braun KM, Niemann C, Jensen UB et al. Manipulation of stem cell proliferation and lineage commitment: visualisation of label-retaining cells in wholemounts of mouse epidermis. Development 2003;130:5241–5255.
14 Braun KM, Watt FM. Epidermal label-retaining cells: background and recent applications. J Investig Dermatol Symp Proc 2004;9:196–201.
15 Rando TA. The immortal strand hypothesis: segregation and reconstruction. Cell 2007;129:1239–1243.
16 Lansdorp PM. Immortal strands? Give me a break. Cell 2007;129: 1244–1247.
17 Kiel MJ, He S, Ashkenazi R et al. Haematopoietic stem cells do not asymmetrically segregate chromosomes or retain BrdU. Nature 2007; 449:238–242.
18 Trempus CS, Morris RJ, Bortner CD et al. Enrichment for living murine keratinocytes from the hair follicle bulge with the cell surface marker CD34. J Invest Dermatol 2003;120:501–511.
19 Horsley V, Alipantis AO, Polak L et al. NFATc1 balances quiescence and proliferation of skin stem cells. Cell 2008;132:299–310.
20 Nowak JA, Polak L, Pasolli HA et al. Hair follicle stem cells are specified and function in early skin morphogenesis. Cell Stem Cell 2008;3:33–43.
21 Kuroki T, Murakami Y. Random segregation of DNA strands in epidermal basal cells. Jpn J Cancer Res 1989;80:637–642.
22 Waghmare SK, Bansal R, Lee J et al. Quantitative proliferation dynamics and random chromosome segregation of hair follicle stem cells. EMBO J 2008;27:1309–1320.
23 Wilson C, Cotsarelis G, Wei ZG et al. Cells within the bulge region of mouse hair follicle transiently proliferate during early anagen: heterogeneity and functional differences of various hair cycles. Differentiation 1994;55:127–136.
24 Brennand K, Huangfu D, Melton D. All beta Cells Contribute Equally to Islet Growth and Maintenance. PLoS Biol 2007;5:e163.
25 Blanpain C, Horsley V, Fuchs E. Epithelial stem cells: turning over new leaves. Cell 2007;128:445–458.
26 Qian H, Buza-Vidas N, Hyland CD et al. Critical role of thrombopoietin in maintaining adult quiescent hematopoietic stem cells. Cell Stem Cell 2007;1:671–684.
27 Yoshihara H, Arai F, Hosokawa K et al. Thrombopoietin/MPL signaling regulates hematopoietic stem cell quiescence and interaction with the osteoblastic niche. Cell Stem Cell 2007;1:685–697.
28 Ito M, Kizawa K, Hamada K et al. Hair follicle stem cells in the lower bulge form the secondary germ, a biochemically distinct but functionally equivalent progenitor cell population, at the termination of catagen. Differentiation 2004;72:548–557.
29 Lowry WE, Blanpain C, Nowak JA et al. Defining the impact of beta-catenin/Tcf transactivation on epithelial stem cells. Genes Dev 2005;19:1596–1611.
30 Conboy MJ, Karasov AO, Rando TA. High incidence of non-random template strand segregation and asymmetric fate determination in dividing stem cells and their progeny. Plos Biol 2007;5:e102.
31 Shackleton M, Vaillant F, Simpson KJ et al. Generation of a functional mammary gland from a single stem cell. Nature 2006;439:84–88.
32 Stingl J, Eirew P, Ricketson L et al. Purification and unique properties of mammary epithelial stem cells. Nature 2006;439:993–997.
33 Barker N, van Es JH, Kuipers J et al. Identification of stem cells in small intestine and colon by marker gene Lgr5. Nature 2007;449:1003–1007.
34 Morris RJ, Potten CS. Highly persistent label-retaining cells in the hair follicles of mice and their fate following induction of anagen. J Invest Dermatol 1999;112:470–475.
35 Armakolas A, Klar AJ. Cell type regulates selective segregation of mouse chromosome 7 DNA strands in mitosis. Science 2006;311: 1146–1149.