SUPPLEMENTARY MATERIAL

SUPPLEMENTARY METHODS

Flow-cytometric isolation of HSCs

Bone marrow cells were flushed from the long bones (tibias and femurs) with Hank's buffered salt solution without calcium or magnesium, supplemented with 2% heat-inactivated calf serum (GIBCO, Grand Island, NY; $HBSS^+$). Cells were triturated and filtered through nylon screen (45 um, Sefar America, Kansas City, MO) to obtain a single cell suspension. For isolation of $Flk2^-lineage^-Sca\text{-}1^+c\text{-}kit^+$ HSCs, whole bone marrow cells were incubated with PE-conjugated monoclonal antibodies against lineage markers including B220 (6B2), CD3 (KT31.1), CD4 (GK1.5), CD8 (53-6.7), Gr-1 (8C5), Mac-1 (M1/70), Flk-2 (A2F10.1), Ter119, and IgM in addition to FITC-conjugated anti-Sca-1 (Ly6A/E) and biotin-conjugated anti-c-kit (2B8) antibodies, followed by streptavidin APC-Cy7. For isolation of $CD150^+CD48^-CD41^-lineage^-Sca\text{-}1^+c\text{-}kit^+$ HSCs, whole bone marrow cells were incubated with PE-conjugated anti-CD150 (TC15-12F12.2; BioLegend, San Diego, California), FITC-conjugated anti-CD48 (HM48-1; BioLegend), FITC-conjugated anti-CD41 (MWReg30; BD Pharmingen, San Diego, California), APC-conjugated anti-Sca-1 (E13-6.7), and biotin-conjugated anti-c-kit (2B8) antibody, in addition to FITC-conjugated antibodies against Ter119, B220 (6B2), Gr-1 (8C5) and CD2 (RM2-5). HSCs were frequently pre-enriched by selecting $c\text{-}kit^+$ cells using paramagnetic microbeads and autoMACS (Miltenyi Biotec, Auburn, CA). All flow-cytometry was performed on a FACSVantage SE-dual laser, three-line flow-cytometer (Becton-Dickinson).

BrdU staining

Staining for BrdU on slides was performed as previously described using an anti-BrdU antibody (clone BU1/75, Accurate Chemical and Scientific Corp, Westbury, NY)[15]. HSCs were sorted and then resorted (to ensure purity) onto glass slides in 25 to 100 cell spots, allowed to dry for 1 hour and stored at –80°C for up to 4 weeks. Slides were thawed at room temperature for 15 minutes, fixed in 70% ethanol at –20°C for 30 minutes, rinsed in 0.1M phosphate buffer (PB) twice, incubated in 2N HCl with 0.8% Triton in PB for 30 minutes, incubated in 0.1M Sodium Borate (pH 8.5) for 15 minutes, then rinsed in 0.1M PB at room temperature, at 37°C, and at room temperature again. The slides were then incubated with 0.3% Triton in 0.1M PB supplemented with 5% Goat Serum for 1 hour at 4°C. Slides were incubated at 4°C overnight with the primary anti-BrdU antibody that specifically recognizes BrdU and CldU but does not recognize IdU (clone BU1/75, Accurate Chemical and Scientific Corp.). Slides were then rinsed in 0.1M PB twice and incubated with Alexa488-conjugated goat anti-rat IgG (Invitrogen-Molecular Probes, Carlsbad, California) for 2 hours at room temperature. Slides were rinsed in 0.1M PB twice and incubated with DAPI for 1 hour at room temperature. Finally, slides were rinsed three times in 0.1M PB, the excess buffer was shaken off and the slides were mounted in 70% glycerol in PBS. Images were gathered using an Olympus BX-51 florescence microscope equipped with a Cooke Pixelfly CCD camera.

In some experiments, BrdU incorporation was measured by flow-cytometry using an antibody directly conjugated to allophycocyanin (APC; APC BrdU flow kit, BD Pharmingen). Sorted samples were fixed and permeablized according to manufacturer's instructions, incubated in 2N HCl for 30 minutes at room temperature, neutralized in 0.1M Sodium Borate (pH 8.5), washed in 0.5% Triton in DPBS, stained with anti-BrdU APC and resuspended in DAPI (10

μg/ml) prior to FACS analysis. All flow-cytometry was performed on a FACSVantage SE-dual laser, three-line flow-cytometer (Becton-Dickinson).

CldU and IdU double-labeling

Slides were processed as for BrdU analysis with the addition of a subsequent set of staining steps using a second anti-IdU antibody (clone B44, BD Pharmingen) that does not recognize CldU. Slides were incubated at room temperature in anti-IdU antibody for 2 to 3 hours. Immunofluorescence was developed using a Cy3-conjugated anti-mouse IgG secondary antibody with minimal cross reactivity to rat IgG (Jackson Immunoresearch). Cells isolated from mice that had only received CldU, or only received IdU, or neither were processed in parallel with experimental samples, and demonstrated no cross-reactivity or background staining from primary or secondary antibodies.

Analysis of cell cycle distribution and cell death in HSCs

Cell cycle distribution was analyzed by Hoechst 33342 (Invitrogen-Molecular Probes, Carlsbad, California) staining. $CD150^{+}CD48^{-}CD41^{-}lineage^{-}Sca\text{-}1^{+}c\text{-}kit^{+}$ HSCs were sorted and resorted into ice-cold 70% ethanol and stored at –20ºC overnight. Cells were resuspended in PBS containing 0.02 mg/ml Hoechst 33342, incubated for 30 minutes and analyzed by flow-cytometry using a UV laser.

For activated caspase-3 staining, frozen slides bearing sorted $CD150^{+}CD48^{-}CD41^{-}lineage^{-}Sca\text{-}1^{+}c\text{-}kit^{+}$ HSCs or sections through E11 mouse forebrain were thawed at room temperature for 10 minutes, fixed at room temperature in 10% buffered neutral formalin (VWR, West Chester, Pennsylvania) for 10 minutes, rinsed in 0.1M PB twice, and blocked with 0.3%

Triton in 0.1M PB supplemented with 5% Goat Serum for 1 hour at 4°C. Slides were then incubated with anti-activated caspase-3 antibody (BD Pharmingen, San Diego, California) at room temperature for 2 hours. Slides were then rinsed in 0.1M PB twice and incubated with Alexa488-conjugated goat anti-rabbit IgG (Invitrogen-Molecular Probes, Carlsbad, California) for 1 hour at room temperature. Slides were rinsed in 0.1M PB twice and incubated with DAPI for 30 minutes at room temperature. Finally, slides were rinsed three times in 0.1M PB, the excess buffer was shaken off and the slides were mounted in 70% glycerol in PBS. Images were collected using a florescence microscope.

To test for the incorporation of BrdU due to DNA repair, BrdU was administered to adult mice for 12 hours followed by irradiation with 100 rad from a Gammacell40 Extractor (MDS Nordion, Ottawa, ON, Canada) followed by 48 hours of further BrdU administration. $CD150^{+}CD48^{-}CD41^{-}lineage^{-}Sca\text{-}1^{+}c\text{-}kit^{+}$ HSCs were then sorted onto slides and stained for BrdU as described above.

Mathematical models of BrdU uptake and retention

To model the uptake and retention of BrdU in a population of stem cells we assumed that for days 0 through T the stem cells are exposed to adequate BrdU so that cells incorporate BrdU when they divide. Based on our data a random 6.0% of HSCs enter cycle each day. At day T, the BrdU supply is removed and the level of BrdU incorporated into the chromosomes decreases for every cell division after day T. The rate at which BrdU is diluted from cells during this chase period depends upon how the cells segregate their chromosomes. If chromosomes segregate randomly, then irrespective of whether stem cells divide asymmetrically or symmetrically with respect to daughter cell fate, BrdU labeled chromosomes will stochastically become diluted over

time: on average the BrdU label will be diluted by half during each round of division and multiple divisions will be required to dilute the BrdU label to the point that it is no longer detectable by immunohistochemistry. This is modeled as case 1 below for asymmetrically dividing cells. In contrast, according to the immortal strand model [2], stem cells divide asymmetrically under steady-state conditions and BrdU is preferentially incorporated into newly synthesized DNA strands that are asymmetrically segregated into differentiating daughter cells with each round of division. Under these assumptions, modeled as case 2 below, stem cells retain only the unlabeled older DNA strands once BrdU is withdrawn.

Case 1: Random segregation of chromosomes (see Figure 1b)

BrdU Incorporation: When chromosomes are allowed to segregate randomly, BrdU could be incorporated into one or two DNA strands within each chromosome, depending on the number of times a stem cell divides during the period of BrdU incorporation and the way in which the chromosomes segregate. To model the rate of BrdU incorporation:

y_0 = fraction of cells without BrdU

y_1 = fraction of cells with one strand $BrdU^+$ after only one division

y_2 = fraction of cells with both strands $BrdU^+$ after two or more divisions

α = the proliferation rate of stem cells (we observed 6.0% of HSCs enter cycle per day)

Note as well that cell death was not incorporated into this model because we did not observe significant cell death or changes in HSC frequency during the experiments.

The equations for uptake:

$$\frac{dy_0}{dt} = -\alpha y_0$$

Equation 1: Cells leave the y_0 population when they divide.

$$\frac{dy_1}{dt} = -\alpha y_1 + \alpha y_0$$

Equation 2: Cells from the y_0 population are added into the y_1 population through the incorporation of BrdU into one of the DNA strands. Cells leave the y_1 population when they divide.

$$\frac{dy_2}{dt} = \alpha y_1$$

Equation 3: Cells from the y_1 population are added into the y_2 population through further incorporation of BrdU through cell division.

To determine the frequency of $BrdU^+$ stem cells at any time after the addition of BrdU we solve the system of ordinary differential equations with all HSCs initially being unlabeled prior to BrdU administration. Note that similar equations have previously been used to model BrdU incorporation and depletion from other cells [22].

The process by which cells lose the BrdU label: At day T (when BrdU is removed), we determine the total number of $BrdU^+$ stem cells by adding the y_1 and y_2 populations. Cells in the y_1 population have a BrdU level of 1, whereas y_2 cells have a BrdU level of up to 2. To model the rate at which these cells lose BrdU during subsequent divisions after removing BrdU:

y_{10} = fraction of cells, initially with one labeled DNA strand that undergo 0 divisions after day T

y_{11} = fraction of cells, initially with one labeled DNA strand that undergo 1 division after day T

…

y_{1N} = fraction of cells, initially with one labeled DNA strand that undergo N divisions after day T

y_{20} = fraction of cells, initially with two labeled DNA strands that undergo 0 divisions after day T

y_{21} = fraction of cells, initially with two labeled DNA strands that undergo 1 division after day T

…

y_{2N}=fraction of cells, initially with two labeled DNA strands that undergo N divisions after day T

Cells move from $y_{1(k-1)}$ to y_{1k} at the proliferation rate α (cells move in y_{2k} similarly). The equations for dilution of the BrdU label are:

$$\frac{dy_{10}}{dt} = -\alpha y_{10}$$
$$\frac{dy_{11}}{dt} = -\alpha y_{11} + \alpha y_{10}$$
...
$$\frac{dy_{1N}}{dt} = \alpha y_{1(N-1)}$$
$$\frac{dy_{20}}{dt} = -\alpha y_{20}$$
$$\frac{dy_{21}}{dt} = -\alpha y_{21} + \alpha y_{20}$$
...
$$\frac{dy_{2N}}{dt} = \alpha y_{2(N-1)}$$

With each cell division, the cell's BrdU level is decreased by half. For instance, cells in y_{10} have BrdU level 1, cells in y_{11} have half as much BrdU on average, cells in y_{12} have one quarter as much BrdU on average, etc. Similarly, cells in y_{20} have BrdU level 2, cells in y_{21} have BrdU level 1, cells in y_{22} have BrdU level 0.5, etc. We can determine the total fraction of cells that have a BrdU level that is above a minimum detection level (which can be set at any desired level in the simulations) by adding all relevant populations at any time after day T.

This set of ordinary differential equations is solved for initial conditions that are determined by the observed results of the BrdU incorporation at time T. In this way, it is possible to plot the frequency of $BrdU^+$ HSCs over time, depending on whether it takes 1, 2, 3, or 4 rounds of division to dilute BrdU to the point at which it is no longer detectable (Figure 3a). Note that 1 round of division corresponds to a 2-fold dilution of BrdU while 4 rounds of division correspond to 16-fold dilution. Our empirical data suggest that approximately 3 rounds of division are required to dilute BrdU to the point that it is no longer detectable in HSCs.

Case 2: Asymmetric chromosome segregation (immortal strand model; see Figure 1a)

BrdU Incorporation: Under this model, only one strand of DNA within each chromosome in stem cells can contain BrdU irrespective of how long BrdU is administered (though the proportion of labeled stem cells will increase over time).

The equations for Case 2 are:

y_0 = fraction of cells without BrdU

y_1 = fraction of cells with one strand $BrdU^+$ (after one or more divisions)

α = proliferation rate of stem cells

The equations for uptake:

$$\frac{dy_0}{dt} = -\alpha y_0$$

Equation 4: Cells leave the y_0 population when they divide.

$$\frac{dy_1}{dt} = \alpha y_0$$

Equation 5: Cells from the y_0 population are added into the y_1 population through the incorporation of BrdU into one of the DNA strands.

As before, in order to determine the proportion of cells within each population at the end of day T we solve the system of ordinary differential equations with initial conditions corresponding to the case where initially all HCSs are unlabeled prior to BrdU administration. Cells in population y_0 do not contain any BrdU, while those in y_1 have a BrdU level of 1.

The process by which cells lose BrdU label: We take all cells containing BrdU at day T (all cells in population y_1 and monitor BrdU loss through cell division in the absence of BrdU). In this case, the asymmetric segregation of chromosomes means that all dividing stem cells will lose all of the BrdU label in a single division in the absence of BrdU. Therefore, the fraction of stem cells that remain $BrdU^+$ at time T, simplifies to the fraction of $BrdU^+$ HSCs that do not divide after removing BrdU:

y_{10} = fraction of cells, initially with one labeled strand that undergo 0 divisions after day T

Cells move from y_{10} (and lose their Brdu label) at the proliferation rate α. The equations for loss of BrdU label:

$$\frac{dy_{10}}{dt} = -\alpha y_{10}$$

This differential equation with initial condition $y_{10}(0) = y_1(T)$ determines the fraction of HSCs that retain BrdU over time, according to the immortal strand hypothesis.

SUPPLEMENTARY FIGURES

Supplementary Figure 1: Few HSCs retain BrdU, and the vast majority of BrdU label-retaining bone marrow cells are not HSCs even when BrdU incorporation is measured by flow-cytometry. In case flow-cytometric detection of BrdU was more sensitive than immunofluorescence microscopy, we repeated all of the experiments using this approach. The percentage of $BrdU^+$ whole bone marrow cells (a) or $CD150^+CD48^-CD41^-lineage^-$ HSCs (b) in negative control mice (no BrdU), or after 10 days of BrdU administration, or after an additional 70 days of chase (without BrdU). Note that fewer HSC markers were used to free up a fluorescence channel to detect BrdU by flow-cytometry; however, this should not introduce significant impurities as 45% of $CD150^+CD48^-CD41^-$ cells give long-term multilineage reconstitution in irradiated mice [17]. Only 1.9% of HSCs detectably retained BrdU after 70 days chase and less than 0.04% of $BrdU^+$ bone marrow cells were HSCs (0.0066% x 1.9%/0.36%). The data are based on 3 independent experiments in which 6,000 to 60,000 bone marrow cells or 400 to 1200 HSCs were analyzed per experiment.

Supplementary Figure 2: HSCs identified as $c\text{-}kit^+Flk\text{-}2^-lineage^-Sca\text{-}1^+$ cells also cannot be reliably identified based on BrdU label-retention. a) In case the isolation of HSCs using different markers would identify a population that is more consistently marked by BrdU label-retention, we isolated $c\text{-}kit^+Flk\text{-}2^-lineage^-Sca\text{-}1^+$ cells, which represent around 0.02% of bone marrow cells (2.0% x 1.0%; a), and which are highly enriched for HSCs [23]. b) $c\text{-}kit^+Flk\text{-}2^-lineage^-$

Sca-1$^+$ cells were isolated from mice that had been administered BrdU for 10 days followed by a 70 day chase without BrdU. Cells were sorted onto microscope slides and stained with DAPI (to identify nuclei) and BrdU. c) The frequency of BrdU$^+$ c-kit$^+$Flk-2$^-$lineage$^-$Sca-1$^+$ cells after 10 days of BrdU administration or after an additional 70 days of chase in normal young adult mice or neonatal mice was very similar to what we observed when HSCs were isolated as CD150$^+$CD48$^-$CD41$^-$lineage$^-$Sca-1$^+$c-kit$^+$ cells (Fig. 3c, d). The frequency of BrdU$^+$ c-kit$^+$Flk-2$^-$ lineage$^-$Sca-1$^+$ cells after 4 days of BrdU administration followed by 70 days of chase in cyclophosphamide/G-CSF treated mice was also similar to what we observed in CD150$^+$CD48$^-$ CD41$^-$lineage$^-$Sca-1$^+$c-kit$^+$ HSCs (Fig. 3d). Note that these cells were isolated from some of the same mice analyzed in Figure 3 c, d, so the frequency of BrdU$^+$ bone marrow cells from the same mice are presented in Figure 3c, d. These results were based on two independent experiments in which 6 mice and 200 to 300 cells per mouse were analyzed per experiment.

Supplementary Figure 3: Administration of CldU and IdU does not affect HSC proliferation or cell death and BrdU incorporation during DNA repair is negligible. a) Cell cycle distribution (based on DNA content) of CD150$^+$CD48$^-$CD41$^-$lineage$^-$Sca-1$^+$c-kit$^+$ HSCs isolated from a mouse that did not receive CldU or IdU (left) or from a mouse that received 4 days of CldU followed by 4 days of IdU (right) (two independent experiments). b) Activated caspase-3 staining of CD150$^+$CD48$^-$CD41$^-$lineage$^-$Sca-1$^+$c-kit$^+$ HSCs sorted onto a microscope slide following 10 days of CldU administration and 10 days of IdU administration. Arrowheads identify activated caspase-3$^+$ cells in a section through embryonic day 11 mouse forebrain (positive control), but no activated caspase-3$^+$ HSCs were detected in either CldU/IdU-treated or control mice (200 to 300 HSCs examined per treatment; two independent experiments). c) BrdU

was administered for 12 hours followed by gamma irradiation with 100 rad and then 48 hours of further BrdU administration. No $BrdU^+$ HSCs were detected in negative control mice (no BrdU). Among mice treated with BrdU for 60 hours, 17.4±1.6% of HSCs from non-irradiated mice were $BrdU^+$ and 18.6±4.5% of HSCs from irradiated mice were $BrdU^+$ (250 to 600 HSCs analyzed per treatment; 3 independent experiments). Since DNA damage would be expected in all cells that receive this dose of irradiation[24], this demonstrates that the amount of BrdU that is incorporated as a result of DNA damage repair is negligible and cannot be detected by immunofluorescence in these assays. Our failure to detect BrdU incorporation from DNA repair is consistent with prior studies that found that nucleotide incorporation as a result of DNA repair is negligible relative to DNA replication, even over many years [25].

Supplementary Figure 4: $CldU^+$ and IdU^+ cells can be distinguished by antibody staining. CldU and IdU staining of $CD150^+CD48^-CD41^-lineage^-Sca\text{-}1^+c\text{-}kit^+$ HSCs from control mice that were administered neither label (left column), only CldU (middle column), or only IdU (right column). These controls were performed side-by-side with all CldU/IdU samples using the same staining conditions. IdU staining was only observed in samples from mice administered IdU and CldU staining was only observed in samples from mice administered CldU.

Supplementary Figure 5: CldU persists in mice for less than a day after administration is discontinued. To test whether the CldU/IdU double labeling of HSCs might simply have reflected a slow clearance of CldU from mice after its administration was discontinued (such that dividing HSCs were inadvertently exposed to both CldU and IdU) we sequentially administered CldU and IdU to mice for only 1 day each (a). If significant residual CldU remained in the mice

for at least a day during IdU administration, all IdU^+ HSCs should also be $CldU^+$ in this experiment. In contrast, if CldU is cleared from mice within hours of being discontinued, then only a minority of IdU^+ HSCs should also be $CldU^+$. b) The frequency of $CD150^+CD48^-CD41^-$ $lineage^-Sca\text{-}1^+c\text{-}kit^+$ HSCs that stained only with CldU, or only with IdU, the total frequencies of $CldU^+$ or IdU^+ cells, and the frequency of $CldU^+IdU^+$ HSCs. Nearly two-thirds of IdU^+ HSCs (6% $IdU\text{-}only^+$ versus 9% total IdU^+) were able to incorporate IdU without incorporating CldU over this brief period of administration, indicating that CldU does not persist in mice for longer than 8 hours after administration. The data are based on 3 separate mice analyzed in two independent experiments with 162 to 238 HSCs analyzed per mouse. The fact that some residual CldU remains in these mice during the first several hours of IdU administration can account for the consistent 2 to 3% increase in the frequency of $CldU^+IdU^+$ HSCs above that predicted by the product of single positive HSCs (27% observed versus 24% predicted with 10 day pulses (Fig. 4c); 3% observed versus 1.3% predicted based on 1 day pulses (panel b above)).

SUPPLEMENTARY REFERENCES

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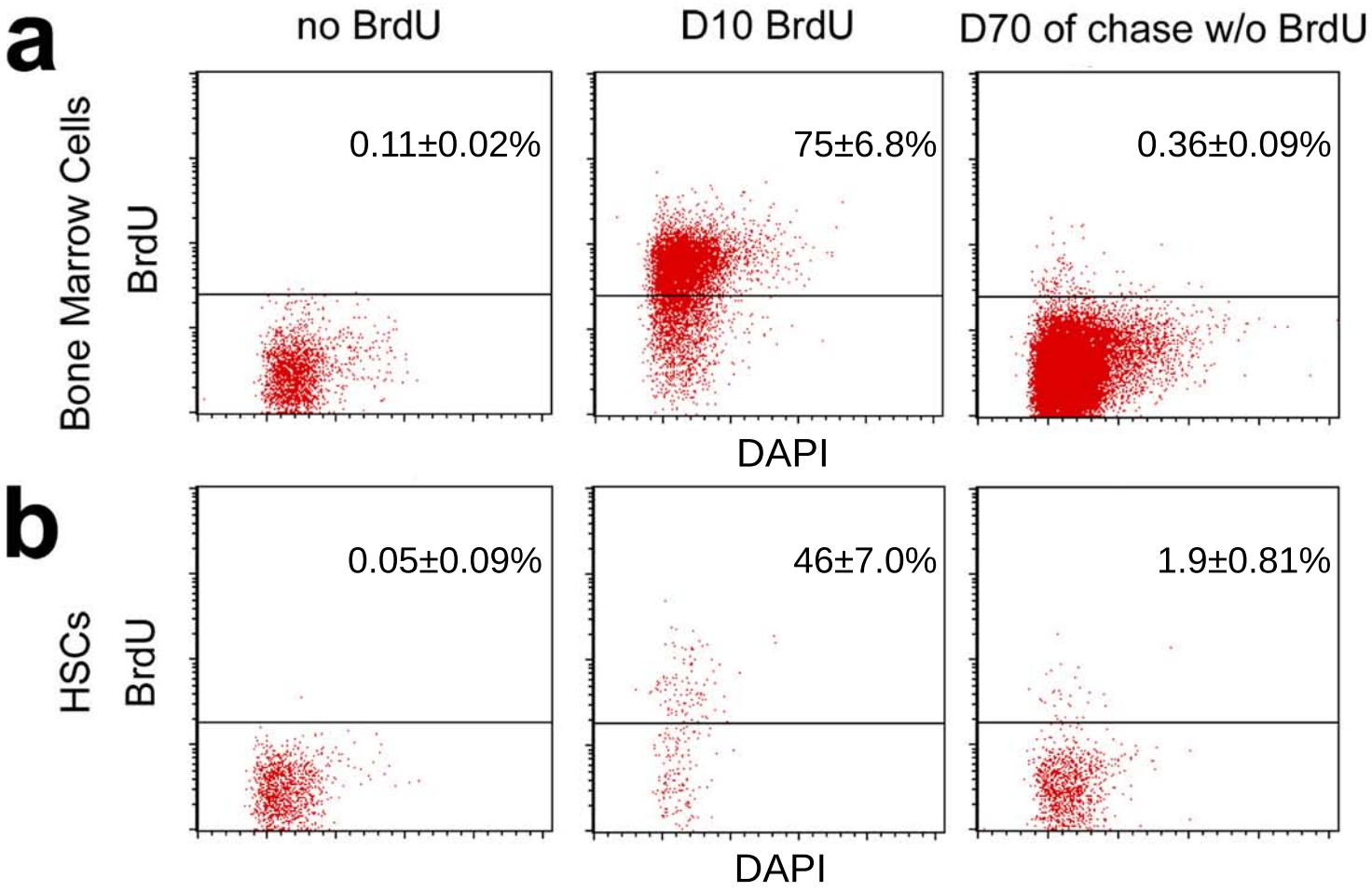
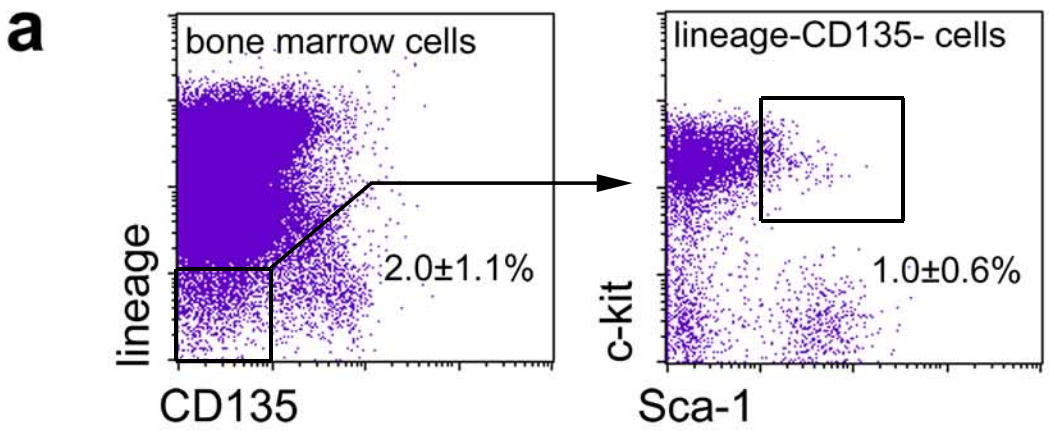
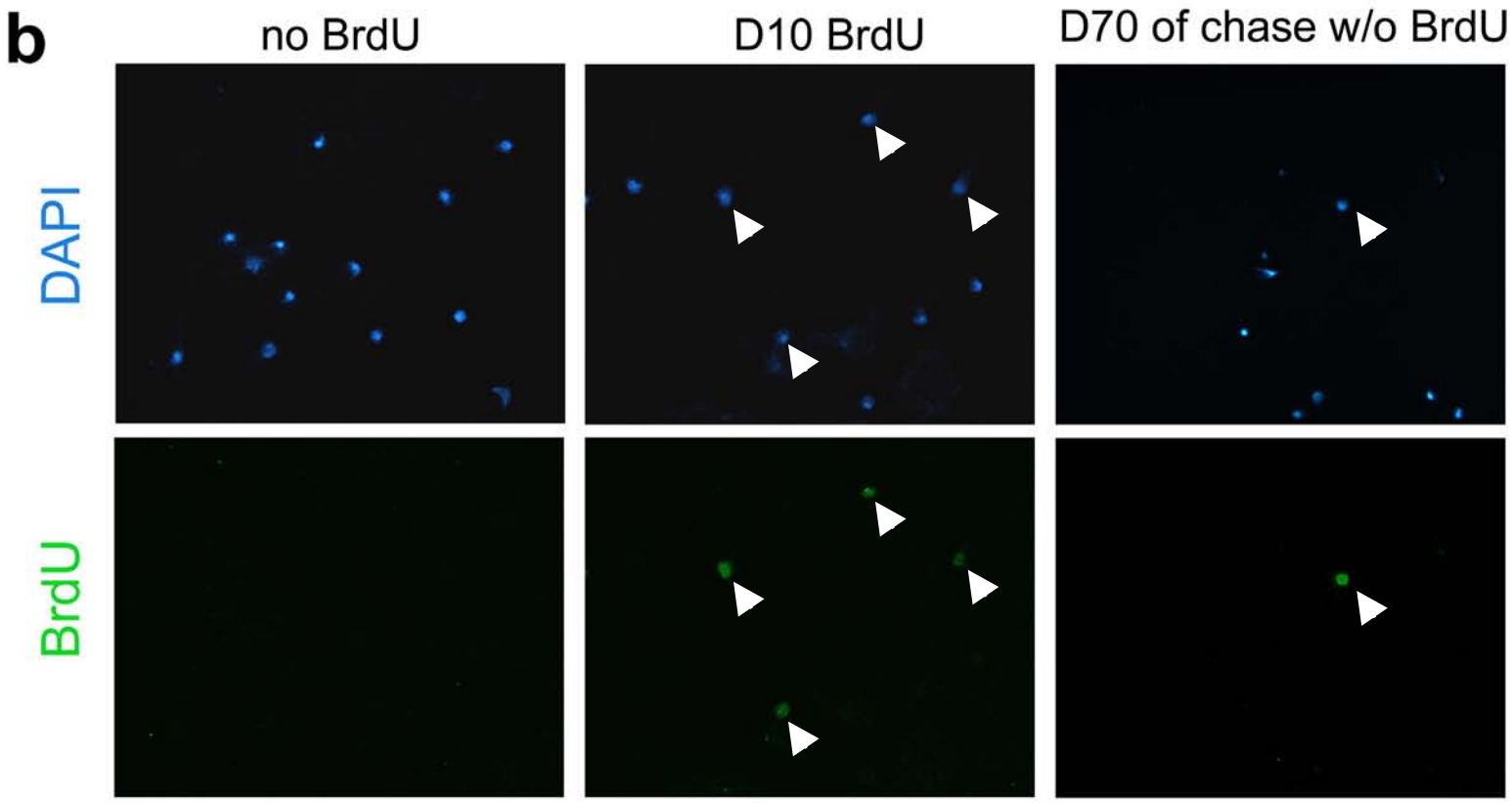


Supplementary Figure 1. Kiel et al.

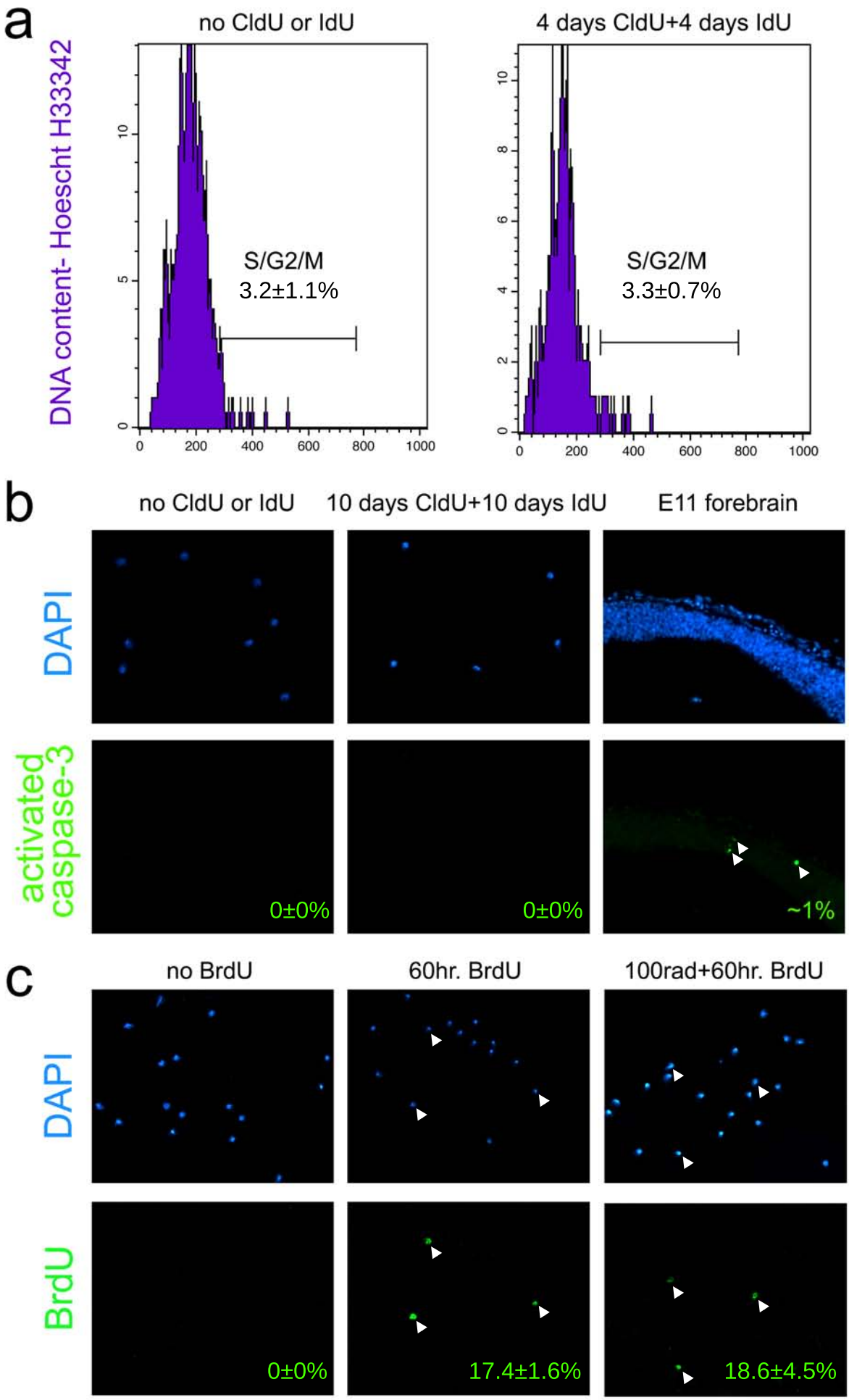




c % BrdU+ HSCs

	D0 of BrdU adult	D10 of BrdU adult	D70 of chase adult	D70 of chase neonate	D70 of chase mobilized
1	0.3	53.3	3.9	8.2	4.4
2	0.0	31.5	4.6	4.6	2.4
3	0.0	46.4			
Mean±SD	0.1±0.2	44±11	4.3±0.5	6.3±2.7	3.4±1.4

Supplementary Figure 2. Kiel et al.



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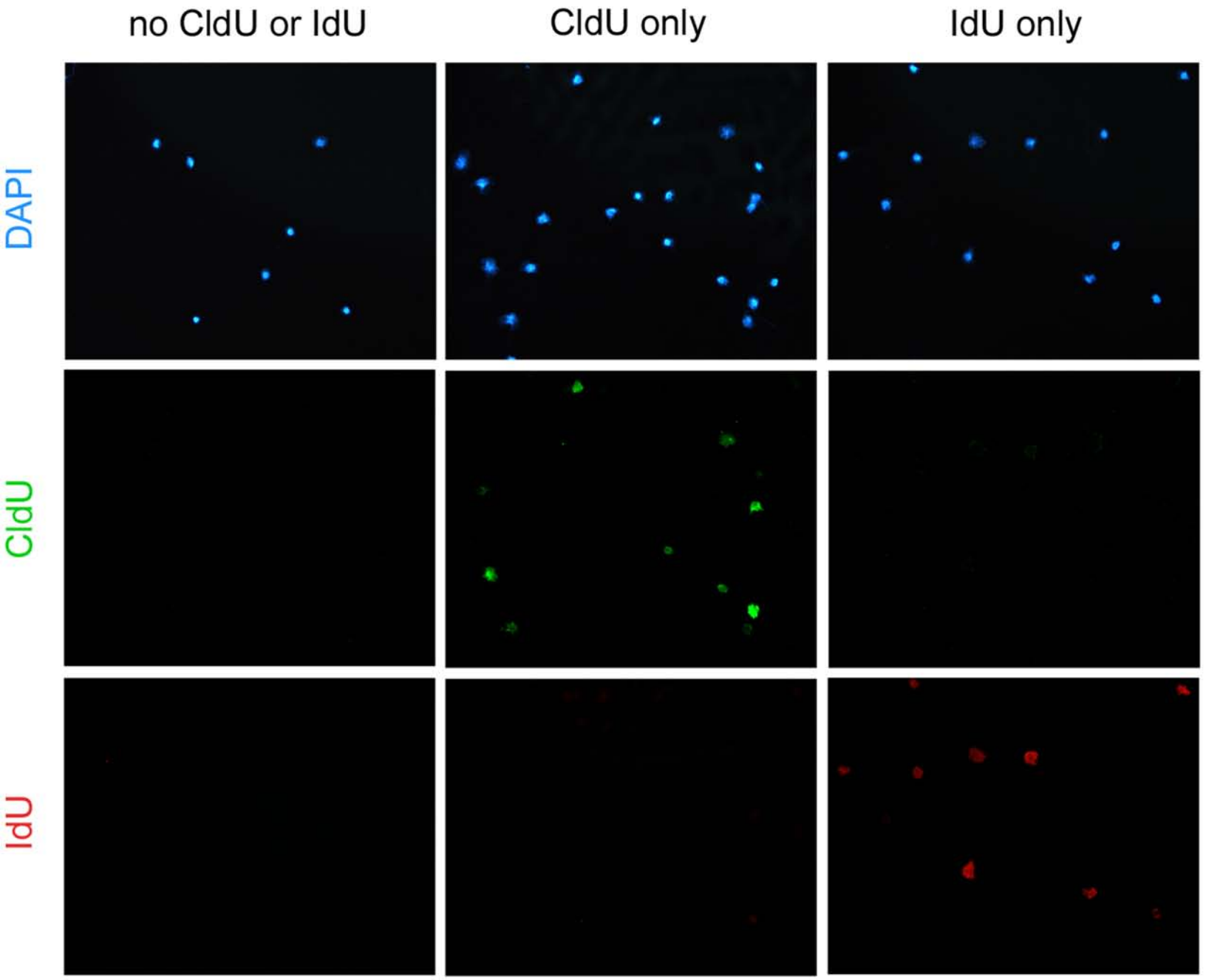

Supplementary Figure 4. Kiel et al.

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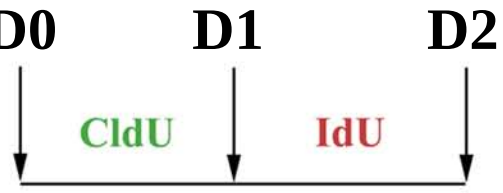

b % HSCs positive for CldU and/or IdU

	CldU-only+	IdU-only+	Total CldU+	Total IdU+	CldU+IdU +
1	11.7	6.2	16.0	10.5	4.3
2	10.2	5.4	13.6	8.8	3.4
3	13.4	6.0	14.8	7.4	1.4
Mean±SD	12±2	6±0.4	15±1	9±2	3±2

Supplementary Figure 5. Kiel et al.