Mammalian heart renewal by pre-existing cardiomyocytes

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Although recent studies have revealed that heart cells are generated in adult mammals, the frequency of generation and the source of new heart cells are not yet known. Some studies suggest a high rate of stem cell activity with differentiation of progenitors to cardiomyocytes1. Other studies suggest that new cardiomyocytes are born at a very low rate2–4, and that they may be derived from the division of pre-existing cardiomyocytes. Here we show, by combining two different pulse-chase approaches—genetic fate-mapping with stable isotope labelling, and multi-isotope imaging mass spectrometry—that the genesis of cardiomyocytes occurs at a low rate by the division of pre-existing cardiomyocytes during normal ageing, a process that increases adjacent to areas of myocardial injury. We found that cell cycle activity during normal ageing and after injury led to polyploidy and multinucleation, but also to new diploid, mononucleate cardiomyocytes. These data reveal pre-existing cardiomyocytes as the dominant source of cardiomyocyte replacement in normal mammalian myocardial homeostasis as well as after myocardial injury.

Despite intensive research, fundamental aspects of the mammalian heart’s capacity for self-renewal are still being actively debated5,6. Estimates of cardiomyocyte turnover range from less than 1% per year7 to more than 40% per year8. Turnover has been reported to either decrease9 or increase8 with age, and the source of new cardiomyocytes has been attributed both to the division of existing myocytes5 and to progenitors residing within the heart5 or in exogenous niches such as bone marrow10. Controversy persists about the plasticity of the adult heart; in part this controversy is due to methodological challenges associated with studying slowly replenished tissues. Toxicity attributed to radiolabelled thymidine11 and halogenated nucleotide analogues12 limits the duration of labelling and may produce direct biological effects. Tissue autofluorescence can decrease the sensitivity and specificity of immunofluorescent methods of detecting cell cycle activity13,14, such as probing for transient expression of cell cycle markers or the incorporation of halogenated nucleotide analogues. The challenge of measuring cardiomyocyte turnover is further compounded by the faster rate of turnover of cardiac stromal cells relative to that of cardiomyocytes14.

Here we used multi-isotope imaging mass spectrometry (MIMS) to study cardiomyocyte turnover and to determine whether new cardiomyocytes are derived from pre-existing myocytes or from a progenitor pool (Fig. 1a). MIMS uses ion microscopy and mass spectrometry to generate high-resolution quantitative mass images and localize stable isotope reporters in domains smaller than 1 μm2 (refs 15–17). MIMS generates 14N quantitative mass images by measuring the atomic composition of the sample surface with a lateral resolution of less than 50 nm and a depth resolution of a few atomic layers. Cardiomyocyte cell borders and intracellular organelles were easily resolved (Fig. 1b). Regions of interest could be analysed at higher resolution, revealing cardiomyocyte-specific subcellular ultrastructure, including sarcomeres (Fig. 1c and Supplementary Fig. 1a). In all subsequent analyses, cardiomyocyte nuclei were identified by their location within sarcomere-containing cells, distinguishing them from adjacent stromal cells.

A great advantage of MIMS is the detection of nonradioactive stable isotope tracers. As an integral part of animate and inanimate matter, they do not alter biochemical reactions and are not harmful to the organism18. MIMS localizes stable isotope tracers by simultaneously quantifying multiple masses from each analysed domain; this enables the generation of a quantitative ratio image of two stable isotopes of the same element19. The incorporation of a tracer tagged with the rare stable isotope of nitrogen (15N) is detectable with high precision by an increase in 14N:15N above the natural ratio (0.37%). Nuclear incorporation of [15N]thymidine is evident in cells that have divided during a 1-week labelling period, as observed in the small-intestinal epithelium, which turns over completely in 3–5 days10 (Fig. 1d); in contrast, [15N]thymidine-labelled cells are rarely observed in the heart (Fig. 1e) after 1 week of labelling. In subsequent studies, small intestine was used as a positive control to confirm label delivery.

Figure 1 | Use of MIMS to study cardiomyocyte turnover. a, Primary question: are new cardiomyocytes derived from progenitors or from pre-existing cardiomyocytes? b, 14N mass image. Subcellular details are evident, including cardiomyocyte nuclei (white arrows). Scale bar, 20 μm. c, MIMS resolves periodic sarcomeres (black arrows) in cardiomyocytes. Non-cardiomyocytes (white arrows) are seen outside cardiomyocyte borders. Scale bar, 5 μm. d, Right, 14N:15N hue-saturation-intensity image of small-intestinal epithelium after labelling with [15N]thymidine. The scale ranges from blue, where the ratio is equivalent to natural ratio (0.37%, expressed as 0% above natural ratio (enrichment over natural ratio)), to red, where the ratio is 150% above natural ratio. 15N labelling is concentrated in nuclei in a pattern resembling chromatin. Scale bar, 15 μm. e, Right, 14N:15N hue-saturation-intensity image of heart section (left ventricle). [15N]Thymidine was administered for 1 week. Asterisk, rare 15N interstitial cells. Cardiomyocyte nuclei (white arrows) are unlabelled. Scale bar, 15 μm.

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To evaluate an age-related change in cell cycle activity, we administered $[^{15}\text{N}]$thymidine for 8 weeks to three age groups of C57BL6 mice starting at day 4 (newborn), at 2 months (young adult) and at 22 months (old adult) (Supplementary Fig. 2). We then performed MIMS analysis (Fig. 2a, b and Supplementary Fig. 3). In the newborn group, 56% ± 3% (mean ± s.e.m.; $n = 3$ mice) of cardiomyocytes showed $^{15}$N nuclear labelling, consistent with the well-accepted occurrence of cardiomyocyte DNA synthesis during postnatal development. We observed a marked decline in the frequency of $^{15}$N-labelled cardiomyocyte nuclei ($^{15}$N$^{+}$ CM) in the young adult (100.00% ± 0.05% $^{15}$N$^{+}$ CM per day; young adult, 0.015% ± 0.003% $^{15}$N$^{+}$ CM per day (means ± s.e.m.); $n = 3$ mice per group, $P < 0.001$) and the old adult (0.007% ± 0.002% $^{15}$N$^{+}$ CM per day (means ± s.e.m.); $n = 3$ mice per group, $P < 0.05$) (Fig. 2c). The observed pattern of $^{15}$N labelling in cardiomyocyte nuclei is consistent with the known chrotatin distribution pattern in cardiomyocytes and was measured at levels that could not be explained by DNA repair (Supplementary Fig. 4). Extrapolating DNA synthesis measured in cardiomyocytes over 8 weeks yields a yearly rate of 5.5% in the young adults and 2.6% in the old mice. Given that cardiomyocytes are known to undergo DNA replication without completing the cell cycle, these calculations represent the upper limit of cardiomyocyte generation under normal homeostatic conditions, indicating a low rate of cardiogenesis.

To test whether cell cycle activity occurred in pre-existing cardiomyocytes or was dependent on a progenitor pool, we performed $[^{15}\text{N}]$thymidine labelling of double-transgenic MerCreMer/ZEG mice, previously developed for genetic lineage mapping (Fig. 3a). We administered $[^{15}\text{N}]$thymidine for 8 weeks to mice of different ages: newborn, starting at postnatal day 4; young adult, starting at 2 months; old adult, starting at 22 months. Top, $^{15}$N$^{+}$:14N mass images show histological details. Bottom, $^{15}$N$^{+}$:14N hue–saturation–intensity images show $^{15}$N$^{+}$ nuclei. Mosaics are constructed from nine tiles, 60 μm each. Scale bar, 30 μm.

Figure 2 | Cardiomyocyte DNA synthesis decreases with age.

Figure 3 | New cardiomyocytes are derived from pre-existing cardiomyocytes during ageing. a, Experimental strategy. MerCreMer$^+/ZEG^+$ (MCM$^+$ ZEG$^+$) mice ($n = 4$) were treated for 2 weeks with 4-OH-tamoxifen to induce cardiomyocyte-specific GFP expression. $[^{15}\text{N}]$Thymidine was administered continuously during a 10-week chase, then cycling cells were identified by $^{15}$N$^{+}$ labelling. New cardiomyocytes ($^{15}$N$^{+}$) derived from pre-existing cardiomyocytes should express GFP at a rate similar to that of the surrounding quiescent ($^{15}$N$^{-}$) cardiomyocytes. New cardiomyocytes ($^{15}$N$^{+}$) derived from progenitors should be GFP$^+$, whereas cardiomyocytes arising from pre-existing cardiomyocytes should express GFP at a frequency similar to the background rate induced by 4-OH-tamoxifen. We administered 4-OH-tamoxifen for 2 weeks to 8-week-old mice ($n = 4$); during a subsequent 10-week chase, mice received $[^{15}\text{N}]$thymidine by means of an osmotic minipump (20 μg h$^{-1}$). With MIMS analysis, we identified 35 $^{15}$N$^{+}$ cardiomyocytes (of 4,190 analysed) over 10 weeks, yielding a projected yearly rate of cardiomyocyte DNA replication of 4.4%. Extrapolating from previous reports of high stem-cell-dependent cardiomyocyte turnover, we had expected to detect more than 320 cardiomyocytes entering the cell cycle; these results exclude such a high rate of turnover (expected $^{15}$N$^{+}$ CM = 321; observed = 35, Fisher’s exact $P = 0.0001$). Immunofluorescent staining for GFP was performed on adjacent sections, and an observer unaware of MIMS analysis results assessed GFP status. Of new cardiomyocytes resulting from DNA synthesis by progenitors, 15N$^{+}$ cardiomyocytes, 77% expressed GFP, a frequency essentially identical to that of surrounding 15N$^{-}$ cardiomyocytes (15N$^{+}$ CM, 77% 15N$^{-}$ CM, 84%, Fisher’s exact $P = n.s.$) (Table 1). If new cardiomyocytes were derived from progenitors, 15N$^{+}$ cardiomyocytes would have been GFP$^-$ (expected = 0/35; observed = 27/35, Fisher’s exact $P < 0.0001$). These data show that 15N$^{+}$ cardiomyocytes resulted from DNA synthesis by pre-existing cardiomyocytes and exclude a substantial contribution from stem cells in cardiomyocyte replacement in the uninjured heart.

Cardiomyocytes can undergo DNA replication without completing the cell cycle. Although multinucleation and polypliodization occur during early postnatal development and in response to myocardial...
stress⁹, we considered the possibility that these processes could account for ¹⁵N⁺ cardiomyocytes in the uninjured adult mouse. We performed fluorescent in situ hybridization in adjacent sections to assess the ploidy state of each ¹⁵N⁺ cardiomyocyte and surrounding ¹⁴N⁻ cardiomyocytes, and an observer unaware of the results of MIMS analysis identified fluorescently labelled chromosomes (Supplementary Fig. 7). The mononucleate, diploid, ¹⁵N⁺ cardiomyocytes in sham-operated mice was similar to that in previous experiments in unoperated mice (yearly projected rates: sham, 6.8%; unoperated, 4.4%), but increased significantly adjacent to infarcted myocardium (total ¹⁵N⁺ cardiomyocyte nuclei: myocardial infarction, 23.0%; sham, 1.1%; Fig. 4a, b and Supplementary Fig. 8). We examined the nucleation, ploidy status and GFP expression of ¹⁵N⁺ cardiomyocytes and surrounding unlabelled cardiomyocytes. We found a significant dilution of the GFP⁺ cardiomyocyte pool at the border region, as previously shown²³,²⁴ (67% versus 79%, P < 0.0001; Table 2 and Supplementary Fig. 9); however, ¹⁵N⁺ myocytes demonstrated a similar frequency of GFP expression to that in unlabelled myocytes (71% versus 67%, Fisher’s exact P = n.s.), suggesting that DNA synthesis was primarily occurring in pre-existing cardiomyocytes. Of ¹⁵N⁺ cardiomyocytes, about 14% were mononucleate and diploid, consistent with division of pre-existing cardiomyocytes (Supplementary Figs 6 and 7). We observed a higher DNA content (more than 2N or greater), we observed a higher frequency of diploid nuclei in the ¹⁵N⁺ pool than in surrounding ¹⁴N⁻ cardiomyocytes (¹⁵N⁺ diploid:polyploid = 22:12; ¹⁴N⁻ diploid:polyploid = 9:56, Fisher’s exact P < 0.0001), consistent with ongoing cell division. We then assessed each cell for multinucleation by using serial 0.5-µm sections adjacent to the section used for MIMS analysis (Supplementary Fig. 6). We observed that 49% of ¹⁵N⁺ cardiomyocytes were mononucleate, in contrast with a frequency of 24% for surrounding ¹⁴N⁻ cardiomyocytes (Fisher’s exact P < 0.01), also consistent with cell division. Most cardiomyocyte DNA synthesis occurred in polyploid and/or multinucleate cardiomyocytes, as might be expected with a physiological hypertrophic response, and was thus unlikely to indicate cardiomyocyte division; however, 17% (6 of 35 ¹⁵N⁺ CM) were diploid and mononucleate, consistent with newly generated cardiomyocytes (Supplementary Fig. 7). The mononucleate, diploid, ¹⁵N⁺ cardiomyocytes were also predominantly GFP⁺ (5 of 6 = 83%, compared with 82% background frequency, Fisher’s exact P = n.s.), suggesting that they arose from pre-existing cardiomyocytes at a slow annual rate of 0.76% (n = 6 of 4,190 over 10 weeks).

We next used MIMS and genetic fate mapping to study myocardial injury. Cardiomyocyte GFP labelling was induced in MerCreMer/ZEG mice with 4-OH-tamoxifen. Mice then underwent experimental myocardial infarction or sham surgery followed by continuous labelling with [¹⁵N]thymidine for 8 weeks. The frequency of ¹⁵N⁺ cardiomyocytes in sham-operated mice was similar to that in previous experiments in unoperated mice (yearly projected rates: sham, 6.8%; unoperated, 4.4%), but increased significantly adjacent to infarcted myocardium (total ¹⁵N⁺ cardiomyocyte nuclei: myocardial infarction, 23.0%; sham, 1.1%; Fig. 4a, b and Supplementary Fig. 8). We examined the nucleation, ploidy status and GFP expression of ¹⁵N⁺ cardiomyocytes and surrounding unlabelled cardiomyocytes. We found a significant dilution of the GFP⁺ cardiomyocyte pool at the border region, as previously shown²³,²⁴ (67% versus 79%, P < 0.0001; Table 2 and Supplementary Fig. 9); however, ¹⁵N⁺ myocytes demonstrated a similar frequency of GFP expression to that in unlabelled myocytes (71% versus 67%, Fisher’s exact P = n.s.), suggesting that DNA synthesis was primarily occurring in pre-existing cardiomyocytes. Of ¹⁵N⁺ cardiomyocytes, about 14% were mononucleate and diploid, consistent with division of pre-existing cardiomyocytes (Supplementary Figs 6 and 7). We observed a higher DNA content (more than 2n) in the remaining cardiomyocytes, as expected with compensatory hypertrophy after injury. Thus, in the 8 weeks after myocardial infarction, roughly 3.2% of the cardiomyocytes adjacent to the infarct had unambiguously undergone division (total ¹⁵N⁺ × mononucleate diploid fraction = 23% × 0.14 = 3.2%). The low rate of cardiomyocyte cell cycle completion was further supported by the absence of detectable aurora B kinase, a transiently expressed cytokinesis marker, which was detected in rapidly proliferating small intestinal cells but not in cardiomyocytes

Figure 4 | Myocardial injury stimulates division of pre-existing cardiomyocytes. a, Myocardial infarction leads to extensive DNA synthesis within and adjacent to a scar (arrows). MerCreMer+/ZEG⁺ mice were treated for 2 weeks with 4-OH-tamoxifen to induce cardiomyocyte-specific GFP expression before myocardial infarction or sham surgery, then [¹⁴N]thymidine was administered continuously for 8 weeks. Mosaics of 70 MIMS tiles (each 60 µm × 60 µm) are shown. Trichrome-stained adjacent section (right) shows the scar (black arrows). Scale bars, 90 µm. b, [¹⁵N]Thymidine-labelled cardiomyocyte nucleus (white arrows) from myocardial infarction border region. Immunofluorescent staining shows that the cardiomyocyte is GFP⁺. Scale bars, 10 µm. c, Mean percentage of ¹⁵N⁺ cardiomyocyte nuclei after myocardial infarction (MI; n = 4) in the scar border region compared with that in sham-operated mice (n = 3). Error bars indicate s.e.m.

Table 1 | ¹⁵N⁺ cardiomyocytes (¹⁴N⁻ CM) during normal ageing

| ¹⁵N⁻ CM | Total counted | ¹⁵N⁻ CM | Percentage ¹⁵N⁺ |
|---------|--------------|---------|-----------------|
| 1,111   | 933          | 84      |
| 35      | 27           | 77      |
| Diploid CM | 10            | 83      |
| Multinucleate CM | 17       | 82      |
| Mononucleate CM | 6           | 83      |

The results include a new diploid/mononucleate fraction. The data also account for ¹⁵N⁻ cardiomyocytes (¹⁴N⁻ CM).
Table 2 | GFP analysis of $^{15}$N$^*$ cardiomyocytes ($^{15}$N$^*$ CM)

|                  | Total counted | GFP$^+$ | Percentage GFP$^+$ |
|------------------|---------------|---------|-------------------|
| Sham             | 3,411         | 2,693   | 79                |
| $^{15}$N$^*$ CM  | 23            | 19      | 83                |
| Myocardial infarction | 7,063        | 4,766   | 67                |
| $^{15}$N$^*$ CM  | 205           | 146     | 71                |
| Polycloid CM     | 126           | 97      | 77                |
| Diploid CM       | 65            | 47      | 72                |
| Multinucleate CM | 59            | 42      | 71                |
| Mononucleate CM  | 41            | 29      | 71                |
| Diploid/mononucleate CM | 16          | 11      | 69                |

Pooled analysis of four mice in the myocardial infarction group and three mice in the sham group. Myocardial infarction led to dilution of GFP$^+$ cardiomyocytes (CM) adjacent to the scar. New cardiomyocytes generated after myocardial infarction (the $^{15}$N$^*$ diploid/mononucleate pool) were predominantly GFP$^+$, which is consistent with the division of pre-existing cardiomyocytes. (Supplementary Fig. 10). We also considered the possibility that a subset of $^{15}$N$^*$ myocytes that were multinucleate and/or polyploid resulted from division followed by additional rounds of DNA synthesis without division. However, quantitative analysis of the $^{15}$N$^*$ population did not identify a subpopulation that had accumulated additional $^{12}$N-label, in contrast with what would be expected in such circumstances (Supplementary Fig. 11). Taken together, these data suggest that adult cardiomyocytes retain some capacity to re-enter the cell cycle, but that most DNA synthesis after injury occurs in pre-existing cardiomyocytes without completion of cell division.

If dilution of the GFP$^+$ cardiomyocyte pool cannot be attributed to division and differentiation of endogenous progenitors, do these data exclude a role for progenitors in the adult mammalian heart? The data could be explained by preferential loss of GFP$^+$ cardiomyocytes after injury, a process that we have previously considered but for which we have not found supporting evidence\(^1\). Such an explanation excludes a role for endogenous progenitors in cardiac repair and would be consistent with data emerging from lower vertebrates\(^8,26\) and the neonatal ageing experiment (Charles River). Double transgenic male MerCreMer/ZEG mice were used in accordance with the Guide for the Use and Care of Laboratory Animals and experiments were approved by the Harvard Medical School Standing Committee on Animals. Male C57Bl/6j mice were used for the ageing experiment (Charles River). Double transgenic male MerCreMer/ZEG mice\(^2\) were used in all other experiments.

METHODS SUMMARY

Multi-isotope imaging mass spectrometry (MIMS) was performed as described previously\(^3\). Mice were used in accordance with the Guide for the Use and Care of Laboratory Animals and experiments were approved by the Harvard Medical School Standing Committee on Animals. Male C57Bl/6j mice were used for the ageing experiment (Charles River). Double transgenic male MerCreMer/ZEG mice\(^2\) were used in all other experiments.

Full Methods and any associated references are available in the online version of the paper.

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Supplementary Information is available in the online version of the paper.
METHODS

Mice. All experiments were conducted in accordance with the Guide for the Use and Care of Laboratory Animals and approved by the Harvard Medical School Standing Committee on Animals. C57Bl/6 male mice were obtained from Charles River. We generated double transgenic MerCreMer/ZEG male mice by crossing breeding cardiomyocyte-specific MerCreMer mice and ZEG mice (Jackson Laboratory). β-Galactosidase–GFAP–FP is under the control of a cytomegalovirus (CMV) enhancer/chicken actin promoter (Achb); the background strain was C57BL/6J (N7). The background strain of the MerCreMer mice was C57Bl/6Sv129. Genotyping was performed by PCR on tail DNA using the following primers: MerCreMer, 5’-GTGTCAGCTGGTGTCCTCTCTCTGTA TAG-3’ (forward) and 5’-AATGTCATCTGCGACACCG-3’ (backward); ZEG, 5’-GGTTGAAAGATGTTGCCG-3’ (backward); ZEG control, 5’-CTAGGCGCACAGAATGGAAAGATCCTGCA CATCAGCC-3’ (backward). To induce Cre recombination and GFP labelling in cardiomyocytes, we injected 4-OH-tamoxifen (a gift from Bio-Technology), labelling was started at postnatal day 4 with subcutaneous injections of 50 mg/kg 5-[15N]Thymidine labelling.

Experimental myocardial infarction. Mice were subjected to experimental myocardial infarction as described. Surgeries were performed by a single operator with more than 20 years of experience in the performance of coronary ligation in rodents. In brief, the left coronary artery was permanently ligated roughly 2 mm more than 20 years of experience in the performance of coronary ligation in rodents. In brief, the left coronary artery was permanently ligated roughly 2 mm behind the left atrial appendage. For sham operations, the thoracic cavity was opened and the heart exposed, but no intramyocardial sutures were placed. 

[15N]Thymidine (Cambridge Isotopes) was administered at a rate of 20 mg/kg with an osmotic minipump (Alzet), implanted subcutaneously at the time of experimental myocardial infarction after a single intraperitoneal bolus dose of 500 μg.

MIMS data acquisition. The factory protocol of the NanoSIMS 50 as well as a standard NanoSIMS 50 and a large-radius NanoSIMS 50L (Cameca) was used for MIMS data acquisition. A standard NanoSIMS 50 and a large-radius NanoSIMS 50L (Cameca) was used for MIMS data acquisition.

MIMS data analysis. From a single field image acquisition, we first extracted four image files: the four original quantitative mass images (QMIs; 12C, 13C, 12C14N and 12C15N) and the two ratio images 12C/14C and 12C15N/12C14N, derived from the pixel-by-pixel division of the 13C QMI by the 12C QMI and of the 12C15N QMI by the 12C14N QMI, respectively. We then used a hue–saturation–intensity transformation of the ratio image to map 15N-labelled regions. The hue corresponds to the ratio value, and the intensity at a given hue is an index of statistical reliability. [15N]Thymidine labelling. For the neonatal cohort in the ageing experiment, labelling was started at postnatal day 4 with subcutaneous injections of 50 μg/kg [15N]thymidine at a rate of 20 μg/h.

Multinucleation analysis. Serial adjacent sections (0.5 μm) were stained to identify cardiomyocyte borders. A given cardiomyocyte was tracked in the vertical axis by locating it in serial sections. Uninjured hearts were stained by using a modified PAS protocol with standard solutions (Electron Microscopy Services), but with longer incubation times optimized for LR white embedding. Slides were incubated for 1 h in xylene at 37 °C, rehydrated through graded alcohols, incubated in periodic acid for 2 h and then in Schiff’s reagent for two nights. Sections were counterstained with haematoxylin and with Scott’s Blueing for 1 h each. Injured hearts were stained with a modified Trichrome staining protocol with standard solutions (Fisher Scientific), but with longer incubation times. Slides were incubated for 1 h in xylene at 37 °C, rehydrated through graded alcohols, incubated for 1 h in Bouin’s fluid at 56 °C, rinsed in tap water, incubated for 1 h in Weigert’s iron haematoxylin stain, rinsed in tap water, incubated for 1 h in scarlet–acid fuchsin solution, rinsed in distilled deionized water, incubated for 30 min in phophotungstic–phphomolybdic acid solution, incubated for 30 min in aniline blue stain solution, and incubated for 20 min in 1% acetic acid.

Fluorescent in situ hybridization. Sections were incubated for 15 min in proteinase K (50 μg/ml) at 60 °C. After a wash with PBS containing 45 mM MgCl2, slides were postfixed in 4% paraformaldehyde (in PBS/MgCl2) and dehydrated through graded alcohols. Biotinylated-labelled chromosome Y paint (StarFISH; Cambio) in hybridization mix was applied to sections and sealed under glass with rubber cement (some samples were analysed with chromosome 18 paint because of product discontinuation of chromosome Y paint). Samples were heated to 90 °C for 15 min. After incubation overnight at 37 °C, slides were washed three times with 50% formamide/2× standard saline citrate at 45 °C, three times with 2× standard saline citrate at room temperature (25 °C), and twice with 4× standard saline citrate/0.1% Tween at room temperature. Samples were blocked for 10 min with 4× standard saline citrate/0.1% Tween/0.05% milk and incubated for 2 h in streptavidin-conjugated Alexa Fluor 488 (Invitrogen) before being washed and mounted. An observer unaware of the MIMS images or [15N]thymidine labelling status of the nuclei assigned ploidy status.

Immunofluorescent staining. Sections were incubated for 5 min in glycine/Tris (50 mM glycine/0.05 M Tris) at room temperature. After a brief wash with 0.05 M Tris, sections were incubated overnight with both chicken anti-GFP (Abcam) and rabbit anti-c-Kit (Abcam) or rat anti-Scal (Abcam) with fresh 0.1% BSA in TBS/0.1% Tween at 4 °C. After a brief wash with TBS, sections were incubated with anti-chicken Alexa Fluor 488 (Invitrogen) before being washed with TBS and then mounted. An observer unaware of the MIMS images or [15N]thymidine labelling status of the nuclei assigned GFP status.

Fluorescence image analysis. We used a custom-written script in IP Lab version 4.0 (Scalable) Imaging software for serial image acquisition. Tissue sections were auto-imaged with an Olympus IX-70 microscope with a digital charge-coupled-device camera (CoolSNAP EZ; Roper Scientific), using an automated stage with a piezoelectric objective positioner (Polytec PI; Auburn MA) to compensate for deviations in the z axis. Images were compressed and stitched into a mosaic with stitching software (Canon Photostitch). Multichannel images were merged in ImageJ before stitching.

Statistical analysis. Statistical testing was performed with Prism 3.0 (Graphpad). Results are presented as means ± s.e.m. and were compared by using t-tests (significance was assigned for P < 0.05). Data comparing event rates were tested with a Fisher exact test.