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Olaf Bergmann, et al.
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Evidence for Cardiomyocyte Renewal in Humans

Olaf Bergmann,1* Ratan D. Bhardwaj,1* Samuel Bernard,2 Sofia Zdunek,3 Fanie Barnabé-Heider,2 Stuart Walsh,3 Joel Zupich,5 Kanar Alkass,5 Bruce A. Buchholz,5 Henrik Druid,4 Stefan Jovinge,3,6 Jonas Frisén1†

It has been difficult to establish whether we are limited to the heart muscle cells we are born with or if cardiomyocytes are generated also later in life. We have taken advantage of the integration of carbon-14, generated by nuclear bomb tests during the Cold War, into DNA to establish the age of cardiomyocytes in humans. We report that cardiomyocytes renew, with a gradual decrease from 1% turning over annually at the age of 25 to 0.45% at the age of 75. Fewer than 50% of cardiomyocytes are exchanged during a normal life span. The capacity to generate cardiomyocytes in the adult human heart suggests that it may be rational to work toward the development of therapeutic strategies aimed at stimulating this process in cardiac pathologies.

Myocardial damage often occurs in chronic heart failure due to loss and insufficient regeneration of cardiomyocytes. This has prompted efforts to devise cardiomyocyte replacement therapies by cell transplantation or by the promotion of endogenous regenerative processes. The development of cell transplantation strategies is advancing rapidly, and some are currently being evaluated in clinical trials (1, 2). Stimulation of endogenous regenerative processes is attractive as it potentially could provide a noninvasive therapy and circumvent the immunosuppression required for allografts. However, it is unclear whether such regenerative strategies are realistic because it has been difficult to establish whether cardiomyocytes can be generated after the perinatal period in humans.

Stem/progenitor cells with the potential to generate cardiomyocytes in vitro remain in the adult rodent and human myocardium (3, 4). Moreover, mature cardiomyocytes have been suggested to be able to reenter the cell cycle and replicate (5). However, studies over several decades in rodents with labeled nucleotide analogs have led to conflicting results, ranging from no to substantial generation of cardiomyocytes postnatally (6). A recent genetic labeling study, which enabled detection of cardiomyocyte generation by stem/progenitor cells (but not by cardiomyocyte duplication), demonstrated cardiomyocyte renewal after myocardial injury, but not during 1 year in the healthy mouse (7).

It is possible that humans, who live much longer than rodents, may have a different requirement for cardiomyocyte replacement. Cell turnover has been difficult to study in humans because the use of labeled nucleotide analogs and other strategies commonly used in experimental animals cannot readily be adapted for studies in humans owing to safety concerns. The limited functional recovery after loss of myocardium and the fact that primary cardiac tumors are very rare indicate limited proliferation within the adult human heart (8). Several studies have described the presence of molecular markers associated with mitosis in the human myocardium (5), but this provides limited information because it is difficult to deduce the future fate of a potentially dividing cell in terms of differentiation and long-term survival.

We have measured carbon-14 (14C) from nuclear bomb tests in genomic DNA of human myocardial cells, which allows retrospective birth dating (9–11). 14C concentrations in the atmosphere remained relatively stable until the Cold War, when aboveground nuclear bomb tests caused a sharp increase (12, 13). Even though the detonations were conducted at a limited number of locations, the elevated amounts of 14C in the atmosphere rapidly equilibrated around the world as 14CO2. After the Limited Nuclear Test Ban Treaty in 1963, the 14C concentrations dropped exponentially, not primarily because of radioactive decay (half-life of 5730 years), but by diffusion from the atmosphere (14). Newly created atmospheric 14C reacts with oxygen to form 14CO2, which is incorporated by plants through photosynthesis. Humans eat plants, and animals that live off plants, so the 14C concentration in the human body mirrors that in the atmosphere at any given time (15–18). Because DNA is stable after a cell has gone through its last cell division, the concentration of 14C in DNA serves as a date mark for when a cell was born and can be used to retrospectively date birth cells in humans (9–11).

We first carbon-dated left ventricle myocardial cells, including cardiomyocytes and other cell types, to determine the extent of postnatal DNA synthesis in the human heart. DNA was extracted, and 14C concentrations were measured by accelerator mass spectrometry (see tables S1 and S2 for 14C values and associated data). The cellular birth dates can be inferred by determining the time at which the sample’s 14C concentration corresponded to the atmospheric concentration (Fig. 1A). 14C concentrations from all individuals born around or after the nuclear bomb tests corresponded to atmospheric concentrations several years after the subjects’ birth (Fig. 1B), indicating substantial postnatal DNA synthesis. Analysis of individuals born before the period of nuclear bomb tests allows for sensitive detection of any turnover before 1955, due to the marked increase in 14C concentrations. By analyzing individuals born at different times before 1955 it is possible to establish the age up to which DNA synthesis occurs, or whether it continues beyond that age.
In all studied cases, born up to 22 years before the onset of the nuclear bomb tests, $^{14}$C concentrations were elevated compared to the levels before the nuclear bomb tests (Fig. 1). Thus, DNA of myocardial cells is synthesized many years after birth, indicating that cells in the human heart do renew into adulthood.

Because cardiomyocytes constitute only about 20% of all cells within the human myocardium (19), it is not possible to infer from these data whether there is postnatal renewal of cardiomyocytes, or whether cell turnover in the myocardium is limited to other cell populations. We therefore set out to specifically birth date cardiomyocytes. Many cardiomyocytes are binucleated, and it is difficult to distinguish a binucleated cell from two aggregating mononucleated cells (of which one could be a noncardiomyocyte) in the flow cytometer. Hence, rather than separating myocardial cells on the basis of cell surface or cytoplasmic markers, we developed a strategy to isolate cardiomyocyte nuclei by flow cytometry.

We found that the well-characterized cardiomyocyte-specific proteins cardiac troponin I (cTroponin I, also known as TNNI3) and cardiac troponin T (cTroponin T, also known as TNNT2) (for review, see (20)) have evolutionar-

![Fig. 1. Cell turnover in the heart.](image)

**Fig. 1.** Cell turnover in the heart. (A) Schematic figure demonstrating the strategy to establish cell age by $^{14}$C dating. The black curve in all graphs shows the atmospheric concentrations of $^{14}$C over the decades since 1930 [data from (14)]. The vertical bar indicates the date of birth of the individual. The measured $^{14}$C concentration (1) is related to the atmospheric $^{14}$C concentration by use of the established atmospheric $^{14}$C bomb curve (2). The average birth date of the population can be inferred by determining where the data point intersects the x axis (3). $^{14}$C concentrations in DNA of cells from the left ventricle myocardium in individuals born after (B) or before (C) the nuclear bomb tests correspond to time points substantially after the time of birth, indicating postnatal cell turnover. The vertical bar indicates the date of birth of each individual, and the similarly colored dots represent the $^{14}$C data for the same individual. For individuals born before the increase in $^{14}$C concentrations, it is not possible to directly infer an age because the measured concentration can be a result of $^{14}$C incorporation during the rising and/or falling part of the atmospheric curve, and thus the concentration is indicated by a dotted horizontal line.

![Fig. 2. Isolation of cardiomyocyte nuclei.](image)

**Fig. 2.** Isolation of cardiomyocyte nuclei. (A to C) Flow cytometric analysis of cardiomyocyte nuclei from the left ventricle of the human heart with an isotype control antibody or antibodies to the cardiomyocyte-specific antigens cTroponin I or T. Boxes denote the boundaries for the positive and negative sorted populations. (D) cTroponin I and T are present in the same subpopulation of heart cell nuclei. (E) Western blot analysis of flow cytometry–isolated nuclei demonstrates nearly all detectable cTroponin T (analyzed with two different antibodies) and I protein in the cTroponin T–positive fraction. Brain and heart tissue were used as negative and positive controls, respectively. (F) The cardiac troponin T–positive population is enriched for the cardiomyocyte-specific transcription factors Nkx2.5 and GATA4. Both fractions contain similar amounts of the nuclear protein histone 3 (loading control). (G) Gene expression analysis of flow cytometry–isolated nuclei shows high expression of cardiomyocyte–specific genes in the cTroponin T–positive fraction (cTroponin I and T, Nkx2.5), whereas marker genes for endothelial cells (vWF), fibroblasts (vimentin), smooth muscle (ACTA2), and leukocytes (CD45) are highly expressed in the cTroponin T–negative fraction (H). Bars in (G) and (H) show the average from three independent experiments (±SD).
ily conserved nuclear localization signals and are partly localized in the nuclei of cardiomyocytes (figs. S1 and S2). Antibodies to cTroponin I and T identify the same subpopulation of nuclei in the myocardium (Fig. 2, A to D), and retrospective birth dating of nuclei isolated with antibodies against either epitope gave similar results (table S1). Western blot and quantitative reverse transcription polymerase chain reaction analysis of sorted nuclei demonstrated a high enrichment of cTroponin I and T in the positive fraction and a depletion in the negative, validating the efficiency of the strategy (Fig. 2, E to H). We assessed the potential transfer of cTroponin I and T during tissue processing by mixing cardiac tissue with another tissue devoid of these proteins, and found that there was negligible transfer of cTroponin I or T to noncardiomyocyte nuclei during tissue dissociation, nuclear preparation, or flow cytometric sorting (fig. S2).

We assessed the specificity of the isolation procedure with known cardiomyocyte-specific markers and markers of noncardiomyocytes present in the myocardium. There was a high enrichment of nuclei containing the known cardiomyocyte-specific nuclear markers Nkx2.5 and GATA4 in the cTroponin-positive fraction, with little contamination of nuclei expressing markers for fibroblasts, smooth muscle cells, endothelial cells, or hematopoietic cells (Fig. 2, F to H). Conversely, cardiomyocyte markers were depleted in the cTroponin-negative fraction (Fig. 2, F to H), indicating that nearly all cardiomyocytes were isolated in the positive fraction. Sorting whole cells with antibodies to a nonnuclear cardiomyocyte-specific epitope confirmed that nuclear cTroponin I and T are specific to cardiomyocytes, but resulted in lower purity compared to sorting nuclei (fig. S3). Flow cytometric reanalysis of all sorted samples demonstrated a DNA content-corrected cardiomyocyte purity of 96 ± 1.8% (mean ± SD; table S1 and fig. S4). Thus, flow cytometry with antibodies against cTroponin I or T allows specific isolation of cardiomyocyte and noncardiomyocyte nuclei.

We extracted DNA from cardiomyocyte nuclei ([5 ± 2) × 10^7, mean ± SD] and measured the 14C concentration in genomic DNA. By analyzing the 14C concentration also in unsorted myocardial nuclei (>10⁶), we mathematically compensated for any contamination in the cardiomyocyte fraction in the individual cases, reducing the risk that contamination with a cell population with a different turnover rate would skew the result for cardiomyocytes. In all individuals born before the onset of the nuclear bomb tests, the 14C concentrations in cardiomyocyte genomic DNA were higher than the pre-bomb atmospheric concentrations, demonstrating DNA synthesis after 1955 (Fig. 3A). Similarly, in all individuals born near or after the time of the nuclear bomb tests, the 14C concentrations in cardiomyocyte DNA corresponded to the concentrations several years after their birth, establishing postnatal cardiomyocyte DNA synthesis (Fig. 3B).

There is no increase in the number of cardiomyocytes after the postnatal period but rather a slow, continuous decrease with age (21). About 25% of cardiomyocytes are binucleated in humans at birth, and this proportion stays constant throughout life (22). Thus, the postnatal cardiomyocyte DNA synthesis detected by 14C analysis cannot be explained by an increase in cardiomyocyte number or binucleation. However, the heart grows during childhood, as the increasing demand of contractile capacity is met by hypertrophy of cardiomyocytes. Almost all cardiomyocyte nuclei are diploid at the time of birth, but the DNA of most nuclei is duplicated to become tetraploid in childhood when the cells undergo hypertrophy (Fig. 3C and fig. S5) (23–25). After the age of 10, there is no further increase in the DNA content of cardiomyocyte nuclei (R = 0.135, P = 0.384, Fig. 3C). The DNA synthesis associated with polyploidization of cardiomyocyte DNA results in incorporation of 14C concentrations corresponding to the atmospheric levels during childhood.

Three of the individuals born before the nuclear bomb tests were more than 10 years old at the onset of the increase in atmospheric 14C. That their 14C concentration in cardiomyocyte DNA was above the prenuclear bomb test levels (Fig. 3A) cannot be explained by DNA synthesis associated with polyploidization, but indicates cardiomyocyte renewal after 1955. Moreover, in the individuals born after the nuclear bomb tests, the difference between the birth date of the person and the date corresponding to the 14C concentration in cardiomyocyte DNA increased with the age of the individual (fig. S6 and table S1), demonstrating that cardiomyocyte DNA synthesis is not restricted to a limited period in childhood but continues in adulthood.

Polyploidization of cardiomyocyte DNA occurs in a stereotypical manner during a rather short period in childhood (Fig. 3C) (23–25), making it possible to calculate its impact on 14C values in each individual (see supporting online text and (26)). By subtracting the childhood polyploidization-associated 14C incorporation from the measured value in each case, we could estimate polyploidization-independent 14C values. In all cases, the polyploidization-independent 14C values corresponded to time points after birth for each individual (Fig. 3D), indicating cardiomyocyte renewal. In the five oldest individuals, who all were born before or at the onset of the nuclear bomb tests, the 14C values were lower than contemporary values (Fig. 3D), establishing that not all cardiomyocytes had been exchanged after 1955 but that a substantial fraction remains from early in life, even in the elderly.

Fig. 3. Cardiomyocyte turnover in adulthood. (A) The 14C concentrations in cardiomyocyte DNA from individuals born before the time of the atmospheric radiocarbon increase correspond to time points after the birth of all individuals. The vertical bar indicates year of birth, with the correspondingly colored data point indicating the ∆14C value. (B) 14C concentrations in cardiomyocyte DNA from individuals born after the time of the nuclear bomb test. (C) Average DNA content (2n = 100%) per cardiomyocyte nucleus from individuals (without severe heart enlargement; see fig. S5) of different ages. Ploidy was measured by flow cytometry. Colored data points identify individuals analyzed for 14C (n = 33). Black data points are from individuals analyzed only with regard to ploidy level (n = 23), and white data points are taken from Adler et al. (n = 26) (24, 26). The dashed lines indicate the 95% confidence interval for the regression curve. (D) 14C values corrected for the physiologically occurring polyploidization of cardiomyocytes during childhood for individuals born before and after the bomb-induced spike in 14C concentrations, calculated on the basis of the individual average DNA content per cardiomyocyte nucleus. The 14C content is not affected in individuals where the polyploidization occurred before the increase in atmospheric 14C concentrations.
Increased cardiac workload in pathological situations often results in cardiomyocyte hypertrophy and heart enlargement, and at late stages can result in polyploidization in adulthood (fig. S5) (24). Although few subjects had cardiac pathology (table S2), none had severe heart enlargement nor a pathological cardiomyocyte ploidy profile, and there was no significant difference in 14C integration in cardiomyocyte DNA in the subjects with cardiac pathology (table S1, Fig. 3C, and fig. S5). Moreover, mathematical modeling of the kinetics of DNA synthesis and 14C integration showed that the measured 14C concentrations in cardiomyocyte DNA could not be a result of polyploidization during adulthood (see supporting online text). Furthermore, analysis of 14C concentrations in DNA from only diploid or only polyploid cardiomyocyte nuclei demonstrated similar degrees of 14C integration after childhood in both compartments, providing further evidence for cardiomyocyte renewal independently of polyploidization (see supporting online text, fig. S7, and table S3).

Several studies of sex-mismatched transplant recipients have indicated fusion of human cardiomyocytes with other cells (27). However, fusion appears to mainly occur transiently after transplantation, and even in the acute phase the fusion rate is too low to explain the 14C data (fig. S8). DNA damage and repair are very limited in differentiated cells (28) and, at least in neurons, are well below the detection limit of the method used (10, 11). Although cell fusion and DNA repair may affect 14C concentrations in cardiomyocyte DNA, available data suggest that the magnitude of these processes makes them negligible in the current context and that the 14C data we report here (after compensation for polyploidization) likely accurately reflects cell renewal.

Mathematical modeling of 14C data from individuals born both before and after the nuclear bomb tests, which provides slightly different and complementary information, as well as of subjects of different age within these groups, can provide an integrated view on cell turnover (9). We used an analytical model that includes polyploidization in childhood to assess which one of many scenarios for cell birth and death best describes the data. Times at which cells are born, ploidize, and die are tracked. The atmospheric 14C values corresponding to DNA synthesis events are integrated to yield a calculated 14C level, on the basis of each subject’s birth date, age at death, and DNA content. The calculated 14C levels were fitted to the purity-corrected values to find the best renewal rates for each scenario (see supporting online text for a comprehensive description of the modeling). We first calculated what the annual turnover rate would be in each individual if the rate was constant throughout life. This indicated annual turnover rates of 0.2 to 2% (Fig. 4A). However, there was a clear negative correlation to age (R = −0.84; P = 0.001), establishing that the turnover rate declines with age. The strong negative correlation to age also indicates that there is limited interindividual variation in the cardiomyocyte turnover rate and its decrease with age.

We next tested a series of different models allowing turnover rates to change with age. The best fit was found with an inverse-linear declining turnover rate (Fig. 4B), in which younger cardiomyocytes were more likely than older ones to be replaced (see supporting online text). This model predicts that cardiomyocytes are renewed at a rate of ~1% per year at the age of 25 and 0.45% at the age of 75 (Fig. 4B). With this turnover rate, most cardiomyocytes will never be exchanged during a normal life span (Fig. 4C). At the age of 50, 55% of the cardiomyocytes remain from the time around birth and 45% have been generated later (Fig. 4C). The age of cardiomyocytes is on average 6 years younger than the individual (Fig. 4D).

The 14C data indicate a substantially higher renewal rate for noncardiomyocytes, with a median annual turnover of 18% and a mean age of 4.0 years (see supporting online text). Our data do not allow us to identify whether new cardiomyocytes derive from cardiomyocyte duplication or from a stem/progenitor pool, because both would result in similar 14C integration in DNA.

Analysis of cell proliferation in the human myocardium has previously indicated a cardiomyocyte proliferation rate that could result in the exchange of all cardiomyocytes within 5 years (29), but the 14C concentrations in DNA exclude such a high mitotic renewal rate. We asked whether cardiomyocytes may be heterogeneous, with an identifiable subpopulation turning over relatively fast and the rest not turning over at all. This scenario is incompatible with the data, and it is most likely that the vast majority of cardiomyocytes have a similar probability of being exchanged at a given age (see supporting online text).

The limited functional recovery in humans after myocardial injury clearly demonstrates insufficient regeneration of cardiomyocytes. The renewal of cardiomyocytes, indicated by the continuous integration of 14C, suggests that the development of pharmacological strategies to stimulate this process may be a rational alternative or complement to cell transplantation strategies for cardiomyocyte replacement.

Fig. 4. Dynamics of cardiomyocyte turnover. (A) Individual data fitting assuming a constant turnover (see supporting online text) reveals an almost linear decline of cardiomyocyte turnover with age (R = −0.84; P = 0.001). A constant-turnover hypothesis might therefore not represent the turnover dynamics accurately. (B) Global fitting of all data points (see supporting online text, error sum of squares = 1.2 × 10^10) shows an age-dependent decline of cardiomyocyte turnover. (C) The gray area depicts the fraction of cardiomyocytes remaining from birth, and the white area is the contribution of new cells. Estimate is from the best global fitting. (D) Cardiomyocyte age estimates from the best global fitting. The dotted line represents the no-cell-turnover scenario, where the average age of cardiomyocytes equals the age of the individual. The black line shows the best global fitting. Colored diamonds indicate computed data points from 14C-dated subjects. Error bars in (A) are calculated from the errors on 14C measurements. Error bars in all other graphs are calculated for each subject individually and show the interval of possible values fitted with the respective mathematical scenario.
S-Nitrosylation of Drp1 Mediates β-Amyloid–Related Mitochondrial Fission and Neuronal Injury

Dong-Hyung Cho,†1‡ Tomohiro Nakamura,1∗ Jianguo Fang,1 Piotr Cieplak,2 Adam Godzik,2 Zezong Gu,3‡ Stuart A. Lipton3§

Mitochondria continuously undergo two opposing processes, fission and fusion. The disruption of this dynamic equilibrium may herald cell injury or death and may contribute to developmental and neurodegenerative disorders. Nitric oxide functions as a signaling molecule, but in excess it disrupts the balance between mitochondrial fission and fusion can lead to excessive mitochondrial fragmentation. Fragmentation triggered by dysfunction of the fragmentation protein Drp1 (dynamin-related protein 1), for example, contributes to synaptic damage and subsequent neuronal loss because of nitrosative/oxidative stress and impaired bioenergetics (1–6). Excessive fission results in abnormally small mitochondria with fragmented cristae (2), as observed in electron microscopy studies of neurons in the Alzheimer’s disease (AD) (7). Drp1 homologs are S-nitrosylated, which regulates their activity (8, 9). Furthermore, β-amyloid protein (Aβ) oligomers induce excessive mitochondrial fission and neuronal damage in a nitric oxide (NO)–mediated fashion (2, 10). We sought to determine whether Drp1 is S-nitrosylated and thereby activated in AD.

Cerebrocortical neurons transfected with the mitochondrial marker mito-DsRed2 were exposed to the NO donor S-nitrosocysteine (SNOC) (11) and morphological changes in mitochondria were monitored by 3D-deconvolution fluorescence microscopy (2). Mitochondria normally displayed an elongated filamentous morphology, but addition of SNOC induced fragmented, smaller mitochondria in a dose-dependent manner, due to fission (Fig. 1A and B) (2, 11). Using a biotin-switch assay (12), we found that SNOC induced S-nitrosylation of Drp1 (forming SNO-Drp1) in neurons before inducing fission (Fig. 1C).

To investigate whether endogenously generated NO can induce SNO-Drp1, we used human embryonic kidney (HEK) 293 cells stably expressing neuronal NO synthase (nNOS). These cells were subjected to bimtin-switch assay after incubation with the calcium ionophore A23187 to activate nNOS. Endogenous Drp1 was S-nitrosylated by endogenous NO; this reaction was blocked by the NOS inhibitor N-nitro-l-arginine (NNA; Fig. 2, A and B). SNO-Drp1 was not detected in controls performed without ascorbate to remove NO, thus preventing replacement of NO by bimtin (which is detected in this assay), or without bimtin-HPDPE (N-[6-(bimtinamidomethyl)hexyl]-3′-(2-pyrrolidinylthio)-propionamide).

Using the same conditions under which Aβ causes mitochondrial fragmentation and consequent neuronal damage (2), we found that Aβ could induce SNO-Drp1 formation. Cerebrocortical neurons were exposed to oligomers of the pathologically active fragment Aβ25-35 or, as a control, reverse-sequence Aβ35-25. Formation of SNO-Drp1 was observed only in Aβ25-35–treated neurons, not in the control (Fig. 2C). Additionally, we tested the effect of endogenously produced Aβ, generated from amyloid precursor protein (APP) in conditioned medium of N2a/APP695 stable cell lines or in conditioned medium of N2a cells exposed to oligomers of the pathologically active fragment Aβ25-35 or, as a control, reverse-sequence Aβ35-25. Formation of SNO-Drp1 was observed only in Aβ25-35–treated neurons, not in the control (Fig. 2C). Additionally, we tested the effect of endogenously produced Aβ, generated from amyloid precursor protein (APP) in conditioned medium of N2a/APP695 stable neuronal cell lines or CHO cells stably expressing human APP with the Val717 → Phe mutation (designated 7PA2 cells). Exposing N2a cells to SNOC or conditioned medium resulted in SNO-Drp1 formation (Fig. 2C). We also found elevated levels of SNO-Drp1 in vivo in brains of the AD transgenic mouse model Tg2576, which expresses high levels of the Swedish APP mutation (Lys670 → Asn, Met671 → Leu) (fig. S1).

To extend these findings to humans, we examined brains obtained shortly after death from patients manifesting AD (table S1). We found increased SNO-Drp1 levels in 17 of 17 AD brains studied, but not in brains of deceased Parkinson’s disease patients or controls who died of non-CNS causes (Fig. 2, D and E, and fig. S2). To determine whether the level of SNO-Drp1 in AD human

1Center for Neuroscience, Aging, and Stem Cell Research, Burnham Institute for Medical Research, 10901 North Torrey Pines Road, La Jolla, CA 92037, USA. 2Bioinformatics and Systems Biology Program, Burnham Institute for Medical Research, 10901 North Torrey Pines Road, La Jolla, CA 92037, USA. 3Department of Neuroscience, Columbia School of Medicine, Columbia, MO 65212, USA. 4To whom correspondence should be addressed. E-mail: slipton@burnham.org

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26. Materials and methods are available as supporting material on Science Online.
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Supporting Online Material

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Materials and Methods SOM Text Figs. S1 to S8 Tables S1 to S3 References 14 August 2008; accepted 29 January 2009 10.1126/science.1164680

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