LETTERS

Identification of sister chromatids by DNA template strand sequences

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It is generally assumed that sister chromatids are genetically and functionally identical and that segregation to daughter cells is a random process. However, functional differences between sister chromatids regulate daughter cell fate in yeast[1] and sister chromatid segregation is not random in *Escherichia coli*[2]. Differentiated sister chromatids, coupled with non-random segregation, have been proposed to regulate cell fate during the development of multicellular organisms[3]. This hypothesis has not been tested because molecular features to reliably distinguish between sister chromatids are not obvious. Here we show that parental 'Watson' and 'Crick' DNA template strands can be identified in sister chromatids of murine metaphase chromosomes using CO-FISH (chromosome orientation fluorescence *in situ* hybridization[4]) with unidirectional probes specific for centromeric and telomeric repeats. All chromosomes were found to have a uniform orientation with the 5′ end of the short arm on the same strand as T-rich major satellite repeats. The invariable orientation of repetitive DNA was used to differentially label sister chromatids and directly study mitotic segregation patterns in different cell types. Whereas sister chromatids appeared to be randomly distributed between daughter cells in cultured lung fibroblasts and embryonic stem cells, significant non-random sister chromatid segregation was observed in a subset of colon crypt epithelial cells, including cells outside positions reported for colon stem cells[5]. Our results establish that DNA template sequences can be used to distinguish sister chromatids and follow their mitotic segregation *in vivo*.

Major satellite repeats have a uniform head-to-tail orientation on mouse chromosomes relative to the centromere[6,7]. To determine whether this polarity is fixed relative to chromosome ends, we hybridized unidirectional probes specific for major satellite and telomere repeats to single-stranded metaphase chromosomes using CO-FISH (Fig. 1a). For the CO-FISH procedure, cells are treated with BrdU for one round of DNA replication resulting in BrdU incorporation exclusively into the newly formed DNA[4,8]. After treatment with Hoechst 33258 (a DNA dye) and ultraviolet irradiation, nicks are created exclusively at sites of BrdU incorporation, which are then used to remove newly formed DNA by exonuclease treatment and DNA denaturation. The resulting single-stranded chromosomes (containing template DNA only) are hybridized with strand-specific probes (Fig. 1a).

Notably, all chromosomes except the Y chromosome showed a uniform orientation of major satellite relative to telomeric repeats (Fig. 1b). On each chromosome, the 5′ end of the short arm (characterized by C-rich telomere repeats) is adjacent to T-rich major satellite repeat sequences, and the 3′ end of the short arm (characterized by G-rich telomere repeats) is adjacent to A-rich major satellite repeat sequences. All template strands (except those in chromosomes 4 and 18)[9] show mutually exclusive staining with fluorescently labelled peptide-nucleic acid (PNA) probes specific for either A-rich or T-rich major satellite DNA (Fig. 1b, c and Supplementary Figs 1 and 9). Because the orientation of major satellite DNA relative to telomeric DNA is fixed, probes hybridized to major satellite repeats were used to arbitrarily define Watson (red fluorescence, Fig. 1d) and Crick (green fluorescence, Fig. 1d) DNA template strands. A similar chromosomal polarity was observed in *Mus spretus* fibroblasts, with the 5′ end of the short arm adjacent to T-rich minor satellite repeats in most chromosomes (Supplementary Fig. 2). As CO-FISH can differentially label sister chromatids, we adapted the CO-FISH technique to allow us to directly follow chromatid segregation *in vivo* (Fig. 1e).

Non-random segregation of DNA strands in mammalian cells was first reported using indirect pulse-chase experiments with nucleotide analogues in dividing murine intestinal crypt epithelial cells[10]. To study directly the pattern of sister chromatid segregation in such cells, we injected adult mice for 12 h at 1-h intervals with BrdU before the collection of colon tissue, which was fixed, sectioned and subjected to CO-FISH with major satellite probes. Only a minority of cells in colon crypts were actively dividing, as shown by BrdU incorporation (Fig. 2a, right and inset). These BrdU-positive cells showed discrete, non-overlapping red and green fluorescent signals (herein referred to as CO-FISH signals) from the strand-specific probes (Fig. 2b, white arrowheads) indicating successful generation of single-stranded chromosomes. In contrast, most non-mitotic cells showed overlapping red and green fluorescence from the major satellite probes hybridizing to both strands of double-stranded chromosomes (Fig. 2b, yellow arrowhead, Fig. 2c). Cell pairs showing apparent template strand asymmetry were found at different positions within the colon crypt, including high within the crypt axis (Fig. 2c, d and Supplementary Fig. 3). Sister nuclei showing reciprocal, asymmetric CO-FISH fluorescence are compatible with non-random distribution of sister chromatids containing either Watson or Crick DNA template strands (Figs 1e, 2e, Supplementary Fig. 4 and Supplementary Movie 1). We confirmed that CO-FISH signals in mitotic colon cells from mice subjected to 12 h of BrdU treatment were exclusively derived from cells after only one round of DNA replication (Supplementary Fig. 5)[11]. Of note, DNA template strand asymmetry was also observed in colon tissue sections of *M. spretus* using probes specific for minor satellite repeats (Supplementary Fig. 6).

Chromosomes aligned at the metaphase plate *in vivo* displayed what appeared to be a polar arrangement of Watson and Crick sister chromatids (Fig. 2f and Supplementary Movie 2). Furthermore, major satellite DNA template strands appeared to be clustered after mitosis (Fig. 2g), and often had a marked 'mirror-image' asymmetry with territories of red and green fluorescence in one daughter cell

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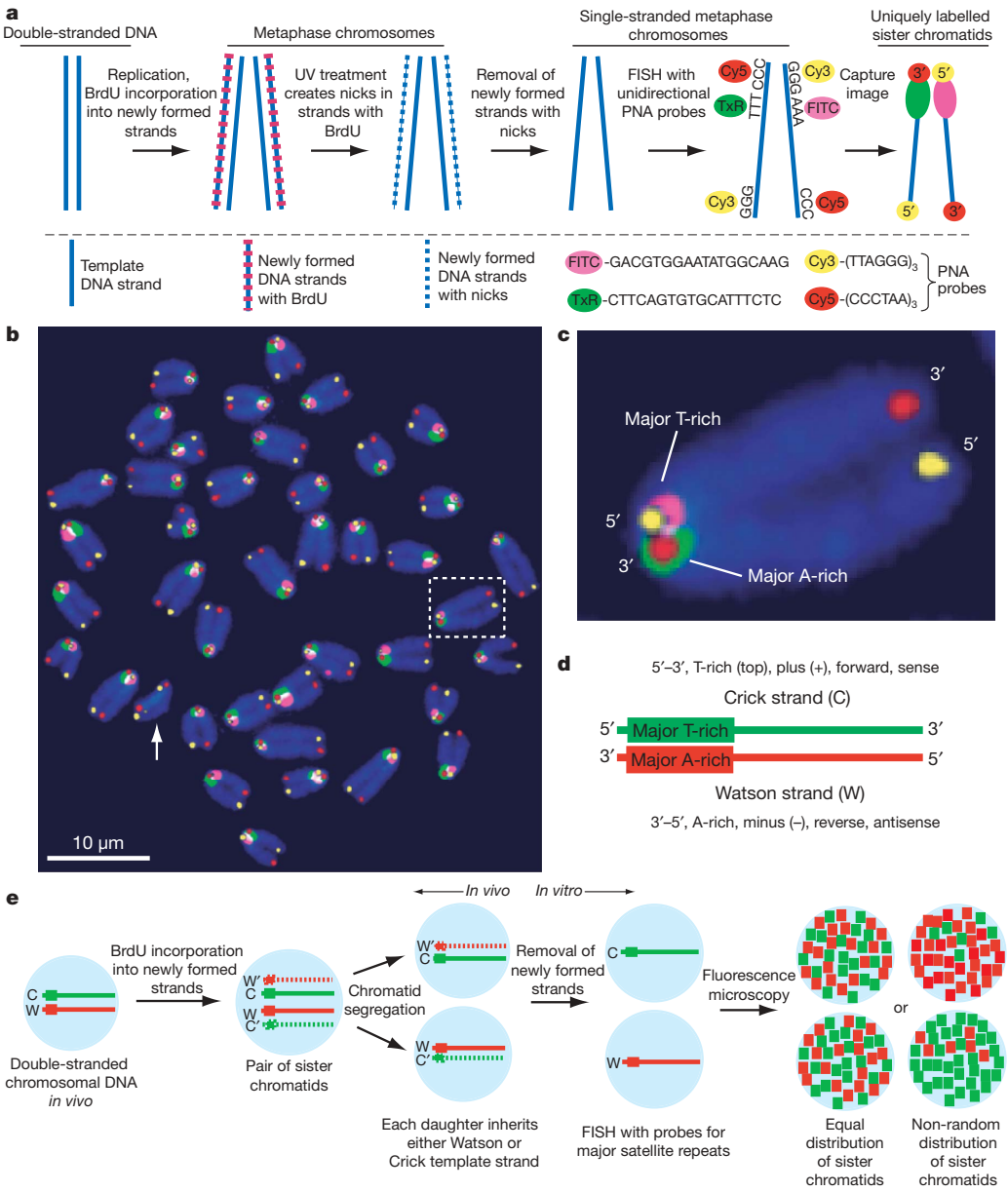

Figure 1 | Highly conserved orientation of telomeric and major satellite DNA in murine chromosomes revealed by four-colour CO-FISH. **a**, Schematic diagram of the CO-FISH procedure. UV, ultraviolet. **b**, Pseudo-colour CO-FISH image of murine metaphase chromosomes. Note that major satellite repeats on all chromosomes except the Y chromosome (arrow, no major satellite DNA) have the same orientation. **c**, Magnification of the boxed chromosome shown in **b**. **d**, Definition of Watson and Crick DNA template strands based on the uniform orientation of major satellite DNA. **e**, The relative distribution of Watson and Crick major satellite fluorescence can be used to study sister chromatid segregation patterns *in vivo*.

mirrored by territories of the opposite colour in the other daughter cell (Fig. 2g and Supplementary Movies 3 and 4). These observations indicate that pericentric regions of several chromosomes cluster in at least some post-mitotic colon cells on the basis of parental DNA template strand sequences. To exclude major rearrangements in nuclear architecture by our CO-FISH procedure, we performed three-colour CO-FISH with both major satellite probes and a telomeric probe (Supplementary Fig. 7). Telomeric signals were observed at expected positions adjacent to centric regions (the terminus of the short chromatid arms) and adjacent to the division plane (the terminus of the long chromatid arms) in support of the notion that the CO-FISH procedure does not grossly alter the general morphology and positioning of segregating chromosomes.

Our qualitative observations suggested that sister chromatids of most chromosomes are segregating non-randomly in a subset of dividing colon epithelial cells. To test whether our observations could nevertheless be explained by chance, we quantified the relative Watson and Crick fluorescence in each daughter cell using dedicated software (see Supplementary Fig. 8 and Supplementary Methods for details). The measured fluorescence was converted to a relative fluorescence ratio (Fig. 3a) based on the reasoning that the total fluorescence from both daughters is the outcome of redistributing a fixed number of DNA template strands from a mother cell to the two daughter cells (Fig. 1e). Reciprocal ratios of Watson and Crick fluorescence are in agreement with the expected distribution of chromatids between daughter cells.

We compared the measured CO-FISH fluorescence signals from sectioned colon and preparations of isolated colon cells to two cultured cell types not expected to show non-random segregation patterns: pluripotent embryonic stem (ES) cells and lung fibroblasts (Fig. 3a and Supplementary Data Table). To avoid selection bias for asymmetry, every cell pair with clear non-overlapping CO-FISH signals was analysed (Fig. 3b). Although this impartial acquisition of data ensures that the measured sister chromatid segregation patterns are not influenced by cell selection, the results will include all recently divided cells, which may complicate data analysis if chromatid segregation patterns differ between cell types. Nevertheless, cell pairs from colon section and isolated colon cells showed a broader distribution of Watson and Crick fluorescence, compared to cultured ES cells and lung fibroblasts (Fig. 3b, grey boxes), reflecting a higher frequency of sister chromatid asymmetry. Up to 50% of cell pairs from all cell types showed an excellent reciprocal ratio of measured fluorescence values between daughter cells, with Watson fluorescence distribution ratios mirrored within 5% by complementary Crick fluorescence distribution

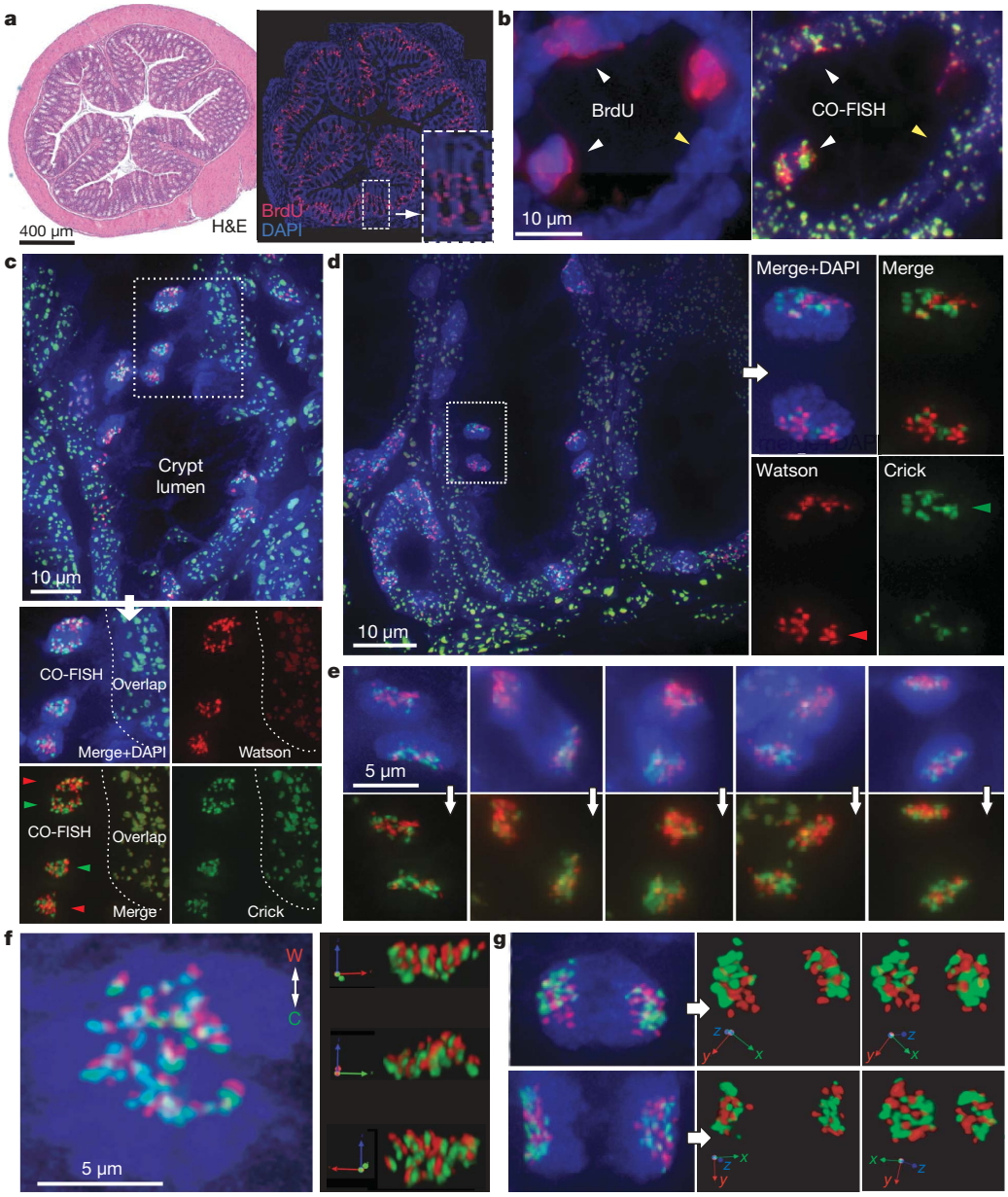

Figure 2 | CO-FISH to study sister chromatid segregation patterns. **a**, Low magnification of adjacent colon sections stained with haematoxylin and eosin (H&E; left), and with DAPI and an anti-BrdU antibody (right). **b**, High magnification of a section stained for BrdU (left) that was subsequently subjected to CO-FISH (right). BrdU-labelled cells show non-overlapping red and green fluorescence (white arrowheads), non-mitotic cells without BrdU show overlapping probe signals (yellow arrowhead). **c**, Example of CO-FISH (non-overlapping) signals in pairs of post-mitotic cells in colon crypts. **d**, Post-mitotic cell pairs relatively high in colon crypt with asymmetric CO-FISH fluorescence. **e**, Examples of asymmetric CO-FISH fluorescence in paired colon cells. **f**, Non-random alignment of sister chromatids at metaphase (right: different projections from Supplementary Movie 2). **g**, Mirror-image symmetry and clustered CO-FISH fluorescence in paired daughter cells (see also Supplementary Movies 3 and 4).

ratios (Fig. 3b, filled squares, and Supplementary Data Table). Cell pairs showing reciprocal fluorescence outside this arbitrary cutoff (Fig. 3b, open circles) most probably reflect noise in CO-FISH measurements due to loss of DNA, non-specific fluorescence and other causes.

To test whether the measured asymmetry in colon cells was non-random, we superimposed our observed fluorescence distributions of cell pairs within the arbitrary 5% reciprocal cutoff value to 95% and 99% confidence intervals calculated from simulated random segregations, representing the range of fluorescence values expected by chance (see Supplementary Figs 9–11 and Supplementary Materials for full discussion of simulated random segregation and statistical analysis). The distribution of Crick template strand fluorescence from sectioned colon tissue and isolated colon cells was outside the 95% or 99% confidence intervals calculated for random sister chromatid segregation (Fig. 3c; $P < 0.05$ open arrowheads, $P < 0.01$ solid arrowheads). This includes a higher frequency of cell pairs with extreme asymmetry, as well as a lower frequency of cell pairs with a symmetrical distribution, than predicted by simulated random segregation. Although fewer cell pairs with extreme asymmetry were present or preserved in colon cell suspensions, the results were nevertheless significant ($P < 0.01$). In contrast, in ES cells and lung fibroblasts the measured fluorescence intensity values were within the 95% and 99% confidence intervals calculated for random segregation. The one exception was in lung fibroblasts at the symmetrical 55% fluorescence value, suggesting a skewing of segregation towards a 50:50 distribution of chromatids (Fig. 3c, bottom, arrowhead). These results support the conclusion that the observed asymmetry of DNA template strand fluorescence in paired colon cells results from non-random segregation of sister chromatids rather than from rare random segregation events. We consider it unlikely that this conclusion is flawed by errors in our methods or fluorescence measurements. The inevitable measurement noise from various sources is not expected to affect adjacent daughter cells in opposite ways (skewing for red fluorescence in one daughter and for green fluorescence in the other). On the other hand, we cannot exclude that BrdU incorporation itself somehow affected sister chromatid segregation and further studies are needed to confirm our findings. Of note, we did not observe 100% asymmetric segregation of sister chromatids in any pair of mitotic colon cells. Most likely, a subset of colon cells selectively segregates sister chromatids from most but not all chromosomes. Alternatively, a small number of specific chromatids could be selectively captured in a larger proportion of cells. The strand-specific probes in this study are unable to detect minor deviations from random sister chromatid

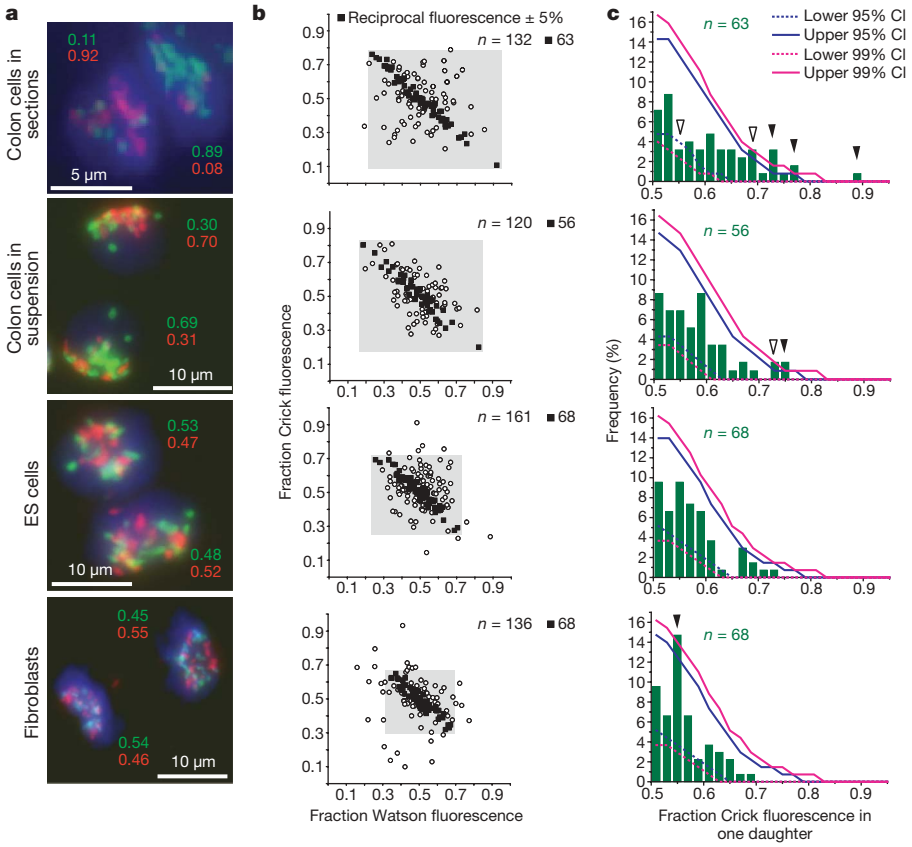

Figure 3 | Measurements of Watson and Crick DNA template strand fluorescence in post-mitotic cells. **a**, Examples of fluorescence measured in the indicated cell types. **b**, For *n* cell pairs, the ratio of Watson and Crick fluorescence in one of the daughter cells (arbitrary selection) is plotted. Solid black squares show cells with reciprocal Watson and Crick fluorescence ratios ± 5%, whereas open circles represent cells with Watson/Crick fluorescence ratios outside this arbitrary cutoff. **c**, The observed Crick fluorescence distributions in selected individual cells (*n* = black squares in **b**) were compared to fluorescence distribution values obtained by simulated random segregation. The observed frequency (*y* axis) of Crick fluorescence (*x* axis, green histograms) in one daughter cell (with the brightest Crick fluorescence) is plotted. The upper and lower 95% and 99% confidence intervals (CI, solid and dashed blue and magenta lines, respectively) represent the range of fluorescence distributions expected by chance. The values measured in colon tissue sections as well as colon cell suspensions fall outside the range for simulated random segregation ($P < 0.05$: open arrowheads; $P < 0.01$: solid arrowheads).

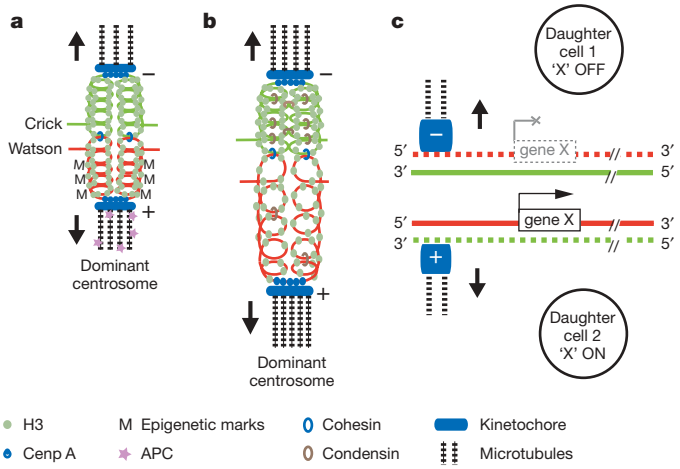

Figure 4 | Models for the mechanism and function of asymmetric sister chromatid segregation. Only the template strand of double-stranded DNA in sister chromatids is shown. **a**, Uneven distribution of epigenetic marks (M) between sister chromatid centromeres could result in asymmetric nucleation of microtubules or selective capture of microtubules coming from the 'dominant' centrosome[15,16]. **b**, Differences in higher-order chromatin structure could alter the elastic properties of (peri)centric chromatin[30] and select specific sister chromatids by microtubules originating from the dominant centrosome. **c**, Regulation of cell fate by selective segregation of sister chromatids that differ in epigenetic marks at centromeres and selected genes.

segregation or detect selective segregation of a few or single chromosomes[12].

Our results provide the first direct data supporting non-random segregation of DNA template strands in mammalian cells *in vivo*. Non-random segregation of sister chromatids has previously been observed in *E. coli*[2] and has been suggested from indirect measurements in various eukaryotic cells[13,14]. Neither the mechanism nor the function of selective sister chromatid segregation is known at present. To enable non-random segregation, sister chromatid centromeres as well as the two centrosomes of the mitotic spindle must have distinct marks or properties that enable specific connections (Fig. 4). Asymmetry at centrosomes[15,16] could result in differences in the timing, the number or the dynamic behaviour of microtubules radiating from each pole. Alternatively, such differences could result from proteins enriched at a specific pole (Fig. 4a). The adenomatous polyposis coli (APC) tumour suppressor protein could be an example of the latter given its involvement in several cellular processes including chromosome segregation and spindle assembly[17–21]. How sister chromatid centromeres are distinguished is equally enigmatic, but probably depends on differences in (peri)centric chromatin, perhaps by differences in the loading[22] or retention[23] of (peri)centric proteins or strand-specific replication[24], methylation[25] or transcription of centromeric DNA[26,27]. Centromeric RNA is known to regulate the assembly of centromeres[28] and strands of major satellite DNA are differentially transcribed during murine development[29]. Chromatin differences between sister chromatids could either be directly recognized by factors at asymmetric spindles (Fig. 4a) or favour selective

attachment to microtubules by changes in elastic properties[30] (Fig. 4b). We propose that the observed non-random segregation of sister chromatids contributes to cell fate decisions as predicted by the 'silent sister' hypothesis[3] (Fig. 4c). Further studies will test the predictions of this hypothesis that chromatin differences between sister chromatids contribute to differences in gene expression between cells, and thus regulate cell fate in asymmetrically dividing cells.

METHODS SUMMARY

CO-FISH analysis. Metaphase chromosomes were prepared from mouse ES cells (C57BL/6J background) incubated with 40 μm BrdU for 12 h before collection using standard cytogenetic procedures. Cytospin preparations of binucleated cells (3 μg ml^{-1} of cytochalasin B for 2 h before collection) were prepared from cultured ES cells and cultured adult lung fibroblasts. For CO-FISH, BrdU-treated cells or chromosomes were treated with pepsin, RNase A, Hoechst 33258 and irradiated with ultraviolet light[8]. Nicked DNA was removed by denaturation after digestion with exonuclease III, and remaining DNA (template) strands were hybridized to directly labelled fluorescent PNA probes specific for C- and G-rich telomere repeats and T- and A-rich major satellite DNA. Slides were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). For *in vivo* studies, C57BL/6J mice (2–3 months old) received intraperitoneal injections of BrdU at 12 × 1-h intervals. Colon tissue was formaldehyde-fixed and paraffin-embedded using standard procedures. Deparaffinized tissue sections (6 μm) were used for CO-FISH as described earlier, with further treatment at pH 6.0 and 80 °C for 45 min. Suspensions of viable colon cells were prepared with collagenase, then dropped onto slides after hypotonic treatment and fixation with methanol/acetic acid to control for possible artefacts from tissue sectioning.

Image analysis. Fluorescence images captured using a digital camera mounted on a fluorescence microscope were combined and processed to provide pseudo-colour images using Adobe Photoshop software. For tissue sections, a stack of images at 0.2–0.5 μm intervals was acquired and projected into a single plane. In some cases deconvolution software (SoftWoRx, Applied Precision) was used to create projection images. For image and data analysis, the fluorescence intensities within individual nuclei of paired daughter cells were measured using in-house software, and a combination of Bootstrap inference and Monte Carlo simulations was applied to build a random-segregation model and calculate 95% and 99% confidence intervals of expected fluorescence distribution profiles.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Author Contributions E.F. helped with the design of the experiments, image acquisition, data analysis, interpretation of results and writing of the paper. E.A.C. performed most of the CO-FISH experiments. A.H. performed most of the mouse work. L.B. acquired some images for this study. S.S.S.P. performed analysis of digital data and helped with statistical analysis that was performed by S.M. D.G.H. helped with the design of the study and interpretation of results. P.M.L. conceived the study, helped with image acquisition, interpretation of results and writing of the paper.

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METHODS

Preparation of cells for CO-FISH analysis. Undifferentiated wild-type murine embryonic stem cells (C2, C57BL/6NTac background) and R1-derived wild-type embryonic stem cells ($C1^{+/+}$, 129S1 background)[31] were obtained from A. Nagy (Samuel Lunenfeld Research Institute, Toronto) and cultured on gelatin-coated plastic culture dishes in DMEM containing 20% FCS in the presence of 100 ng ml^{-1} of leukaemia inhibitory factor as described[32]. Murine embryonic fibroblasts were grown in DMEM-FCS. BrdU (Invitrogen) was added to semi-confluent cultures at a final concentration of 40 μM for 12 h before collection. Binucleated cells were prepared by adding cytochalasin B (Sigma-Aldrich, 3 μg ml^{-1}) to cultures 2 h before collection of cells by trypsinization. Cells were spun onto microscope slides using a Shandon Cytospin 4 (Shandon Scientific) and fixed with 3:1 methanol/acetic acid. For preparation of metaphase cells colcemid (Sigma-Aldrich, 0.1 μg ml^{-1}) was added for 1 h before collection. Trypsinized cells were treated with 0.075 M KCl (Stem Cell Technologies, Inc.) for 10 min before fixation with 3:1 methanol/acetic acid using standard cytogenetic procedures. Fixed cells were stored at −20 °C. To obtain metaphase spreads for CO-FISH, cells were dropped onto wet microscope slides and dried overnight at room temperature.

CO-FISH. Slides were rehydrated in PBS, pH 7.4 (Stem Cell Technologies) for 15 min and fixed for 2 min in 4% formaldehyde in PBS followed by three washes in PBS for 5 min each. Slides were treated with freshly prepared pepsin (P7000, Sigma-Aldrich) at 1 mg ml^{-1} in acidified water (pH 2.0) at 37 °C for 10 min followed by two washes for 2 min each in PBS, a rinse in 2×SSC and treatment with RNase A (0.1 mg ml^{-1} in PBS) for 10 min at 37 °C. Slides were washed twice with PBS for 5 min each, and stained with 100 μl Hoechst 33258 (Sigma-Aldrich) at 1 mg ml^{-1} under parafilm for 15 min at room temperature. The slides were rinsed with 2×SSC, transferred to a tray, covered with a glass coverslip and irradiated with ultraviolet light for 30 min in a UV Stratalinker 1800 (calculated dose 5.4×10^3 J m^{-2}). BrdU-substituted DNA strands were digested with 50 μl exonuclease III (New England Biolabs) at 3,000 U ml^{-1} in buffer supplied by the manufacturer (50 mM Tris-HCl, 5 mM $MgCl_2$ and 5 mM dithiothreitol (DTT), pH 8.0) at 37 °C for 10 min under a coverslip. Slides were rinsed three times for 5 min each in 2×SSC before denaturation in 70% formamide in 2×SSC for 1 min at 70 °C and dehydration in ice cold 70%, 90% and 100% ethanol for 2 min each. Cells were rehydrated in PBS for 10 min, fixed in 4% formaldehyde in PBS for 2 min, washed three times for 5 min each in PBS, dehydrated in ethanol again and air dried. Hybridization mixture (20 μl) was added to each slide, covered with a coverslip, and cells were denatured on a hot plate at 80 °C for 2 min. The hybridization mixture consisted of 10 mM Tris-HCl, pH 7.6, 1 mM $MgCl_2$, 70% formamide, 0.25% blocking reagent (New England Nuclear), 0.5 μg ml^{-1} Cy5-labelled $(CCCTAA)_3$ PNA and 0.5 μg ml^{-1} Cy3-labelled $(TTAGGG)_3$ PNA (specific for the G- and C-rich telomeres, respectively), 1 μg ml^{-1} fluorescein-labelled GACGTGGAATATGGCAAG PNA specific for the T-rich strand of mouse major satellite DNA[33] and 1 μg ml^{-1} TexasRed-labelled CTTCAGTGTGCATTTCTC PNA specific for the A-rich strand of mouse major satellite DNA. Fluorescently labelled PNA probes were obtained from Applied Biosystems, Panage Inc. or Biosynthesis Inc. without noticeable differences in results. After hybridization for 1 h at room temperature, slides were washed twice for 15 min each in 70% formamide, 10 mM Tris-HCl, 1% BSA, and for 3 × 5 min in TNT (0.1 M Tris-HCl, 0.15 M NaCl, 0.08% Tween-20, pH 7.5). After dehydration in ethanol, slides were air dried and counterstained with DAPI at 200 ng ml^{-1} in DABCO antifade solution[34].

CO-FISH on paraffin-embedded tissue sections. C57BL/6J mice (2–3 months old) were injected intraperitoneally with BrdU for 12 h at 1-h intervals as described[35]. For metaphase analysis, BrdU was injected for 0, 8, 12 or 16 h, with an extra injection of colcemid to arrest cells at metaphase 1 h before tissue collection. Colon tissue was fixed overnight in 4% formaldehyde in PBS, and embedded in paraffin using standard procedures. Tissue sections (6 μm) were baked overnight at 60 °C, deparaffinized in xylene three times for 15 min each at room temperature before dehydration in 100% ethanol (twice for 10 min). The previously described CO-FISH protocol was used for metaphase spreads from cultured cells with the following modifications. Slides were treated in 10 mM citric acid buffer, pH 6.0 at 80 °C for 45 min, washed at room temperature in PBS twice for 5 min each, and water for 5 min followed by pepsin and RNase treatment as described earlier. After incubation with Hoechst and treatment with ultraviolet irradiation as earlier, slides were rinsed with 2×SSC for 5 min and denatured in 70% formamide, 2×SSC for 2 min at 72 °C, dehydrated, air dried and rehydrated in PBS for 10 min before treatment with RNase A and exonuclease III as above. Slides were washed twice for 5 min each in 2×SSC, denatured in 70% formamide, 2×SSC for 1 min at 70 °C, dehydrated in ethanol and air dried. For hybridization, 40 μl of hybridization mixture containing 1 μg ml^{-1} Cy5-labelled GACGTG GAATATGGCAAG or Cy5-labelled GAAGGACCTGGAATATGG PNA specific for the T-rich Crick strand of mouse major satellite DNA[33] and 1 μg ml^{-1} Cy3-labelled CTTGCCATATTCCACGTC specific for the A-rich Watson strand of mouse major satellite DNA was used. After denaturation for 3 min at 80 °C and hybridization overnight at room temperature, slides were washed and counterstained with DAPI at 10 ng ml^{-1} in PBS for 5 min, rinsed three times for 5 min in PBS, dehydrated in ethanol and covered under DABCO antifade solution for fluorescence microscopy.

Isolation of paired cells from colon. C57BL/6J mice (2–3 months old) were injected intraperitoneally with BrdU for 12 h at 1-h intervals and paired cells from colon were isolated by modification of a published method[35]. In brief, colon tissues were dissected from BrdU-treated mice and placed in ice-cold PBS. Faeces were cleared by flushing the colon with a syringe filled with ice-cold PBS. Colons were subsequently cut longitudinally and minced into 1-cm pieces then incubated in 15 ml of predigestion solution (5 mM EDTA, 1 mM DTT, 1× PBS) for 30 min at 37 °C. The resulting cell suspension was centrifuged for 5 min at 350*g* at 20 °C. Supernatant was aspirated and the cell pellet was resuspended in 15 ml of digestion solution (prepared by mixing 50 mg of collagenase type XI (Sigma-Aldrich) and 100 mg of dispase II (Sigma-Aldrich) into 100 ml of PBS) for 90 min at 37 °C. After incubation, the cell suspension was centrifuged for 5 min at 350*g* at 20 °C. Supernatant was aspirated and discarded. Cell pellet was resuspended in PBS, vortexed for 20 s and passed through a 100-μm cell strainer (BD Falcon). Cells were treated with 0.075 M KCl for 10 min at 37 °C before fixation with 3:1 methanol/acetic acid. Fixed cells were stored at −20 °C, then spun onto microscope slides and subject to CO-FISH analysis as above.

Fluorescence microscopy, image acquisition and selection. Fluorescence signals were captured on an Axioplan microscope (Zeiss) equipped with filters for DAPI, FITC, Cy3, Cy5 and Texas Red (Chroma Technology and Semrock) using an Axiocam MRm digital camera controlled by Metasystems ISIS software (Altlussheim). Alternatively, images were acquired on a Coolsnap HQ digital camera attached to an inverted microscope (IX70 Olympus) fitted to an imaging system (DeltaVision RT, Applied Precision) equipped with similar filter sets. Grey-scale (12 bit) images at the wavelengths of interest were acquired through a high-numerical-aperture ×63/1.4 or ×60/1.4 oil immersion lens. For tissue sections, a stack of images at 0.15–0.5 μm intervals were acquired to cover the entire thickness of the section. Fluorescence signals in individual image planes were projected onto a single image plane using ISIS software (Metasystems) or SoftWoRx (Applied Precision) software before or after deconvolution.

Acquisition of image stacks was limited to informative cell pairs defined as cell pairs in which both nuclei appeared to be intact and did not overlap with neighbouring nuclei. To avoid ascertainment bias, image stacks from every informative cell pair were acquired. Because only a few informative cell pairs were present on individual slides, images were acquired from several slides to generate sufficient data for statistical analysis. Details of quantitative image analysis and statistical analysis are provided as Supplementary Information.

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