The EMBO Journal (2011) 30, 1104–1109 | © 2011 European Molecular Biology Organization | All Rights Reserved 0261-4189/11
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THE EMBO JOURNAL

Lgr5 intestinal stem cells have high telomerase activity and randomly segregate their chromosomes

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Somatic cells have been proposed to be limited in the number of cell divisions they can undergo. This is thought to be a mechanism by which stem cells retain their integrity preventing disease. However, we have recently discovered intestinal crypt stem cells that persist for the lifetime of a mouse, yet divide every day. We now demonstrate biochemically that primary isolated Lgr5 + ve stem cells contain significant telomerase activity. Telomerase activity rapidly decreases in the undifferentiated progeny of these stem cells and is entirely lost in differentiated villus cells. Conversely, asymmetric segregation of chromosomes has been proposed as a mechanism for stem cells to protect their genomes against damage. We determined the average cell cycle length of Lgr5 + ve stem cells at 21.5 h and find that Lgr5 + ve intestinal stem cells randomly segregate newly synthesized DNA strands, opposing the 'immortal strand' hypothesis.

The EMBO Journal (2011) **30,** 1104–1109. doi:10.1038/emboj.2011.26; Published online 4 February 2011
Subject Categories: development; molecular biology of disease
Keywords: asymmetric segregation; immortal strand; Lgr5; stem cells; telomerase

Introduction

The intestinal epithelium provides an attractive system to study adult stem cells. It is the fastest self-renewing tissue in mammals with a turnover time of 5 days. It has a simple, repetitive architecture of crypts and villi and a defined hierarchical organization. Epithelial renewal is driven by stem cells located near the base of the crypts of Lieberkühn. These stem cells divide to fill a Transit Amplifying (TA) compartment. TA cells divide with a cell cycle time of 12 h and go through 4–5 rounds of division after which they differentiate into one of several cell types. Enterocytes and goblet cells move up flanks of the villi and are eventually shed into the lumen, while Paneth cells settle at the bottom of the crypts to live for up to 2 months (Ireland *et al*, 2005).

Several stem cell markers have been proposed that either identify cells at position +4, the original stem cell proposed by Potten (Potten, 1998; He *et al*, 2004), or the mark cells that are localized near the base of the crypt (Barker *et al*, 2007; Snippert *et al*, 2009). Three years ago, our laboratory identified Lgr5 as a definitive marker of crypt stem cells. Lgr5 + ve cells are located between Paneth cells, and had previously been named crypt base columnar cells (CBCs) (Cheng and Leblond, 1974a, b). Using lineage tracing experiments, we have demonstrated that these cells represent genuine stem cells, giving rise to all the different epithelial cell types throughout life (Barker *et al*, 2007).

With the identification of the Lgr5 + ve cells, we were able to demonstrate that the intestinal stem cells do not divide asymmetrically. Rather, the 14 Lgr5 + ve stem cells of each crypt are maintained by neutral competition for Paneth cell surface after the stem cells have divided (Lopez-Garcia *et al*, 2010; Snippert *et al*, 2010). The continuously and symmetrically dividing stem cells are dependent on niche signals from the adjacent Paneth cells (Sato *et al*, 2010), and have equal potential to maintain their stemness after each division.

Our data suggest that the Lgr5 + ve stem cells are not quiescent. Their cell cycle time, previously estimated at 1 day (Barker *et al*, 2007), implies that they go through 700–1000 divisions in the lifetime of a mouse. This feat is mirrored by the unimpeded expansion that can be accomplished *in vitro* with these cells (Sato *et al*, 2009). This unexpected behaviour challenges current dogmas and requires many logistical adaptations, in particular regarding maintenance of the integrity of the genome. Telomeres, highly repeated sequences located at chromosome ends, are believed to shorten with each somatic cell division, effectively limiting its lifespan. Telomere shortening is counteracted by telomerase, a RNA-dependent DNA polymerase complex. Telomerase activity has been detected in germline cells and embryonic stem cells, cancer cells (Kim *et al*, 1994) and most human fetal somatic tissues (Wright *et al*, 1996; Ulaner *et al*, 1998). However, in adult tissues telomerase activity has only been demonstrated in stem cells of the hair follicle (Ramirez *et al*, 1997) and in bone marrow-derived haematopoietic stem cells (Morrison *et al*, 1996).

In the intestine, expression of telomerase has been published for mouse and human (Greenberg *et al*, 1998; Saleh *et al*, 2008), although the expression of telomerase as identified by a mTert-GFP expressing transgenic was found in only 1 cell of every 157 crypts (Breault *et al*, 2008). In a follow-up paper, Breault *et al* provide evidence that these rare Tert-GFP + cells represent quiescent stem cells and that actively cycling crypt cells (that include Lgr5 + stem cells) do not contain telomerase activity (Montgomery *et al*, 2010). It is not directly clear why a quiescent cell would require

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Received: 19 November 2010; accepted: 7 January 2011; published online: 4 February 2011

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telomerase activity. Conversely, antibody staining on human tissue located telomerase expressing cells in the bottom third of every colonic crypt, though only minor telomerase activity was found for whole colon extracts (Hiyama *et al*, 2001).

Another way by which stem cells protect their DNA was suggested to be the so-called 'immortal strand' mechanism (Cairns, 1975). During mitosis, stem cells are suggested to retain the 'old' template DNA strand and transfer the newly synthesized strand to their daughter cells. Such label retention and selective chromosome segregation have been reported to occur post injury in hypothetical stem cells located at position +4 counting from the small intestinal crypt bottom (Potten *et al*, 2002). Recently, Nathke and co-workers described the occurrence of symmetric and asymmetric label retention in the stem cell compartment of mouse and human intestine (Quyn *et al*, 2010). On the other hand, double labelling experiments indicated that haematopoietic stem cells and hair follicle stem cells do not asymmetrically segregate their chromosomes (Kiel *et al*, 2007; Sotiropoulou *et al*, 2008).

To address aspects of the logistical challenge of the maintenance of genome integrity in the face of a thousand consecutive cell divisions, we have studied telomerase activity and asymmetric chromosome segregation in Lgr5 stem cells in small intestinal crypts.

Results

Telomerase in Lgr5+ve stem cells

In order to study the presence and activity of telomerase in intestinal stem cells, we isolated Lgr5+ve epithelial cells by FACS from isolated crypts of Lgr5-EGFP knock-in mice. With our sorting procedure we distinguished three different GFP-expressing populations (Figure 1B). Previous experiments have shown that the GFP-Hi cells are the real stem cells, which can generate ever-expanding epithelial organoids with all hallmarks of *in vivo* epithelial tissue (Sato *et al*, 2009). Micro array analysis indicates that the GFP-Hi population represents the stem cells and the other two GFP-positive populations represent their daughters (Muñoz *et al*, in preparation). The fold change between the populations is shown in Figure 1E for a selection of genes that mark the different lineages.

The stem cells and their progeny were analysed for the expression of the catalytic component of Telomerase Tert by RT–PCR (Figure 2A). We find that the Lgr5+ve populations all contain Tert mRNA at levels significantly higher then whole crypt samples. No Tert expression was detected in villus cells.

To test for telomerase activity, cell extracts were analysed using a TRAPeze telomerase detection assay (Figure 2B). This shows that the activity of telomerase diminishes during differentiation. GFP-Hi Lgr5+ve stem cells have the highest level of activity, whereas extracts from the GFP-Lo population exerted telomerase activity at comparable levels to extracts from isolated crypts. Differentiated villus cells did not show telomerase activity anymore. The colorectal cancer cell line DLD1 was used as a positive control for telomerase activity (Hiyama *et al*, 2001).

Stem cell telomeres are shorter in old mice

After showing that Lgr5+ve stem cells contain telomerase activity, we were interested to see if this activity is sufficient to maintain the lengths of telomeres in these cells. Previous

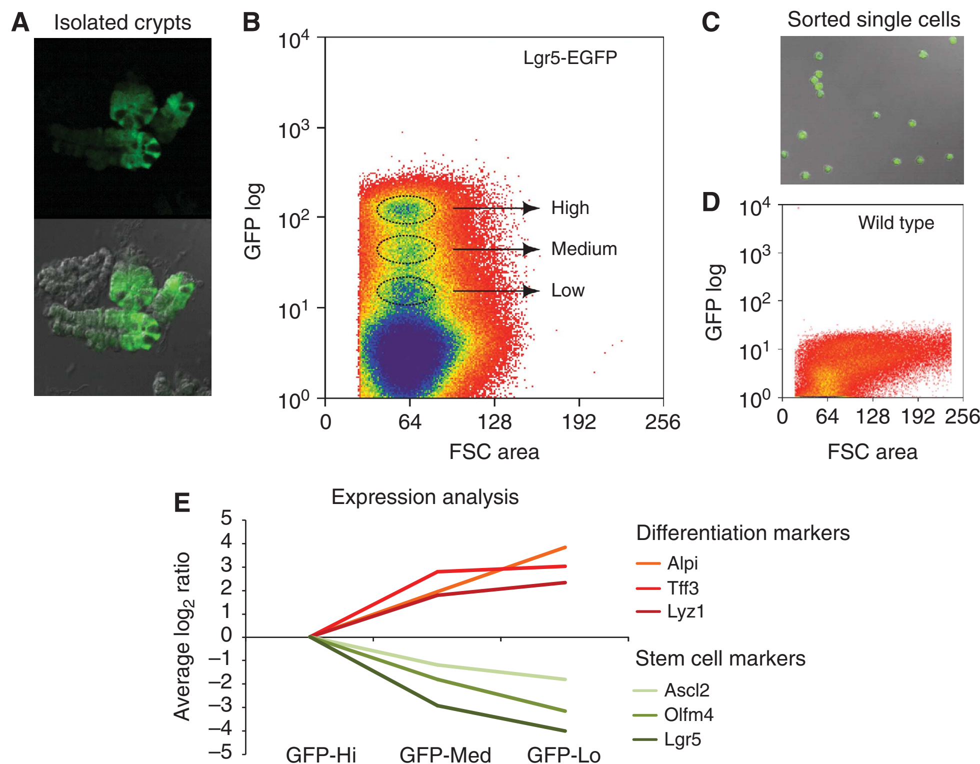

Figure 1 Three different Lgr5-EGFP populations can be distinguished. (**A**) Isolated EGFP-Lgr5+ crypts. (**B**) FACS isolation of three different Lgr5+ve populations. (**C**) Single sorted cells are all GFP-positive. (**D**) FACS analysis of wild-type intestinal cells. (**E**) Gene expression analysis comparing the three sorted GFP populations. Expression of stem cell markers is highest in the GFP-Hi population.

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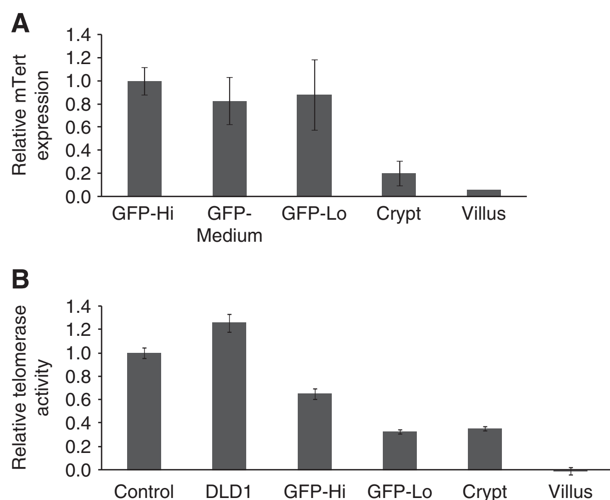

Figure 2 Telomerase activity in stem cells but not in villus cells. (**A**). mTert expression analysed by RT–PCR. Stem cells express higher levels of mTert than average crypt or villus cells. Normalized for Lgr5-high stem cells, error bars represent s.e.m. (**B**) Telomerase activity of cell extracts measured by TRAPeze reaction, normalized for the telomerase-positive control cell line provided with the kit. Extract from the colorectal cancer cell line, DLD1, gives high activity. Lgr5-high cells have a higher telomerase activity than Lgr5-low cells, whereas villus cells possess no telomerase activity. Error bars represent s.e.m.

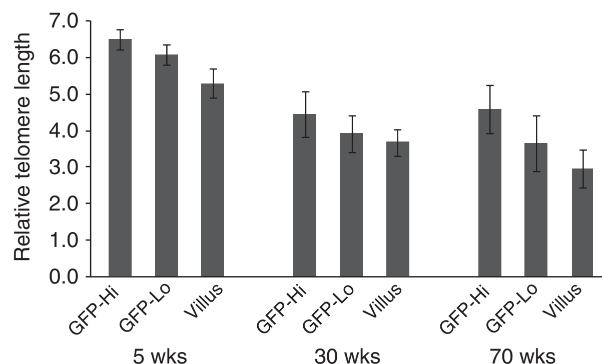

Figure 3 Telomeres in stem cells and daughters shorten over time. Telomere length is represented relative to telomere length of DLD1. Bars represent average values of relative telomere length. Error bars represent s.e.m.

publications have shown that telomeres of epithelial cells in the intestine (Wang *et al*, 2009) and in stem cells of many other organs (Flores *et al*, 2008) shorten over time. Both studies used Q-FISH to quantify telomere length, but the epithelium was separated only into crypt and villus fractions (Wang *et al*, 2009), or the $+4/+5$ position was taken as the stem cell compartment (Flores *et al*, 2008).

We analysed telomere length of sorted Lgr5 + ve cells and villi of mice of 5, 30 and 70 weeks old by Flow Cytometry. Telomere length is presented relative to the length in DLD1 cells as shown in Figure 3. The data show that the telomeres shorten with age also in the Lgr5 + ve stem cells. However, at any given time point, the telomeres of differentiated villus cells were shorter than the telomeres in Lgr5-high stem cells and their immediate daughters. Moreover, the average telomere length of Lgr5-low cells was substantially lower than in Lgr5-high cells.

Determination of the average cell cycle length

In order to determine what other measures the intestinal stem cells use to maintain the integrity of their genome we set out to validate the model of the immortal strand hypothesis. To do this, first the length of the cell cycle was determined accurately. Mice were injected with EdU and sacrificed at different time points after injection (Figure 4A). Agarose sections were stained for the mitosis marker phospho-histon H3 (PH3) and differential interference contrast was used to show the morphology of the tissue. As there is an almost geometrical distribution of Lgr5 + ve stem cells and post-mitotic Paneth cells in the base of the crypt (Sato *et al*, 2010; Snippert *et al*, 2010), stem cells were identified as dividing cells adjacent to a Paneth cell. A quadruple staining for Lgr5-GFP, PH3, EdU and DAPI is given in Figure 4B.

Cells incorporate EdU label only in S phase. Therefore, analysis at different time points after injection should reveal successive waves of EdU-positive mitotic nuclei. Indeed, such waves were observed upon counting the percentage of EdU-positive stem cells in mitosis (Figure 4D). The lapsed time between the first two peaks was found to be 21.5 h, representing the average cell cycle length of a stem cell. Sequential double labellings were performed with EdU and BrdU with a 21.5-h interval to show that cells in the second wave of mitosis indeed reentered the cell cycle (Supplementary Figure S1).

Symmetric segregation of EdU label

After determining the cell cycle length, we analysed the stem cell divisions in the second round of mitosis after EdU incorporation at 26.5 h. At this time asymmetric segregation of chromatin strands, as proposed by the immortal strand hypothesis, should be visible as asymmetric distribution of EdU label. Agarose sections were stained for incorporated EdU and the epithelial membrane marker E-cadherin, and counterstained with DAPI (Figure 5A).

Three colour overlays clearly showed that dividing cells at all positions in the crypt segregate their chromatin in a symmetric manner. The E-cadherin staining proved to be essential to ensure that the observed condensed chromosomes indeed belonged to a single dividing cell before its cytokinesis. A total of 51 mitotic stem cells were identified for which E-cadherin staining completely lined the contours of the dividing cell in 3-dimensional reconstruction. Nine of these cells are shown in Figure 5A–I, and represent divisions in which DNA separation is orientated parallel as well as perpendicular to the basement membrane. In the 51 dividing cells, no asymmetric segregations of EdU-labelled chromatin and no preferential orientation of the plane of division were observed. Although we did not find asymmetric segregation of label, the possibility of asymmetric divisions in a minority of cells cannot formally be excluded. However, based on the 51 counted events, if 1 of the 14 stem cells in each crypt of the small intestine would asymmetrically segregate its chromatids, the *P*-value for failing to observe such a scenario is <0.025.

Discussion

Stem cells quiescence is often considered to be a central attribute of adult stem cells, as continuous cell divisions may challenge the genomic integrity (Cotsarelis *et al*, 1990; Arai *et al*, 2004). Limiting the number of cell divisions

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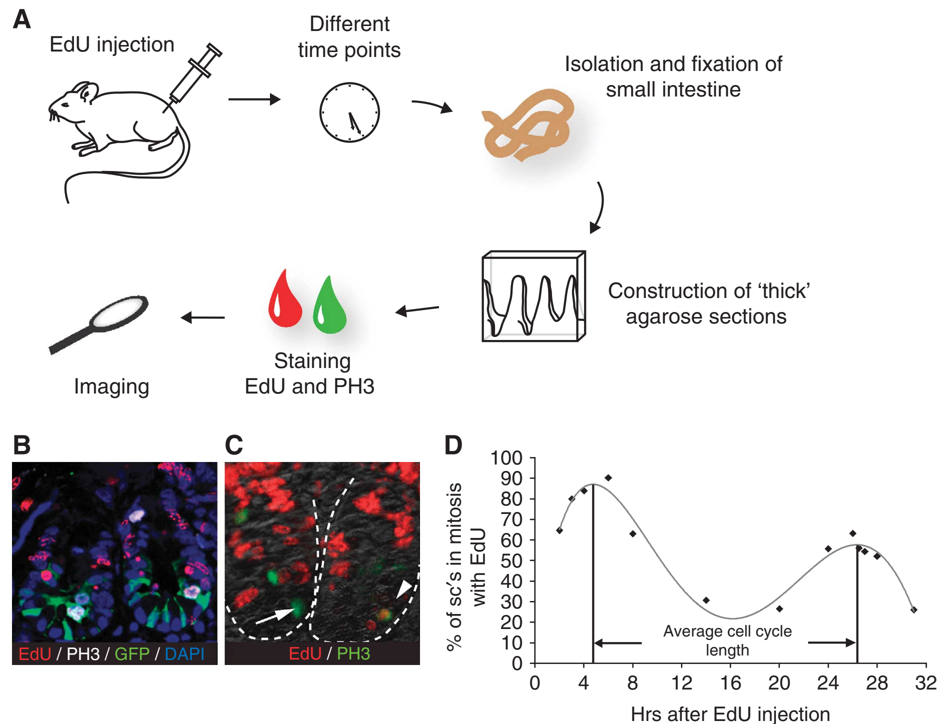

Figure 4 Crypt base columnar cells cycle once every 21.5 h. (**A**) Outline of experiment. (**B**) Co-staining of Lgr5-GFP antibodies (green), PH3 antibodies (white), which marks cells in mitosis, and incorporated EdU (red) together with DAPI. (**C**) Differential interference contrast visualization of cells in two crypts labelled with PH3 antibodies (green), which marks cells in mitosis, and incorporated EdU (red). CBCs in mitosis can be seen with (arrowhead) or without (arrow) incorporated EdU. (**D**) Graphic overview of counted stem cells. Individual data points were obtained from different mice. The line connecting the data points (optimal polynomial, $R^2 = 0.958$) shows two peaks, representing the first and second wave of mitosis. The difference between the peaks gives the average cell cycle length.

would also provide a means to minimize progressive telomere shortening (Forsyth *et al*, 2002). In this study, we find that Lgr5 + ve stem cells divide on average every 21.5 h.

This continuous cycling requires a mechanism to avoid telomere erosion. Indeed, we find that intestinal stem cells contain significant telomerase activity and that this activity is gradually lost in their progeny. The level of telomerase activity is comparable to the immortal diploid colorectal cancer cell line DLD1. This agrees with the finding that telomerase-deficient mice develop atrophies in proliferative organs such as the intestinal epithelium in the third generation (Lee *et al*, 1998). However, despite the presence of telomerase activity in stem cells, telomere shortening is not completely prevented. Interestingly, the average telomere length of Lgr5-low cells was substantially lower than in Lgr5-high cells at all ages. The degree of telomere shortening of several kilobases observed has occurred in only a limited number of cell divisions (4–5). It is unlikely that this telomere shortening is merely caused by an end-replication problem. Indeed, the possibility of active telomere degradation has been suggested before (Flores *et al*, 2008).

Besides telomere erosion, the acquisition of mutations may pose another challenge in rapid cycling cells (Cairns, 1975). Selective segregation of chromosomes has been proposed as a mechanism to avoid replication induced mutations. Asymmetric distribution of label in label-retaining cells in the intestine has been suggested but is still under debate (Potten *et al*, 2002; Quyn *et al*, 2010). In our study, we do not find asymmetric segregation of chromosomes in intestinal stem cells. A possible explanation for this disparity could be that no membrane marking was used in previous studies, which makes it difficult to discriminate between two dividing neighbouring cells and a single dividing cell.

This study demonstrates that the Lgr5-high cells, which represent the 'workhorse' stem cells of the small intestinal crypt, actively express telomerase. The Lgr5 crypt stem cells are likely the first described somatic cells that will complete many hundreds of cell divisions in the lifetime of a mouse, in essence defying the Hayflick limit (Hayflick and Moorhead, 1961). Against this backdrop, it remains mysterious why telomerase-mutant knockout mice only reach 'telomere-crisis' in the intestinal epithelium in the third generation. It should be noted, however, that each generation starts 'afresh' with the telomere length of germline cells, which will be substantially longer than the telomeres of an Lgr5 stem cell after its numerous somatic divisions. How exactly stem cells handle the mutational challenge posed by the hundreds of cell divisions they go through remains to be determined. Asymmetric segregation of chromatids as defined by the 'immortal strand' hypothesis does not appear to represent the mechanism by which these cells accomplish this feat.

Materials and methods

Lgr5 + ve cell sorting

The generation of Lgr5-EGFP mice and isolation of intestinal crypt and villus fractions were described earlier (Barker *et al*, 2007). Freshly isolated small intestines were incised along their length and villi were scraped off. The intestinal tissue was washed in PBS/EDTA (5 mM) for 5 min, and subsequently incubated in fresh PBS/EDTA for 30 min at 4°C. Vigorous shaking yielded free crypts, which were incubated in SMEM supplemented with Trypsine (1 mg/ml)

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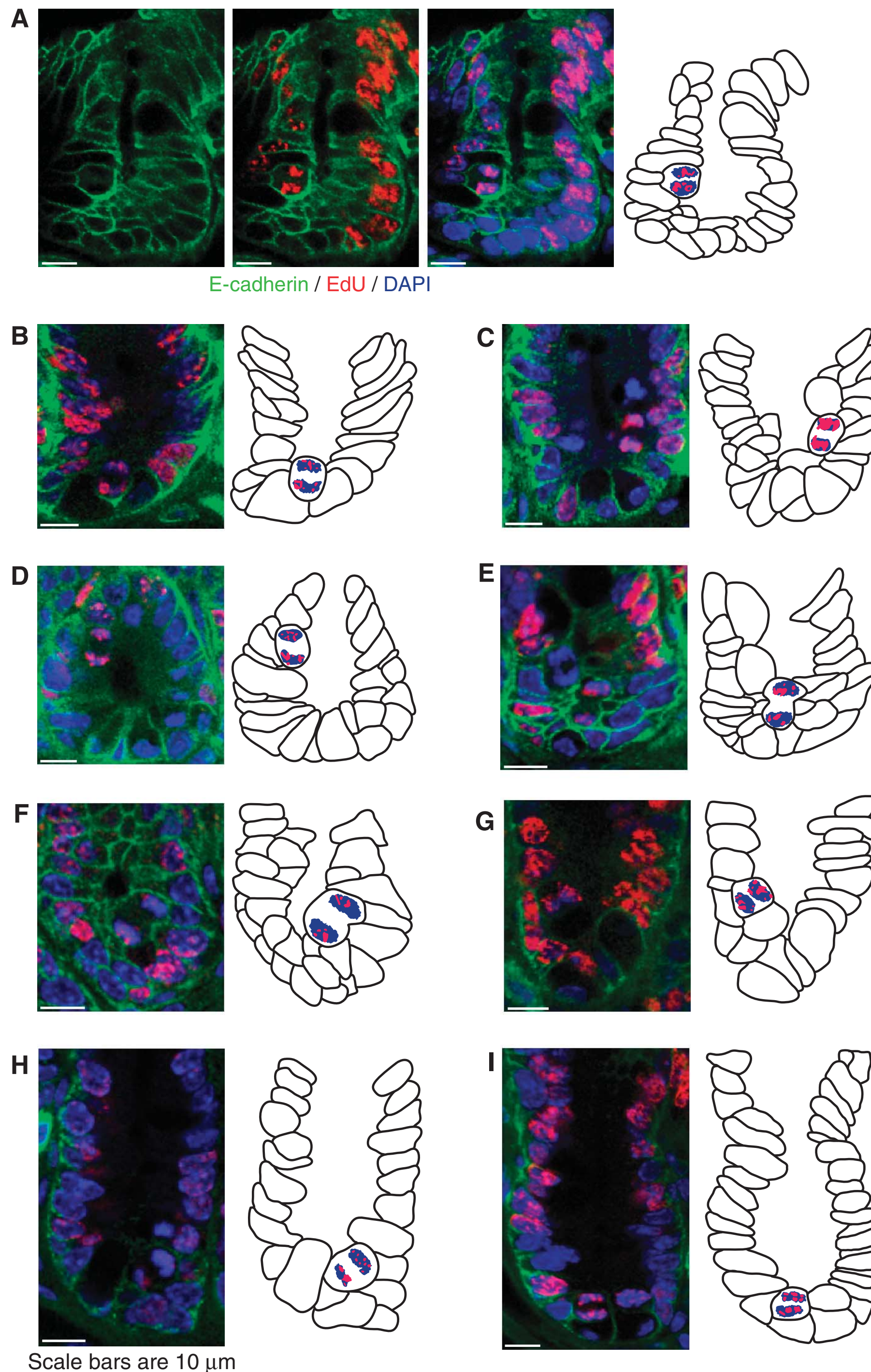

Figure 5 Random segregation of EdU label during stem cell mitosis. (**A**) Three colour overlay of intestinal crypt showing membrane marker E-cadherin (green), incorporated EdU label (red) and DNA (blue). A simplified representation of the division is given to accentuate the cell that is dividing. (**B–I**) Divisions at multiple positions in the crypt all show symmetric segregation of label.

and DNase (0.8 U/μl) for 30 min at 37°C. After incubation, cells were spun down, resuspended in SMEM (Invitrogen) and filtered through a 40-mm mesh. Single GFP-expressing cells were isolated using a MoFlo cell sorter (Beckman Coulter). Propidium iodide was used to exclude dead cells.

EdU injection

Adult mice were injected intraperitoneally with 350 μl 3 mM EdU solution in PBS.

Confocal analysis

For confocal analysis, intestines were fixed in 4% formaldehyde. After washing, the intestines were embedded in 4% agarose and 175 μm sections were prepared using a vibratome. After click-it staining for EdU (Click-iT^{TM} EdU, Invitrogen), antibody staining for phospho-histone H3 (Upstate) or E-cadherin (Transduction Laboratories) and counterstain with DAPI (Vector Vectashield), the sections were analysed by confocal microscopy (Leica SP2 AOBS).

Telomerase activity

TRAPeze: standard TRAP reaction was performed with fluorescent probes (TRAPEZE, Chemicon) on isolated cells from 100 weeks old Lgr5-EGFP mice according to manufacturer's instructions. Briefly, sorted cells were lysed in CHAPS lysis buffer. Protein levels were measured using the Bradford method, and equal amounts of protein

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were evaluated. Cell extracts were incubated for 30 min, adding a varied number of telomeric repeats onto the substrate oligonucleotide corresponding to telomerase activity. Positive and negative controls were examined simultaneously. PCR amplification was then performed with fluorescent probes and fluorescence was measured on a Flexstation2 (Molecular Devices).

Telomere length

Telomere length was analysed using the Telomere PNA Kit/FITC for Flow Cytometry from DAKO, following the manufacturer's protocol. DLD1 colorectal cancer cells were used as a reference. Cells from two mice were pooled before sorting. Three pooled samples of sorted cells were analysed for each age group.

RT–PCR primers

To analyse the expression of Tert mRNA by RT–PCR, the following primers were used:

1. Tert fwd: TGGCTTGCTGCTGGACACTC, rev: TGAGGCTCGTCTTA ATTGAGGTCTG.
2. Actb fwd: GCTTCTTTGCAGCTCCTTC, rev: GACCAGCGCAGC GATATC.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

Conflict of interest

The authors declare that they have no conflict of interest.

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