Random chromosome segregation in mouse intestinal epithelial stem cells

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Abstract The mammalian intestinal epithelium is endowed with a high cell turnover sustained by a few stem cells located in the bottoms of millions of crypts. Until recently, it was generally assumed that the extreme sensitivity to DNA damaging agents leading to cell death and the asymmetric mode of chromosome segregation of intestinal epithelial stem cells prevented the illicit survival of mutated stem cells and guarded against mistakes leading to aneuploidy and neoplastic transformation. Recent evidence points instead to a pool of mutipotent self-renewing stem cells capable of repairing DNA by homologous recombination significantly more efficiently than other crypt cells. Furthermore, the equilibrium between cell division and differentiation is achieved at the level of the cell population obeying to a random mode of chromosome segregation and a predominantly symmetric mode of cell division. This review summarizes the experimental findings on the mode of cell division adopted by intestinal epithelial stem cells.

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Abbreviations

3HdTR	tritiated thymidine
APC	adenomatous polyposis coli
ASCL2	Acheate scute-like 2
BMI1	B-lymphoma Moloney murine leukemia virus insertion region-1
BrdU	5-Bromo-20-deoxyuridine
Chromo. A	chromogranin A
CBCC	crypt base columnar cell
CELSR1	cadherin, EGF LAG-seven-pass G-type receptor 1
Cp4	cell position 4
DBA	*Dolichos biflorus* agglutinin
DCLK1	Doublecortin-like kinase 1
DLL1	Delta-like 1
DT	diphteria toxin
EdU	5-ethynyl-2′-deoxyuridine
G6PD	glucose-6-phosphate dehydrogenate
H-2	mouse major histocompatibility antigen 2
HOPX	homeodomain-only protein
LGR5	leucine-rich repeat-containing G protein-coupled receptor 5
LRC	label-retaining cell
LRIG1	leucine-rich repeats and immunoglobulin-like domains protein 1
MSI1	Musashi-1
NEU	*N*-nitroso-*N*-ethylurea
NEUROG3	Neurogenin 3
NUMA	nuclear mitotic apparatus protein
NUMB	protein numb homolog
OLFM4	olfactomedin 4
PGK-1	phosphoglycerate kinase 1

PROM1	prominin1
PCNA	proliferating cell nuclear antigen
IE	intestinal epithelium
SOX9	SRY-box containing gene 9
SMOC2	SPARC related modular calcium binding 2
SSH	silent sister hypothesis
TA	transit-amplifying (compartment of the crypt)
TAM	tamoxifen
TERT	telomerase reverse transcriptase
VANGL1	van Gogh-like protein 1

Introduction

The epithelium of the small intestine is composed of a folded cell monolayer organized into millions of crypts buried in the connective tissue and finger-like villi protruding in the gut lumen. The crypts correspond to the proliferative compartment containing relatively few differentiated cells, and the villi correspond to the differentiated compartment containing only postmitotic cells. Except for its flat surface (no villi), the epithelium of the colon is similarly compartmentalized. When the relationships among dividing and differentiated cells was investigated in mice and humans, using embryo aggregation chimaeras (Ponder et al. 1985), spontaneous (Fuller et al. 1990; Taylor et al. 2003), or mutagen-induced loss of gene expression (Williams et al. 1992), as well as Y chromosome tracing in colonic crypts of an XO/XY mosaic person (Novelli et al. 1996), it was found that the epithelium of each crypt is monoclonal, whereas each villus receives inputs from several crypts and is therefore polyclonal. According to Cairns (2006), in tissues with very high turnover rates such as the intestine epithelium (IE), the stem cell at the origin of each actively dividing clone could be periodically replaced as a result of asymmetric division of a long-lived, quiescent stem cell, and this may prevent accumulation of mutations resulting from a reduced capacity for repair. Protection could also result from stem cells adopting a non-random mode of chromosome segregation that leaves one template strand of each chromosome free of replication errors (Cairns 1975). Both hypotheses have been tested in various tissues, including the IE. Yet, to this day, the exact nature and location of IE stem cells, the relative importance of self-renewal, hierarchy, and plasticity within the crypt,

and the exact mode of chromosome segregation are intensely debated. We will thus try and clarify the IE stem cell problem before addressing the main issue of chromosome segregation.

The intestinal epithelial stem cell population: singular or plural?

The model that prevailed up until recently places IE stem cells above Paneth cells, at the base of the transit-amplifying (TA) progenitor compartment, which corresponds in average to the fourth cell position (cp4) from the crypt base (Hughes et al. 2012; Marshman et al. 2002; Potten et al. 2002; Sangiorgi and Capecchi 2008). Why have cp4 cells been proposed in the first place? This is because they were functionally defined as the only cells capable of regenerating a crypt after severe damage (Cai et al. 1997; Potten and Hendry 1975; Roberts and Potten 1994). Firstly, β-irradiation targeted to the Paneth cell compartment was shown to induce sterilization of the crypts, indicating that unirradiated cells located higher up in the crypt are not cryptogenic (Hendry et al. 1989). Secondly, the possibility that crypt base columnar cells (CBCCs) interspersed between Paneth cells (Fig. 1a, d) may be more cryptogenic than cp4 cells located immediately above them was excluded, based on the fact that CBCCs are significantly reduced in numbers during the early stages of regeneration following γ-irradiation (Fig.1b) (Potten and Hendry 1975; Potten and Loeffler 1990). However, it is important to note that, at the time, specific markers for cells located immediately above Paneth cells were not available, and therefore, it was impossible to ascertain that cell position +4 (cp4) was occupied by the same type of cell before and after irradiation.

In contradiction with the cp4 model, CBCCs were premised to be the actual IE stem cells based on their low level of morphological differentiation (Behnke and Moe 1964; Troughton and Trier 1969) and the results of a phagosome-tracing experiment following tritiated thymidine (3HdTR)-mediated CBCC death (Cheng and Leblond 1974). CBCCs are known to phagocytose dead cells after which large phagosomes are present in their cytoplasm. The 3HdTR-labeled phagosomes originating from dead CBCCs, and ingested by surviving CBCCs, were shown to be inherited by TA progenitors, followed by all differentiated cell types along the crypt-villus axis (Cheng and
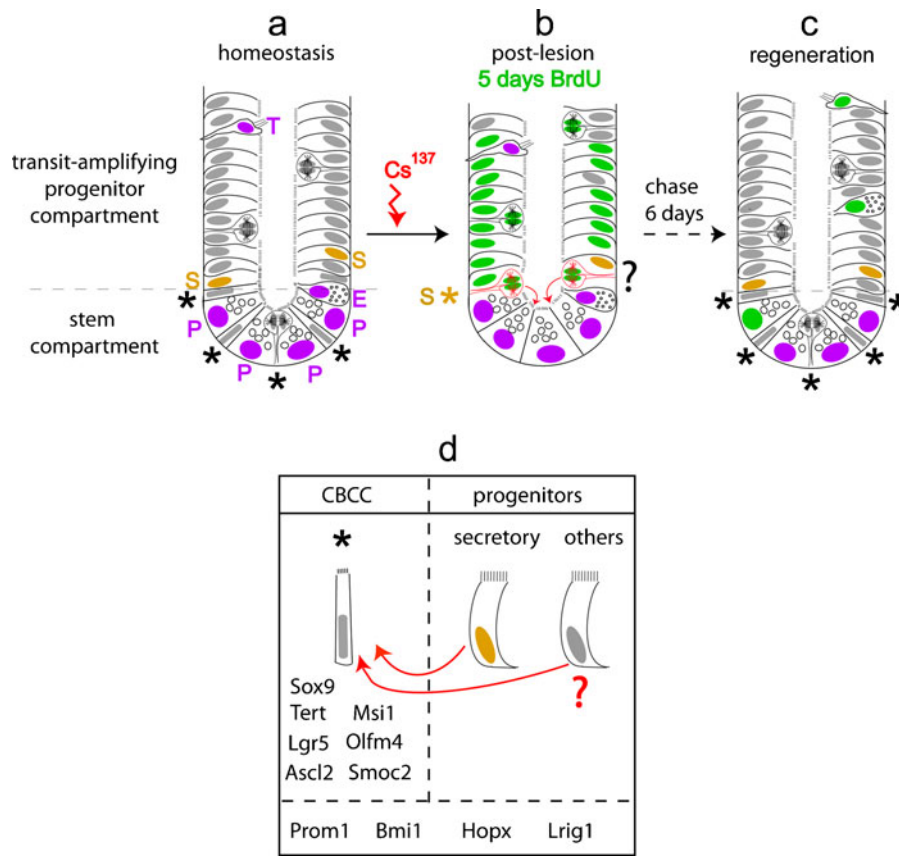
Fig. 1 Schematic representation of self-renewal, hierarchy, and plasticity in the crypt epithelium. **a** Hierarchy. A longitudinal section of a healthy crypt is represented with its stem and TA progenitor compartments, four CBCCs (*asterisk*), four Paneth cells (*P*), one enteroendocrine cell (*E*), one tuft cell (*T*), and various types of progenitors, including two secretory progenitors (*S*). (For the sake of clarity, goblet cells are not represented). Under normal conditions, all the epithelial lineages originate from CBCCs (*asterisk*). **b** Plasticity. In case of CBCC-targeted injury, like sublethal γ irradiation, crypt bottoms are depleted from CBCCs. All surviving actively dividing cells can be labeled with BrdU for 5 days (green nuclei). **c** Long-term label-retaining cells (*LRCs*). After 6 days of chase (11 days post-irradiation), some of the surviving BrdU$^+$ progenitors, including secretory progenitors (*S**), and may be other as yet unidentified progenitors (*question mark*), have produced new CBCCs, new progenitors and new differentiated cells. LRCs (green nuclei) are postmitotic Paneth cells, enteroendocrine or tuft cells. **d** Overview of the stem and progenitors markers. Whereas PROM1, BMI1, LRIG1, and HOPX are detected in most cells within the crypt, SOX9, MSI1, LGR5, OLFM4, ASCL2, TERT, and SMOC2, are highly enriched in CBCCs (see Table 1)

Leblond 1974). Another proof of the multipotent nature of CBCCs came with the finding that 90 % of long-lived clones mutated at the *Dolichos biflorus* agglutinin-binding site 1 locus contained one mutated CBCC (Bjerknes and Cheng 1999). More recently, CBCCs were found to specifically express the leucine-rich repeat-containing G-protein coupled receptor 5 (LGR5) (Barker et al. 2007). The results of *Lgr5*-based expression-reporting and lineage-tracing studies (Table 2) convincingly demonstrated that, under normal conditions, all types of differentiated epithelial cells present along the entire GI tract derive from CBCCs,

both in vivo (Barker et al. 2007) and in vitro where crypt-villus structures can be obtained from a single LGR5$^+$ CBCC (Sato et al. 2009).

The CBCC status of mother cell for all intestinal epithelial cells was subsequently challenged by the results of diphtheria toxin (DT)-mediated ablation of CBCCs followed by lineage-tracing studies based on the B-lymphoma Moloney murine leukemia virus insertion region-1 (*Bmi1*) gene (Table 2). BMI1 is a transcriptional repressor that regulates self-renewal of adult neural and hematopoietic stem cells (Molofsky et al. 2003; Park et al. 2003) and is commonly thought to be

preferentially expressed in the cp4 candidate stem cell population (Hughes et al. 2012; Sangiorgi and Capecchi 2008; Yan et al. 2012). Unexpectedly, intestinal epithelial homeostasis could be maintained for 6–10 days in the total absence of CBCCs, and Lgr5$^+$ CBCCs reappeared in the bottom of the crypts as soon as the DT treatment was stopped (Tian et al. 2011; Yan et al. 2012). Therefore, it looked as if at least two distinct stem cell populations coexisted in the intestinal epithelium, with normal cell turnover relying on a relatively abundant pool of actively cycling LGR5$^+$ BMI1^{neg} CBCCs, and relatively rare, quiescent LGR5^{neg} BMI1$^+$ cp4 cells taking over following destruction of CBCCs. End of the story?

Not quite, since the dose of tamoxifen commonly used to induce lineage tracing is suspected to interfere with the results, and tamoxifen-induced apoptosis of Lgr5^{neg} crypt cells seems to be required for efficient lineage tracing from Lgr5-positive cells (Zhu et al. 2013). Furthermore, several groups have now shown that the endogenous BMI1 messenger RNA and protein are in fact detected in most cells within the crypt (Itzkovitz et al. 2011; Munoz et al. 2012; Takeda et al. 2011). Therefore, BMI1 should no longer be considered as a marker of cp4 quiescent stem cells located above the last Paneth cells, and the term cp4 should only be used to indicate cell position, not cell identity (see Fig. 1d and Table 1). The broad pattern of *Bmi1* gene expression is compatible with the fact that *Bmi1*-LacZ$^+$ tracing events were shown to initiate at any position within the crypt (Munoz et al. 2012; Tian et al. 2011) and may be responsible for the reportedly rapid and drastic destruction of the intestinal epithelium following Bmi1$^+$ cell-targeted death (Sangiorgi and Capecchi 2008). Likewise, several molecules that were previously thought to be enriched in the slow-cycling cp4 candidate stem cell, including TERT (Montgomery et al. 2011), HOPX (Takeda et al. 2011), and LRIG1 (Powell et al. 2012), were recently shown, by several independent approaches, to be present in LGR5$^+$ CBCCs (see Fig. 1d and Table 1), with no specific enrichment in cp4 cells (Itzkovitz et al. 2011; Munoz et al. 2012; Schepers et al. 2011).

From the data collected so far, it is now clear that CBCCs and some cells present in the TA compartment represent distinct clonogenic cell populations that are mobilized under different conditions (Fig. 1). Multiscale integrative models were developed that account for the three-dimensional structure of the

Table 1 List of genes specifically expressed in intestinal epithelial stem cells (CBCCs) or expressed in both stem and progenitor cells (related to Fig. 1)

Stem	
Msi1	Kayahara et al. 2003; Potten et al. 2003
Sox9	Blache et al. 2004
Lgr5	Barker et al. 2007
Ascl2	van der Flier et al. 2009b
Olfm4	van der Flier et al. 2009a
Tert	Schepers et al. 2011
Smoc2	Munoz et al. 2012
Stem + progenitors	
Prom1	Snippert et al. 2009
Bmi1	van der Flier et al. 2009b; Itzkovitz et al. 2011; Takeda et al. 2011
Hopx	Munoz et al. 2012
Lrig1	Wong et al. 2012

crypt, control of cell cycle, and Wnt signaling, so as to predict the behavior of each cell in response to intra-, inter-, and extracellular cues. They describe how cell production and cell fate decisions could be organized in steady state as well as after tissue injury (Buske et al. 2011; van Leeuwen et al. 2009). These models actually predict what could be inferred from clonal fate data derived from transgenic mice (Lopez-Garcia et al. 2010; Snippert et al. 2010) and irradiation-mediated or toxin-mediated CBCC cell death (Bhanja et al. 2009; Hendry et al. 1989; Hua et al. 2012; Potten and Hendry 1975; Tian et al. 2011). The big picture emerging from these studies is that any crypt cell can be clonogenic, but the probability that a certain cell overtakes the entire crypt depends on its progeny reaching the crypt bottom and responding to niche signals produced by Paneth cells in the small intestine (Sato et al. 2011), and a subset of goblet cells in the colon (Rothenberg et al. 2012). Two candidate cells with clonogenic potential following injury have already been identified and both belong to the secretory lineage (Fig. 1b, c). One is quiescent, Lgr5$^+$ and preferentially located close to the bottom of the crypt (Buczacki et al. 2013). The other is LGR5^{neg}, expresses the Notch Delta ligand DLL1 and can revert to an LGR5$^+$ stem cell in vivo upon tissue damage, and in vitro when provided with exogenous Wnt signals (van Es et al. 2012) (Table 2).

Table 2 Overview of the mouse strains used in expression-reporting, lineage-ablation, and lineage-tracing studies of the intestinal epithelium

Mouse strain	Driver insertion site	Treatment	Result	Reference
Expression				
Lgr5-IRES-LacZ	Lgr5 exon 18	–	LGR5-LACZ expressed in CBCCs	Barker et al. 2007
Lgr5[EGFP-IRES-CreERT2/+]	Lgr5 exon 1	–	EGFP expressed in all CBCCs of 25 % crypts; Lgr5 gene function abolished in the knock-in allele	Barker et al. 2007
Lgr5[DTR-EGFP/+]	Lgr5 exon 1	–	DTR-EGFP expressed in CBCCs of 100 % crypts	Tian et al. 2011
Bmi1[EGFP/+]	Bmi1 exon 2	–	EGFP expressed in TA cells (positions +3 to +6); Bmi1 gene function abolished in the knock-in allele	Tian et al. 2011
Smoc2[EGFP-IRES-CreERT2/+]	Smoc2 exon 1	–	EGFP expression in all CBCCs of 25 % crypts	Munoz et al. 2012
Lineage ablation				
Bmi1[IRES-Cre-ER/+]; Rosa26[LSL-DT/+]	Bmi1 3' UTR	1 day TAM / 3 days TAM	Partial destruction of the crypts / Total destruction of the crypts	Sangiorgi and Capecchi 2008
Lgr5 DTR-EGFP/+	Lgr5 exon 1	DT	Total ablation of CBCCs, intact crypt architecture	Tian et al. 2011
Bmi1-CreERt2;R26R; Lgr5[LSL-DTR-EGFP/+]	Bmi1 BAC	6 days DT + TAM + 3 days recovery	Total ablation of CBCCs, new Bmi1-derived CBCCs	Tian et al. 2011
Lineage tracing				
RIII↔ C57BL/6Jlac mouse embryo aggregation chimaeras	-	-	Mosaic patches of fully DBA⁻ or DBA⁺ crypts	Ponder et al. 1985
Female mice mosaic for the X-linked $Pgk-1^a$ and $Pgk-1^b$ alleles	-	-	Isolated crypts express PGK-1[a] or PGK-1[b] (never both)	Ponder et al. 1985
C3H	-	NEU	Drift towards G6PD⁻ crypt clonality	Williams et al. 1992
Dlb-1^{−/−} SWR mice	-	NEU	Drift towards DLB-1⁺ crypt clonality	Bjerknes and Cheng 1999
Lgr5[EGFP-IRES-CreERT2/+]; Rosa26[LSL-LacZ]	Lgr5 exon 1	TAM	Lgr5-LacZ⁺ tracing from CBCCs	Barker et al. 2007
Bmi1[IRES-Cre-ER/+];Rosa26[LSL-LacZ]	Bmi1 3'UTR	TAM	Bmi1-LacZ tracing from cp4 cells	Sangiorgi and Capecchi 2008
Bmi1[IRES-Cre-ER/+];Rosa26[LSL-LacZ]	Bmi1 3'UTR	TAM	Bmi1-LacZ tracing from any crypt cell	Munoz et al. 2012
AhCre; R26R[LSL-Confetti]	-	BNF	Drift towards crypt clonality	Snippert et al. 2010
Lgr5[EGFP-IRES-CreERT2/+];R26R[LSL-Confetti]	Lgr5 exon 1	TAM	Drift towards crypt clonality	Snippert et al. 2010
AhCre[ERt], R26[LSL-βGlu+]; AhCre[ERt]; R26[LSL-YFP]	-	BNF + TAM	Drift towards crypt clonality	Lopez-Garcia et al. 2010
Bmi1[IRES-Cre-ERT2/+]; Rosa26[LSL-LacZ], Lgr5[LSL-DTR-EGFP/+]	3' to Bmi1 STOP codon in frame with Lgr5 ATG	TAM + DT	Bmi1-LacZ tracing from any crypt cell	Tian et al. 2011
Smoc2[EGFP-IRES-CreERT2/+]; Rosa26[LSL-LacZ]	Smoc2 exon 1	TAM	Smoc2-LacZ⁺ tracing from CBCCs	Munoz et al. 2012

BNF β-naphtoflavone, TAM tamoxifen, DT dihteria toxin, NEU N-nitroso-N-ethylurea

Symmetry versus asymmetry during cell division in the crypt: where and when?

The immortal strand hypothesis

A previous observation by Karl Lark of asymmetric template DNA strand segregation in mouse embryonic fibroblasts (Lark et al. 1966) prompted John Cairns, 40 years ago, to put forward the "immortal strand" hypothesis (Cairns 1975). The basic assumption of the immortal strand hypothesis is that, in tissues with high cellular turnover, a subpopulation of stem cells avoids accumulating mutations arising from errors in DNA replication by systematically inheriting, for each chromosome, the chromatid with the older template strand (Cairns 1975). Accumulating evidence from studies of stem cells in various tissues has confirmed that nonrandom chromosome segregation could take place in neural (Karpowicz et al. 2005) and muscle stem cells (Rocheteau et al. 2012; Shinin et al. 2006) (reviewed in (Wakeman et al. 2012)).

Christopher Potten was the first to study the mode of chromosome segregation in the mouse intestinal epithelium (Potten et al. 1978, 2002). He assumed that the switch from a random to a nonrandom chromosome segregation mode, if it happens, should coincide with the epithelium stem cell population reaching equilibrium after the period of developmental growth. In that case, stem cells whose DNA is labeled with a stable isotope reporter like tritiated thymidine (^{3}HTdR), during their penultimate symmetric divisions, should retain the label during all subsequent generations, hence the term of label-retaining cell (LRC). In one model experiment, young mice were given split doses of ^{3}HTdR for 3 days, followed 6 weeks later by split doses of bromodeoxyuridine (BrdU) for 2 days. Single and double-labeled cells were scored for six successive days after the last BrdU dose, and peak cp4 labeling indices were calculated. The labeling indices for non-Paneth (granule-negative) cycling (BrdU$^+$) LRCs (^{3}HTdR$^+$) dropped abruptly from 12 % after the last BrdU injection to 0 % 3 days later (Potten et al. 2002). This result fitted rather well the theoretical non-random scheme (Fig. 2, left). Indeed, in case of nonrandom chromosome segregation, ^{3}HTdR$^+$ BrdU$^+$ stem cells replicating their DNA in the absence of BrdU are expected to remain ^{3}HTdR$^+$ and to become BrdU-negative from the first generation. Similar conclusions were reached after

labeling cells during postirradiation crypt regeneration (Potten et al. 2002). For supporters of the cp4 stem cell model, the fact that after 6 weeks of chase, ^{3}HTdR was more often detected in cp4 cells than in other crypt cells, re-enforced the notion that long-term DNA label retention is a property of intestinal epithelial stem cells.

Since these pioneering experiments, long-term DNA label retention in crypt bottom cells has been widely used as a surrogate marker of stemness (He et al. 2007; Holmberg et al. 2006; Kim et al. 2004; Quyn et al. 2010). However, a significant point of concern regarding these studies is the absence of appropriate test for the detection of specific markers like Ki67 and lysozyme to sort out non-Paneth actively cycling LRCs from fully differentiated postmitotic LRCs. Indeed, in most instances, granules are difficult to detect in newborn lysozyme$^+$ Paneth cells, and depending on the plane of section, they may or may not be present in mature Paneth cells. Furthermore, through fate mapping of individual CBCCs, using mutagens (Williams et al. 1992) or Cre reporters (Lopez-Garcia et al. 2010; Snippert et al. 2010), it was shown that crypts drift toward clonality within a few months. When monoclonal conversion of intestinal epithelial crypts was analyzed at single stem cell resolution, clone-size expansion or contraction was observed before monoclonal conversion was complete, and this early dynamics suggests a scenario of neutral drift within the CBCC population (Lopez-Garcia et al. 2010; Snippert et al. 2010). Therefore, with a population size of 14–18 CBCCs/crypt, symmetric divisions in which both stem cell daughters have the same fate should be the rule rather than the exception, and this argues against long-term template-strand retention in the stem cell lineage.

This observation led several research groups to revisit the concept of maintenance of genome integrity mediated by nonrandom chromosome segregation in the intestinal epithelium. In a pilot experiment, we performed whole-mount immunohistochemistry on small intestines of adult mice, stripped from the muscle layers, following a single injection of BrdU (50 mg/kg). Within 52h (≈2 cell cycles), the BrdU nuclear staining pattern of CBCCs evolved from bright and homogeneous to spotty in the absence of BrdU, and the number of spots steadily decreased, attesting the progressive and random loss of labeled chromosomes through successive divisions (Fig. 3).
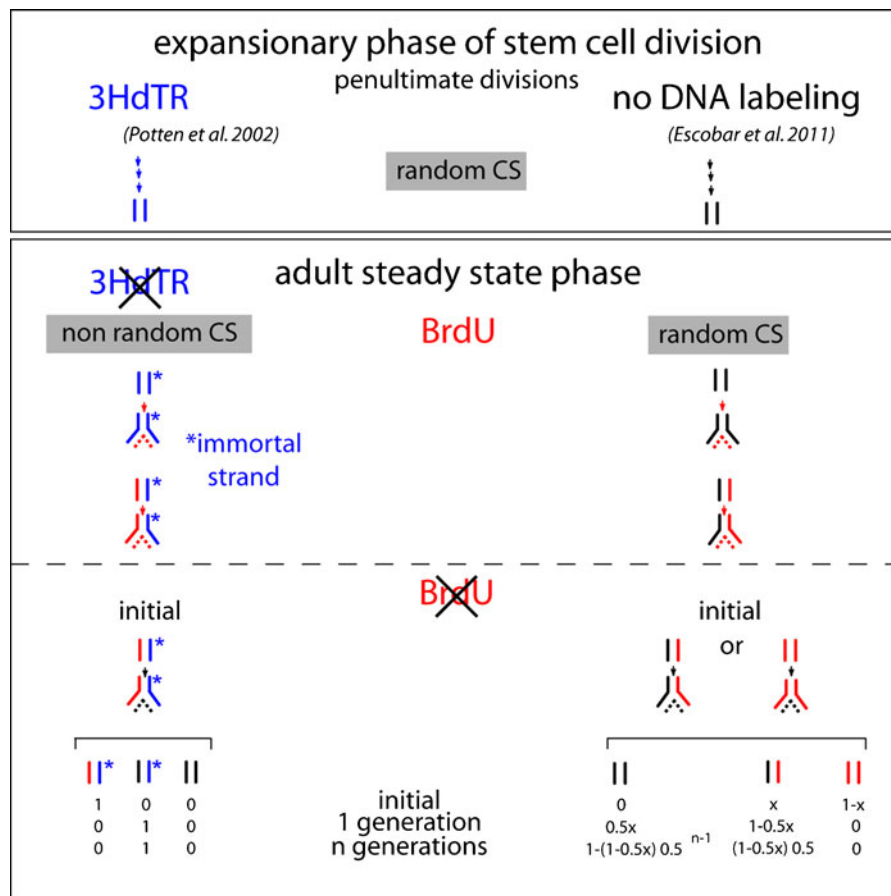
expansionary phase of stem cell division
penultimate divisions

3HdTR
(Potten et al. 2002)

random CS

no DNA labeling
(Escobar et al. 2011)

II

II

adult steady state phase

3HdTR

non random CS

BrdU

random CS

II*

II

*immortal strand

II*

II

B̶r̶d̶U̶

initial

II*

initial or

II   II

II*	II*	II		II		II	II
1	0	0	initial	0		x	1-x
0	1	0	1 generation	0.5x		1-0.5x	0
0	1	0	n generations	$1-(1-0.5x)0.5^{n-1}$		$(1-0.5x)0.5$	0

Fig. 2 Examples of pulse-chase experiments designed to determine the normal, adult mode of chromosome segregation (*CS*) of intestinal epithelial stem cells (only one chromosome is featured). *Left* nonrandom segregation of all chromosomes. Chromosomes are labeled with 3HdTR (*blue*) just before switching to the adult nonrandom CS mode. They inherit the same "immortal" 3HdTR[+] (*asterisk*) DNA strands, generation after generation, and the other strands can be labeled with BrdU (*red*). As soon as the "immortal" strands serve as templates in the absence of BrdU (one generation), all 3HdTR[+] chromosomes become BrdU-negative. *Right* Random segregation of all chromosomes. Provided that the BrdU labeling period is long enough, both DNA strands of most chromosomes can be labeled during the adult phase. Since both DNA strands are transmitted to the next generation with equal probability, the chance that first-generation chromosomes become BrdU-negative is negligible. The number of BrdU[+] chromosomes steadily decreases with each generation of stem cells produced during the chase period

The largest BrdU-labeled spots likely correspond to clusters of major satellite T-rich and A-rich repeats of centromeres that remain associated through interphase and S phase, and dissociate only in prophase.

In another study, when all actively dividing epithelial cells in the ileal crypts of adult mice were DNA-labeled, a clear signal could still be detected in 94–97 % of CBCCs, and 93–96 % of cells located immediately above the last Paneth cells (cp4), after 72 h of chase (Escobar et al. 2011). This is clearly not compatible with a nonrandom mode of chromosome segregation. In case of nonrandom chromosome segregation, this lapse of time would have been sufficient to deplete the bottom of crypts from labeled CBCCs, half of which are expected to become unlabelled after 24 h (one generation) in the absence of BrdU (Fig. 2, left). The same conclusion was reached based on the results of BrdU DNA-labeling during postirradiation crypt regeneration (Escobar et al. 2011) and in another study where the EdU-labeled chromatin of 51 mitotic CBCCs, not yet engaged in cytokinesis (daughter nuclei not yet
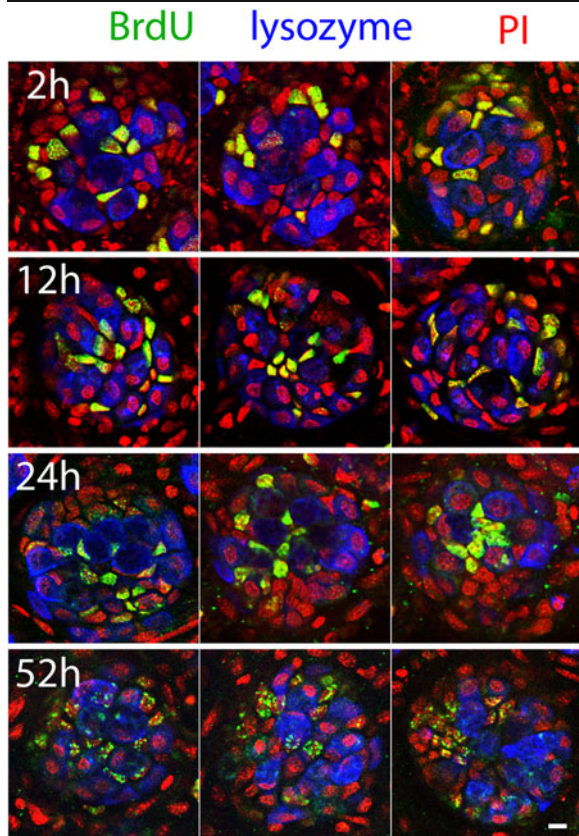
Fig. 3 Random segregation of BrdU-labeled chromosomes in CBCCs. Adult mice were killed at the indicated times following a single injection of BrdU (50 mg/kg). Small intestines were fixed in 3 % paraformaldehyde, followed by immersion in 2 N HCL for 2 h and neutralization. Small segments of 5 mm^2 were treated with 5 mg/mL collagenase IV (15 min at 40 °C) to facilitate removal of the muscle layers. Segments were then incubated with primary antibodies for 24 h at room temperature, followed by secondary antibodies for 24 h at room temperature. Series of optical sections through the crypt bottoms were obtained using a Bio-Rad 1024 CLSM confocal laser-scanning microscope. For each time point, projection views from the abluminal side of three crypt bottoms are illustrated. The BrdU staining pattern evolved from bright and homogeneous at the end of the labeling period (2 h) to spotty after 52 h in the absence of BrdU. *PI* propidium iodide. *Scale bar*, 10μM

separated), was shown to segregate randomly (Schepers et al. 2011). Moreover, when ileum sections, removed 18 days after irradiation and BrdU treatment, were simultaneously stained for BrdU, Ki67, lysozyme (Paneth cells), chromogranin A (enteroendocrine cells), and DCLK1 (tuft cells), none of the 232 BrdU$^+$ LRCs detected by this method in a total of 806 crypt sections was found to be lineage negative (Fig. 1c; Escobar et al.

2011). Similar results were observed 11 days after irradiation, with all BrdU$^+$ LRCs staining positive for a differentiation marker of either the Paneth, enteroendocrine or tuft cell lineage (Fig. 4). The fact that, after 11–18 days of chase, Paneth cells were over-represented in mouse ileum is in line with their higher representation (Ettarh and Carr 1996) and relatively longer lifespan compared with that of goblet, enteroendocrine, and tuft cells (Bjerknes and Cheng 1981; Cheng et al. 1969; Gerbe et al. 2011; Ireland et al. 2005; Thompson et al. 1990; Troughton and Trier 1969). The same
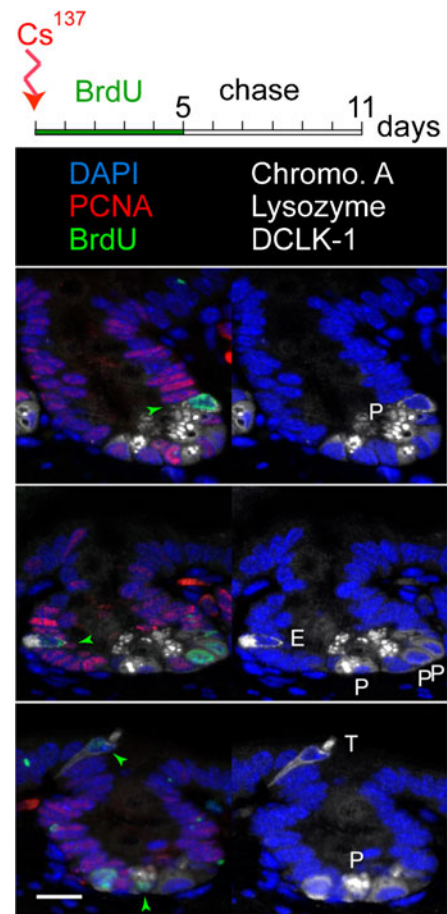

Fig. 4 Intestinal epithelial stem cells are not long-term DNA label-retaining cells (*LRCs*). Following whole-body irradiation of adult mice with a dose of 6 Gy, BrdU (5 mg/mL) was given in drinking water for 5 days, and mice were killed 6 days later. Most BrdU-retaining cells (*arrowheads*) found in the lower half of the crypts after 6 days of chase are postmitotic, terminally differentiated lysozyme$^+$ Paneth cells (*P*) with only very few chromogranin A$^+$ enteroendocrine cells (*E*). At this time point, DCLK1$^+$ tuft (*T*) LRCs are usually found higher up in the crypt, or in villi (not shown). *Scale bar*, 10 μM

conclusion, in direct contradiction with the results of Christopher Potten's experiments, was reached by another group using MIMS, a combination of ion microscopy and mass spectrometry, to track DNA labeling quantitatively with stable isotope reporters like ^{15}N-thymidine (Steinhauser et al. 2012). After DNA labeling during all potential periods of stem cell formation, spanning in utero through postnatal development, the authors did not find LRCs other than Paneth cells in the small intestinal crypt (Steinhauser et al. 2012). Together, these results suggest (1) that CBCCs (stem) and their progeny (TA progenitors) segregate most, if not all, of their chromosomes randomly, both under homeostatic conditions and when recovering from a lesion, and (2) long-term DNA label retention in the bottom of the crypt simply reflects the division history of the cells.

The "Silent Sister" concept

With the type of pulse-chase experiments used to test the immortal strand hypothesis, it is difficult to answer the question of whether rare cells segregate one or a small number of chromosomes asymmetrically, a potential mechanism proposed by others to regulate lineage cell commitment (Lansdorp 2007). According to the "silent sister" hypothesis put forward by Peter Lansdorp, epigenetic marks at centromeres and selected genes could be developmentally controlled, vary between chromosomes and between cells, and lead to the selective DNA sequence orientation-dependent attachment of all, or only some chromatids, to microtubules coming from the mother centrosomes. This possibility was recently tested in the unlesioned mouse colon epithelium in vivo, using the technique of chromosome orientation fluorescent in situ hybridization with major satellite probes to follow the fate of parental BrdU-labeled template strand inheritance in postmitotic cell pairs (Falconer et al. 2010). The results revealed a slightly higher frequency of daughter-cell pairs with asymmetry than predicted by simulated random segregation. However, they did not afford discrimination between the two following scenarios: (1) a subset of colon cells selectively segregate sister chromatids from most chromosomes or (2) a small number of specific chromosomes are selectively segregated in a large number of cells (Falconer et al. 2010).

Spindle orientation and planar cell polarity of the intestinal epithelium

During the S phase of the cell cycle, the centrosome duplicates and the two daughter centrosomes move to opposite sides of the nucleus, defining the axis and the position of the mitotic spindle. The planar spindle positioning that allows both daughter cells to maintain contact with the basement membrane is required for the formation of the central lumen in epithelial tubulogenesis of internal organs like the lungs, kidneys, mammary glands, brain, and gut (Rodriguez-Fraticelli et al. 2011). Past the developmental phase, variation in the orientation of the mitotic spindle was suggested to regulate the balance between stem cell proliferation and differentiation in the small intestine and colon epithelia of adult mice (Quyn et al. 2010). Mitotic spindles were found to be oriented perpendicular to the apical surface, specifically in the stem cell compartments of mouse and human intestine and colon, a feature that was lost in precancerous tissue heterozygous for the adenomatous polyposis coli tumor suppressor (Apc) (Quyn et al. 2010). Different results were reported in two other studies performed in the mouse small intestine where metaphase and anaphase spindles were found to be always planarly aligned with the basal membrane, in both the TA compartment and the base of the crypt (Fig. 1), except during prometaphase when their position is not yet definitive (Bellis et al. 2012; Fleming et al. 2007). Moreover, unlike what is happening in invertebrates (Neumuller and Knoblich 2009), the absence of asymmetric cortical segregation of NUMB, VANGL1, CELSR1, and NUMA in colonic epithelial stem cells is not in favor of a mechanism that coordinates spindle orientation and cell fate determinants (Bellis et al. 2012). In contrast, the APC tumor suppressor has been identified as a spindle-positioning factor (Green et al. 2005) and a connection has been found in certain types of intestinal tumors between spindle misorientation, cytokinetic failure, and mutations in APC (Caldwell et al. 2007; Fleming et al. 2009) and E-cadherin (den Elzen et al. 2009). This may partly be explained by the fact that disrupting cadherin function perturbs the cortical localization of APC (den Elzen et al. 2009). Moreover, most APC mutations result in the loss

of both the microtubule and the end-binding protein 1 interaction domains of the protein (Morrison et al. 1998), thus altering microtubule attachment to kinetochores, spindle positioning, and chromosome segregation (Bruning-Richardson et al. 2011).

Conclusions and perspectives

In closing, what the experiments of the last decade did confirm is the remarkable capacity of the adult mammalian intestinal epithelium to maintain its regenerative power to cope with a large variety of insults. Available data do not support the chromosome-sorting model as a major mechanism of genome integrity preservation in the adult intestinal epithelium. Similar conclusions have previously been reached for the haematopoietic system and the skin (Kiel et al. 2007; Sotiropoulou et al. 2008; Waghmare et al. 2008), two other tissues undergoing continuous turnover during adult life and engaging in wound repair in response to injury. In fact, such mechanisms of genome protection by the segregation of mutations or by the control of lineage (Cairns 2006) may not normally be needed if, as recently reported, intestinal epithelial stem cells repair DNA by homologous recombination significantly more efficiently than any other crypt cell (Hua et al. 2012). Further experiments are needed to provide definitive answers to the question as to which cells could use chromosome sorting to direct cell-fate decisions in the adult unlesioned intestinal epithelium. This might be achieved by adapting the protocol used by Rocheteau et al. (2012) to demonstrate nonrandom chromosome segregation in a subpopulation of muscle satellite stem cells with a lower metabolic state. Such experiments could include DNA labeling of colon crypt epithelial cells, followed by isolation of the first generation of cells produced in the absence of label. The isolated cells should then be stained for markers of cell fate, and analysis of single chromatids by chromosome orientation fluorescent in situ hybridization could be performed on metaphase spreads, which should provide higher image resolution than the images of post-mitotic cell nuclei used in the study of Falconer et al.

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