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Intestinal Stem Cell Replacement Follows a Pattern of Neutral Drift

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With the capacity for rapid self-renewal and regeneration, the intestinal epithelium is stereotypical of stem cell—supported tissues. Yet the pattern of stem cell turnover remains in question. Applying analytical methods from population dynamics and statistical physics to an inducible genetic labeling system, we showed that clone size distributions conform to a distinctive scaling behavior at short times. This result demonstrates that intestinal stem cells form an equipotent population in which the loss of a stem cell is compensated by the multiplication of a neighbor, leading to neutral drift dynamics in which clones expand and contract at random until they either take over the crypt or they are lost. Combined with long-term clonal fate data, we show that the rate of stem cell replacement is comparable to the cell division rate, implying that neutral drift and symmetrical cell divisions are central to stem cell homeostasis.

heories of epithelial cell renewal place stem cells at the apex of proliferative hierarchies, possessing the lifetime property of selfrenewal (1). In homeostasis, the number of stem cells remains fixed, imposing an absolute requirement for fate asymmetry in the daughters of dividing stem cells, such that only half are retained as stem cells. Fate asymmetry can be achieved either by being the invariant result of every division (intrinsic asymmetry) or by being orchestrated from the whole population, where cell fate following stem cell division is specified only up to some probability (population asymmetry) (1, 2). These alternative models suggest very different mechanisms of fate regulation, yet their identification in normal tissues remains elusive.

In the intestinal crypt, the apex of the proliferative hierarchy is associated with the anchored cells of the crypt base (3). Heterogeneity in expression of fate-determining genes and in cell cycle characteristics has suggested that at least two populations (Lgr5⁺ and Bmi1⁺) of crypt stem cells may exist (4, 5). For neither population has the pattern of self-renewal been revealed.

Recent studies have provided evidence in support of intrinsic asymmetry by showing that crypt base cells are more likely to show an oriented spindle than cells higher in the crypt, and this correlates with asymmetric DNA segregation during division (6, 7). However, the phenomenon of monoclonal conversion, whereby crypts become monophenotypic (clonal) with time after genetic marking of individual cells (8-10), presents an apparent experimental paradox. This observation rules out truly invariant modes of cell division involving multiple stem cells and has been explained by rare errors in intrinsic asymmetry (11-13). Hence, the consensus view remains that intestinal stem cells divide asymmetrically due either to inherent properties or environmental cues (*3*). Here we show that the dynamics of clonal growth leading to monoclonal conversion is directly related to the pattern of steady-state self-renewal.

Intestinal clones were induced by low-level Cre-mediated recombination in Ahcre ERt animals (targeting potentially all the proliferative populations within the crypt with a single treatment) crossed to Cre-reporter strains as described previously, and were visualized in tissue whole mounts or sections [Fig. 1, A to J, and supporting online material (SOM) S-I]. With only $1.9 \pm 0.7\%$ of crypts containing labeled cells at 2 weeks after induction, the vast majority of clones were expected to derive from single cells. By imaging the crypt base, we first determined the proportion that have achieved monoclonal conversion (Fig. 1, I and J). Crypts can become monoclonal a short time after induction, such that ~50% are fully labeled within 8 weeks (Fig. 1K). The dynamics of monoclonal conversion can also be tracked by scoring the number of differentiated progeny that emerge from the crypts (Fig. 1, L to N, and fig. S1). After pulse labeling, cells exported from crypts form one to three clear migration streams on villi (Fig. 1, B and C). On the villus, clones comprised both Goblet and absorptive cell lineages, and within the crypt, clones contained Paneth cells. Each fully labeled crypt supports a clone width of $w_{\text{max}} = 6.8 \pm 1.3$ cells (mean \pm SD, n = 155) along the villus. As with the crypt base analysis, we found that 50% of clones were of full width (i.e., six cells or more) within 8 weeks, corresponding to 50% monoclonal crypts at this time point (Fig. 1M).

Monoclonal conversion rules out a simple model of tissue maintenance that originates from a population of long-lived stem cells following a strict pattern of asymmetric division, and can be explained by two classes of behavior. First, crypts could be maintained by a hierarchy in which a single stem cell generates, through a sequence of asymmetric divisions, stem cells with a more limited proliferative potential (14) (Fig. 2A). Second, tissue could be maintained by an equipotent stem cell population, in which stem cell loss is perfectly compensated by the multiplication of others (15-17) (Fig. 2B). Any detailed model of self-

renewal will belong to one of these two classes (SOM S-II).

Although both behaviors predict attrition of surviving clones and drift toward monoclonality, the full range of clonal fate data allows us to discriminate between them. To understand how, we draw upon concepts from statistical physics applied to population dynamics. Within an equipotent cell population, ongoing replacement leads to "neutral drift" of the clonal population (17). In this case, if the stem cell pool were not limited by anatomical constraints, clonal evolution would settle into a characteristic behavior in which the size distribution acquires a "scaling form": defining $P_n(t)$ as the fraction of surviving clones that host *n* stem cells at a time *t* after induction (see SOM S-III) (17, 18)

$$P_n(t) = \frac{1}{\langle n(t) \rangle} F\left(\frac{n}{\langle n(t) \rangle}\right) \tag{1}$$

where $\langle n(t) \rangle$ denotes the average number of stem cells in a surviving clone. The scaling function, F(x), is "universal," dependent only on the spatial organization of stem cells. From Eq. 1, it follows that, if $\langle n(t) \rangle P_n(t)$ is plotted against $n/\langle n(t) \rangle$, the size distributions will follow the same curve irrespective of the time *t*. In the crypt, where the stem cell compartment is limited, scaling is transient and would eventually fail when a noticeable fraction of crypts become monoclonal.

By contrast, if replacement occurs hierarchically, then clones derived from the "master" stem cell will increase steadily in size, whereas those derived from its shorter-lived progeny will exhibit limited growth followed by loss. Crucially, the mixture of these two behaviors leads to binomial size distributions that do not scale (SOM S-V).

Clone size is impossible to measure in absolute terms in the intestine because of the constant migration and loss of cells. However, we can access $P_n(t)$ indirectly: The cohesion of clones within the crypt, and the proportionality of the clone width emerging from the crypt to the villus (SOM S-I), show that clonal expansion is tied to the circumference at the crypt base. Therefore, the clone width, relative to that of fully labeled crypts, serves as a proxy for the fraction of labeled stem cells within the crypt. Formally, a clone of width w on the villus is associated with a clone covering a fraction $f(w) = w/w_{\text{max}}$ of the circumference at the crypt base. The number of stem cells associated with a clone of width w is given by $n = f(w)N_{\text{stem}}$, where N_{stem} denotes the number of stem cells surrounding the crypt base. With this assignment, we find that for $t \le 4$ weeks, where the vast majority of crypts have yet to become monoclonal, the size distributions show scaling behavior (Fig. 2D), an unambiguous signature of neutral drift dynamics.

The growth rate, $\langle n(t) \rangle$, and the form of F(x) have the potential to offer further insight into the pattern of stem cell fate. In particular, if stem cells are organized into a one-dimensional arrangement, with cell replacement effected by neighboring stem cells (Fig. 3, A and B), then the average size of surviving clones grows as a square root of time,

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ysis of sister cell orientation reveals that the frequent transverse cell divisions required for stem

cell replacement occur at the crypt base (fig. S3).

is their rate of loss? Current debate centers on the

relationship between two crypt base populations,

the Bmi1⁺ cells positioned around row 4 (4) and

the columnar Lgr5⁺ cells that reside at the crypt

base (5, 19). Because both cell populations support

long-lived clonal progeny, equipotency requires

that Bmi1⁺ and Lgr5⁺ cells contribute to the same

stem cell pool, with the cells generating each other.

Such heterogeneity would not affect the scaling

behavior in Eq. 2, as its effect would be resolved

rapidly compared to one-dimensional drift around

the crypt circumference (SOM S-III.4). If we esti-

mate the number of stem cells on the basis of the

total number of $Bmi1^+$ and $Lgr5^+$ cells (4, 5, 19),

we can conclude that their total number is >16,

suggesting that stem cells are replaced laterally by

Which cells constitute the stem cells, and what

 $\langle n(t) \rangle \approx \sqrt{\lambda t}$, with λ the stem cell replacement rate, and the scaling function is predicted to take the form (SOM S-III)

$$F(x) = (\pi x/2) \exp[-\pi x^2/4]$$
 (2)

Referring to the experimental data, the coincidence of the scaling behavior with this universal (parameter-free) form, together with the observed square-root growth of the clone width over the same period (Fig. 2D, inset), reveals that stem cells are indeed being replaced laterally by neighboring stem cells in an effectively one-dimensional geometry. This behavior can accommodate both variability in replacement rates and potential stem cell replacement parallel to the crypt axis (SOM S-III.4 and S-III.5).

Because this pattern of stem cell fate emerges from the consideration of (universal) short-time dynamics, the long-term behavior, including the drift toward clonality, presents a powerful test of the model. By fixing just a single parameter, $\lambda N_{\text{stem}}^2 = 0.025 \pm 0.003$ per week (mean \pm SEM), from a quantitative fit to the average clone width (Fig. 1M), we obtained an excellent agreement of theory (detailed in SOM S-IV and S-VI) with the measured monoclonal fraction (Fig. 1K) and clone size distributions over the entire time course (Fig. 3, C to H, and fig. S2). A similar analysis of clone fate as measured from the crypt base in the colon reveals the same neutral drift behavior (Fig. 3, I and J, and SOM S-VI).

These results reveal that stem cells of the small intestine and colon behave as an equipotent population following a pattern of neutral drift in which the loss of a stem cell is compensated by the multiplication of a neighbor. This process may be achieved through stochastic stem cell loss triggering self-renewal, or through overcrowding of the stem cell pool leading to loss. Further, anal-



Fig. 1. Conversion to monoclonality implies that stem cells are replaced in mouse intestinal crypts. (**A**) Experimental schedule; clones were induced by a single pulse of β -naphthoflavone (β NF) and tamoxifen (TM) in adult mice aged 1.5 to 9 months, and then visualized in the small intestine and colon after chase periods of 2 weeks to 1 year through serial sectioning and whole-mount imaging. (**B** and **C**)

Clonal progeny migrate in coherent streams on villi in whole-mounted tissue, emerging from partially labeled (B) and fully labeled crypts (C). Streams from single crypts are seen to split to occupy one (shown) or up to three villi (not shown). (D and E) Clones contain both enterocytes and Goblet cells. (D) Whole mount containing β glu+ clone stained with Alcian blue to visualize Goblet cells (arrowheads); (E) a sectioned EYFP+ (enhanced yellow fluorescent protein) clone stained with Periodic acid-Schiff to visualize Goblet cells (full arrowheads). Positive Paneth cells are initially absent (v-arrows) but ultimately become labeled (G). (F to H) Schematic shows crypt-to-villus axis of clonal migration streams (brown) on the intestinal epithelium (gray). (F) Longitudinal section of a 2-weekold clone; (G and H) serial sections of a clone migration stream showing a labeled 8-week-old clone. Paneth cells are labeled (v-arrows). (I and J) Whole-mounted tissue showing a partly labeled and a fully labeled crypt, respectively. Dashed circles indicate crypt boundaries; dotted lines show villi (v) out of the focal plane; "c" marks adjacent crypts. Diagram shows the basal viewing perspective of the crypts (circles), of which one is clonally labeled (purple). (K) The fraction of fully labeled crypts over time shows conversion to monoclonality. The line shows a fit to neutral drift dynamics (see main text). Monoclonal conversion occurs at comparable rates, irrespective of age at induction (legend). (L) The number of labeled crypts per 10⁴ villi decays over time after labeling. Densities are normalized by their earliest time point (2 and 3 weeks, respectively) to allow comparison of different cohorts [legend as in (K)]. (M) The average clone width increases during monoclonal conversion at all ages [legend as in (K)]. Theoretical curves in (J) and (K) follow from the fit made in (K). (N) Representative stem cell labeling; the total labeled cross section of villi remains constant, indicating that the labeled cells maintain a constant population (error bars, SEM). Scale bars, 25 μm.

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Fig. 2. Short-time clone size distributions reveal a pattern of "neutral drift" in stem cell replacement. (A and B) Models of stem cell turnover in crypt: In (A), monoclonal conversion follows from turnover of a single, slow-cycling, asymmetrically dividing "master" stem cell at the crypt base. Blue arrows show self-renewal through asymmetric division; gray arrows show divisions leading to differentiation and upward migration. In (B), conversion arises from turnover of an equipotent stem cell population in which stem cell loss is compensated by the multiplication of other stem cells (blue arrows), resulting in random clonal expansion and contraction. (C) Distribution of clone widths between 2 and 52 weeks after labeling (each "+" translates to one clone). (D) When plotted against $w/\langle w(t) \rangle$, the short-term ($t \le 4$ weeks) clone size distributions, $\langle w(t) \rangle P_w(t)$, show the collapse predicted by the scaling Eq. 1. A log-log plot of the average clone width $\langle w(t) \rangle$ (inset) shows square-root growth, $log(\langle w(t) \rangle) = 0.5 log(t - T_T)$ irrespective of age (legend). $T_{\rm T} \approx 1$ week is the migration time from the crypt base onto the villi (23). The coincidence of the data with the scaling function Eq. 2 (curve), together with the square-root growth of $\langle w(t) \rangle$, provides a signature of one-dimensional neutral drift dynamics (see main text).

Α

Fig. 3. Model of neutral drift and monoclonal conversion in the small intestine and colon. (A) The neutral drift model in which N_{stem} equivalent stem cells surround the crypt base. The chance loss of a stem cell and its replacement at the clone edge leads either to the expansion of the labeled (blue) clone (case 1) or to its contraction (case 2). (B) A simulation based on this neutral drift model showing monoclonal conversion after the labeling of all stem cells by different colors. (C to H) Clonal width distributions up to 6 months after labeling alongside theoretical curves following from the neutral drift



0.2

0.1

Fraction clones (%)

100





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Time post-labeling (weeks)

0.2

0.

Colon

4 8 12

Clone width (cells)

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0.2

0.

model with $\lambda/N_{\text{stem}}^2 = 0.025$ per week (see fig. S2 for entire data set). Error bars are SEM. (I and J) Clone size distribution as inferred from the fraction of crypt base labeled in the colon. Clones were scored into four size categories as shown in (J). Curves follow from the neutral drift model with $\lambda/N_{\text{stem}}^2 = 0.040 \pm 0.004$ per week. Scale bars, 25 µm.

their neighbors at a rate $\lambda \approx 1$ per day comparable to the measured cell division rate (5, 20–22). Therefore, asymmetric cell division is not the sole, or even the most common, mode of stem cell division in the intestine: Symmetric stem cell division is not a rare event, but is a central aspect of homeostasis. In place of a hierarchical arrangement, our results identify a pool of equipotent stem cells that is regulated by the behavior of neighbors. The pattern of stem cell regulation in the intestine provides an inherently flexible assembly in which any stem cell can be deployed to differentiate into one of a number of cell types, act to replace stem

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Supporting Online Material

www.sciencemag.org/cgi/content/full/science.1196236/DC1 Materials and Methods SOM Text Figs. S1 to S3 References

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Mutational Robustness of Ribosomal Protein Genes

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The distribution of fitness effects (DFE) of mutations is of fundamental importance for understanding evolutionary dynamics and complex diseases and for conserving threatened species. DFEs estimated from DNA sequences have rarely been subject to direct experimental tests. We used a bacterial system in which the fitness effects of a large number of defined single mutations in two ribosomal proteins were measured with high sensitivity. The obtained DFE appears to be unimodal, where most mutations (120 out of 126) are weakly deleterious and the remaining ones are potentially neutral. The DFEs for synonymous and nonsynonymous substitutions are similar, suggesting that in some genes, strong fitness constraints are present at the level of the messenger RNA.

he distribution of fitness effects (DFE) of mutations is important in evolutionary biology; for example, in the the degradation of genetic information due to Muller's ratchet (1), molecular clocks (2), genetic variation at the molecular level (3), and the impact of selection and genetic drift in natural populations (4). The DFE is also of importance for understanding quantitative traits, complex diseases, the evolution of antibiotic resistance, and predicting the minimal populations sizes needed for maintaining healthy populations of endangered species and in breeding programs (5-8). Mutations may be deleterious, neutral, or beneficial, and the proportion of mutations in each category will depend on several factors (9). Advantageous mutations are rare and appear to be exponentially distributed (6, 10-12). The DFE of deleterious mutations often appears to be bimodal, with one low-fitness peak (including lethal mutations) and a second peak close to wild-type fitness, with weakly deleterious mutations (13). Information on the DFE for deleterious mutations comes mostly from analyses of DNA sequence data (2, 5, 14) or mutation accumulation and mutagenesis experiments (15–18). The indirect estimates of fitness values made from sequence data suffer the drawback that strongly deleterious mutations (with $|s|N_e >>1$) (|s|, absolute value of selection coefficient; N_e , effective population size) are poorly represented, and many details of the DFE are unresolved (19). Direct measurements, on the other hand, are difficult at the high-fitness end, where deleterious effects are

smaller than the percent level (in this study, selection coefficients $|s| < 3 \times 10^{-3}$ could not be detected). Nevertheless, experimental studies of the fitness effects of defined single-point mutations have proven useful, because they allow an assumption-free determination of the underlying DFE, including the frequencies of strongly deleterious mutations. A few such studies in viruses have demonstrated a bimodal DFE, with most mutations being either neutral or lethal (13, 20, 21).

We used Salmonella typhimurium to study the DFE of random base-pair substitutions in the protein synthesis machinery. A total of 126 random base-pair substitutions were engineered into the *rpsT* and *rplA* genes, encoding the ribosomal proteins S20 and L1, respectively (22). These two proteins are nonessential, but deletion mutants lacking either of these ribosomal proteins have severely reduced fitness. Thus, putative mutational effects on fitness can be measured over a large range, and the fitness of complete loss-of-function mutations is known and is larger than zero. We used bacterial growth rate to measure the fitness effects of the mutations. The involvement of ribosomal proteins in translation and the direct relation of translation rates to exponential growth

> Fig. 1. Relative exponential growth rates of single-substitution mutants relative to wild-type controls set to 1. (A) Synonymous substitutions (in LB). (B) Nonsynonymous substitutions (in LB). (C) Synonymous substitutions (in M9 medium). (D) Nonsynonymous substitutions (in M9 medium).



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