Defining Stem Cell Dynamics in Models of Intestinal Tumor Initiation
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Cancer is a disease in which cells accumulate genetic aberrations that are believed to confer a clonal advantage over cells in the surrounding tissue. However, the quantitative benefit of frequently occurring mutations during tumor development remains unknown. We quantified the competitive advantage of Apc loss, Kras activation, and P53 mutations in the mouse intestine. Our findings indicate that the fate conferred by these mutations is not deterministic, and many mutated stem cells are replaced by wild-type stem cells after biased, but still stochastic events. Furthermore, P53 mutations display a condition-dependent advantage, and especially in colitis-affected intestines, clones harboring mutations in this gene are favored. Our work confirms the previously theoretical notion that the tissue architecture of the intestine suppresses the accumulation of mutated lineages.

Cancer development involves competition between normal and deviant cell lineages, ultimately resulting in disruptive tissue overgrowth (1). The cellular effects of mutations can increase proliferation or impair response to cell death–inducing signals (2). However, only very limited quantitative data exist on the net effects of oncogenic alterations at the cell population level. In the intestine, cancer arises from an initial transformation event occurring primarily, but not exclusively, in the stem cell compartment (3-4). Because normal intestinal crypt homeostasis is characterized by competition between equipotent stem cells that continuously replace each other in a random fashion (Fig. 1A) (5,6), oncogenic mutations may confer an advantage on the clones in which they arise by acting on these dynamics. We confirmed and used this assumption to quantify the competitive benefit of mutations frequently occurring in colorectal cancer (CRC).

To track the fate of wild-type (WT) and mutated cell lineages, we induced low-level intestinal recombination either specifically in the crypt base using Lgr5-EGFP-CreERT2 mice or more generally in AhCreERT2 mice, both crossed to the R26-Lox-STOP-Lox-ttdTomato (ttTomfl/fl) reporter strain. Clones were visualized and quantified at the bottom of the crypt, allowing robust clone size quantification around the circumference of the crypt (fig. S1). We observed that on average, clones expand, and the number of fixed clones (i.e., crypts within which the whole epithelium is ttTom+) increases with time (Fig. 1, B to E). By quantitative analysis of the clone size distributions using the stochastic master equation and Bayesian inference, we confirmed earlier studies that continuous, one-dimensional neutral replacements govern intestinal stem cell dynamics (figs. S2 and S3) (5,6). This process is fully defined by only two parameters: the number of functional stem cells per crypt (N) and the rate at which these replace each other (λ). Because of the high-quality clone size distribution data we obtained, we can directly infer both N and λ with considerable precision (fig. S2). For the proximal small intestine (SI), we find that N = 5 and λ = 0.1 replacements per stem cell per day (figs. S2 and S3). This inferred number of stem cells is considerably lower than the number of Lgr5 cells (~16) per crypt (5) but agrees with a functional marker-free estimate of stem cell numbers (7). In fact, we directly confirmed a previous suggestion that a sizable fraction of Lgr5 cells are actually more committed progenitor cells and do not function as stem cells in homeostasis (8) (fig. S4).

Next, we crossed AhCreERT2,ttdTomatofl mice with Kras-G12D0 and Apcfl/fl mice. Recombination in these mice will result in activation of an oncogenic Kras variant or inactivation of one or both copies of the negative Wnt regulator Apc, in addition to ttTom expression. We confirmed that ttTom expression is tightly matched to oncogenic recombination events (fig. S5). Furthermore, our analysis was facilitated by the fact that the low-level clone induction of pre-neoplastic lineages does not substantially alter tissue morphology, as has been reported before (9,10) (Fig. 1B and fig. S6). We found that activated KRAS confers an evident clonal advantage as the KrasG12D harboring clones expand and become fixed more rapidly than the WT lineages (Fig. 1, B to E). In order to express this effect quantitatively, we introduced an additional variable to the one-dimensional drift model to capture the biased drift properties: P(D) signifies the chance that a ttTom+ stem cell replaces its nonlabeled neighbor; conversely, 1−P(D) represents the chance that the nonlabeled stem cell replaces the ttTom+ neighboring stem cell. The neutral version of the model is described by unbiased stochastic replacements, and as expected, using the inference method in combination with the neutral clone size distribution data we find P(D)(WT versus WT) = 0.50 (0.48 to 0.52, 95% confidence interval (CI)) (Fig. 1F), indicating that two adjacent stem cells have equal probability to replace each other. Applying this inference strategy to the biased drift data of Kras mutant clones, we found a well-demarcated posterior probability peak: P(D)(KrasG12D versus WT) = 0.78 (0.75 to 0.81, 95% CI) (Fig. 1G). This implies that for replacement events occurring at the interface of a KrasG12D and a WT clone, the Kras mutant stem cell replaces the WT stem cell in ~80% of the cases, and conversely that the WT stem cell replaces the mutant stem cell in ~20% of the replacement events. Thus, interpreting the altered size distribution of clones in terms of individual stem cell fates provides an intuitive estimate of the potency of oncogenic mutations in the context of a stochastic model, by integrating the effects of proliferation rate, cell death frequencies, and self-renewal properties into a single parameter (P(D)).

Because human CRCs arise in the majority of cases after the inactivation of APC, we determined the clonal advantage of lineages harboring either heterozygous or homozygous inactivating mutations within this gene. Apcfl/fl lineages display only a limited, but significant, benefit over WT clones, whereas Apc−/− lineages have a more marked clonal benefit already at 4 days, making it unlikely that secondary effects of Apc loss [such as chromosomal instability (CIN)] are involved (Fig. 2, A to C). After applying the inference strategy to this data to determine P(D), we found that P(D)(Apc−/− versus WT) = 0.62 (0.58 to 0.66, 95% CI) and P(D)(Apc−/− versus WT) = 0.79 (0.75 to 0.82, 95% CI) (Fig. 2, D and E). In reality, Apc−/−...
Fig. 1. Quantifying the clonal benefit of **Kras**^G12D^.

(A) Intestinal stem cells are equipotent and continuously replace each other in a stochastic fashion. (B) Confocal images of SI crypt bottoms of **AhCre**^ER/tdTom^−/−_mice (WT) and **AhCre**^ER/tdTom^−/−/**Kras**^G12D^ (**Kras**^G12D^) at the indicated time points after clone induction. Clone sizes are indicated as fractions (in eighths) of the crypt circumference. Blue, nuclear stain (DAPI); red, tdTom expression; scale bars represent 30 μm. (C) Heat maps depict the relative frequency of clones of the indicated size (columns) at various time points (rows) for both WT and **Kras**^G12D^. (D and E) Graph displays the average size (D) or the percentage of fixed clones (E) of WT and **Kras**^G12D^ clones at different time points after induction. Error bars indicate the SEM. **N** = 5 mice for each group; for each time point and condition, >200 clones were analyzed. (F and G) Bayesian inference of the biased drift parameter **P**^R^ for WT versus WT (F) and **Kras**^G12D^ versus WT (G) clones. The vertical axis indicates the probability density; the horizontal axis indicates the **P**^R^ values. The higher the probability density value, the more likely this **P**^R^ value underlies the observed biased drift. **P**^R^ = 0.5 indicates neutral drift. The cartoons summarize the replacement properties (with color coding as in the graphs).

Fig. 2. Quantifying the clonal benefit of **Apc** loss. (A) Heat maps depict the relative frequency of clones of the indicated size (columns) at various time points (rows) for **Apc**^+/−^ and **Apc**^-/-^. (B and C) Graph displays the average size (B) or the percentage of fixed clones (C) of WT, **Apc**^+/−^, and **Apc**^-/-^ clones at different time points after induction. Error bars indicate the SEM. WT is as in Fig. 1. (A) to (C) **N** = 5 mice for each group; for each time point and condition, >200 clones were analyzed. (D and E) Bayesian inference of the biased drift parameter **P**^R^ for **Apc**^+/−^ versus WT (D) and **Apc**^-/-^ versus WT (E).
mutations usually occur in a clone of Apc<sup>+/−</sup> cells because of loss of heterozygosity (LOH) or an independent additional mutation (10); therefore, we also calculated the benefit of Apc<sup>−/−</sup> stem cells over Apc<sup>+/−</sup> cells: \( p_R(\text{Apc}^{−/−} \text{versus} \text{Apc}^{+/−}) = 0.69 \) (0.65 to 0.72, 95% CI) (Fig. 2E). Both in the case of Kras<sup>G12D</sup> and in the case of the inactivation of Apc, the inferred values for \( p_R \) result in clone size distributions in accordance with the measured data (Fig. S7). A further confirmation of the validity of the model comes from the observation that tdTom<sup>+</sup> clones induced in Apc<sup>Min</sup> mice, which are characterized by a heterozygous germline Apc mutation, display similar dynamics to neutral clones in a WT background \([p_R(\text{Apc}^{\text{Min}} \text{versus} \text{Apc}^{\text{Min}}) = 0.48 \) (0.46 to 0.51, 95% CI)], indicating neutral competition between Apc<sup>+/−</sup> lineages, although clones holding this mutation clearly have an advantage over WT clones (Fig. S8).

Our findings reveal that although Kras<sup>G12D</sup> mutations or the inactivation of Apc result in a marked competitive advantage of the respective cell lineage, this does not mean that the clone will become deterministically fixed. Mutated stem cells are commonly replaced by WT stem cells, thereby evading the accumulation of further mutations. By using the inferred parameters of the biased drift model \((N, \lambda, p_R)\) and the adapted stochastic master equations, we calculated the proportion of mutant clones that reach fixation at a particular clone age (Fig. 3A). This analysis reveals that the majority of stem cells acquiring an Apc<sup>+/−</sup> mutation will be stochastically replaced by WT stem cells, and these mutant lineages will disappear from the tissue. In addition only ~55% of Apc<sup>−/−</sup> mutant stem cells within an Apc<sup>+/−</sup> background will reach fixation. These findings reveal that the accumulation of mutations in the intestinal tissue is an inefficient process because of the particular tissue architecture and the continuous stochastic replacements of stem cells. To illustrate the inefficiency of the process, we determined the contribution of different mutational trajectories in the formation of aberrant crypt foci (ACF); i.e., a crypt with a fixed Apc<sup>−/−</sup> clone (Fig. 3B). Only 22% of the ACFs result from two subsequent Apc hits (or LOH), and a large proportion of ACFs arise in crypts in which multiple Apc hits have
Because TP53 mutations are often seen in patients with CRC (2), we next investigated the effect of the dominant-negative hotspot mutation P53R172H. However, no significant benefit of this mutation could be detected in the SI (fig. S8). Because TP53 is reported to be of particular importance in colitis-associated CRC formation (12), we also studied P53 mutations in a chronic colitis model (Fig. 4). Colitis induces a shift in stem cell dynamics in the colon, mainly by reducing the replacement rate (fig. S2), and although P53R172H did not confer a benefit to colon stem cells under normal conditions, in colitis, it significantly increases fitness: $P_R$ (P53R172H versus WT) = 0.48 (0.45 to 0.51, 95% CI) and $P_R$ (P53R172H versus WT) = 0.58, (0.54 to 0.63, 95% CI), respectively (Fig. 4C). Therefore, P53 mutant clones have an increased likelihood to become fixed in a gut affected by inflammation (Fig. 4D). This presumably reflects the benefit of P53-mutated cells in dealing with colitis-associated reactive oxygen species formation (13). More generally, this result demonstrates that the competitive benefit of mutations during tumor initiation is dependent on the context in which they arise.

Our work presents a quantification of the effects of relevant mutations on stem cell dynamics during the initiation of a solid malignancy. A limited competitive advantage of common mutations in CRC is revealed, and many mutations that occur are lost from the population because of stochastic, albeit biased, replacement by neighboring WT lineages. This finding supports the accepted but so far theoretical notion that the tissue architecture and the features of the “evolutionary graph” representing the intestinal stem cell population prevent deterministic fixation of mutated lineages (14–16). The values now placed on the clonal advantages conferred by common mutations can be used in future modeling studies of tumor initiation in the intestine that so far have not used experimentally derived values for the benefits of individual mutations (17). This will help to assess other phenomena associated with CRC development, such as CIN (18). Furthermore, our work reveals that the potential competitive benefits of mutations are dependent on the environment in which they arise and provides a compelling explanation of why TP53 mutations are found frequently and already at early stages in colitis-associated CRC (12). The method used here provides a powerful tool to investigate therapeutic strategies to specifically eradicate (pre-) neoplastic stem cells while preserving their normal counterparts.

References and Notes

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Unwanted Evolution
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Unwanted Evolution

Ivana Bozic1,2 and Martin A. Nowak1,3

We mostly think of evolution as a process that has the power to build structures of incredible beauty and functionality, like multicellular organisms, the nervous system, and human language. But evolutionary dynamics can also lead to processes that are not wanted, such as cancer, because they oppose the survival interests of the organism. The somatic evolution of cancer is a consequence of our cells being individual replicators. Upon acquisitioning mutations, cells can revert to their primitive program of proliferation, competition for survival, and selection of the fittest. On page 995 of this issue, Vermeulen et al. (1) demonstrate how multicellular organisms keep this unwanted evolution in check. By quantifying the effects of mutations that frequently occur during colorectal tumor development, the authors show that intestinal tissue architecture acts as a suppressor of selection.

One way to prevent unwanted evolution is to hold somatic mutation rates low. The mutation rate in healthy human cells is on the order of $10^{-10}$ to $10^{-9}$ per nucleotide base pair per cell division (2), perhaps as low as possible given reasonable energy constraints. What about selection—can it be suppressed? There are population structures where this is the case, such as when a large population of differentiated cells is continuously renewed by the asymmetric division of stem cells (3, 4). It has been hypothesized that many epithelial tissues, which are especially susceptible to cancer because of their many cell divisions, are designed to suppress selection (3). Intestinal epithelium is replenished by equipotent stem cells residing at the bottom of crypts. These stem cells are continuously replacing each other in a random fashion (3, 6). Most intestinal tumors are thought to initiate from a transformed (mutated) stem cell, because genomic alterations occurring in differentiated cells would be “flushed out” as a result of the specific tissue architecture (differentiated cells are continuously pushed upward in the crypt and eventually undergo programmed cell death at the top of the crypt) (3, 7). The initiating genetic event in the majority of colorectal cancers is an inactivation of the APC tumor suppressor gene, often followed by mutations in the oncogene KRAS and the tumor suppressor P53, among other genes (8).

To measure the effect of initial genetic changes occurring in colorectal cancer, Vermeulen et al. visualized and quantified the dynamics of stem cell replacement in the mouse intestine when one of the stem cells in the crypt contains a particular change, such as an inactivation of one or both copies of Apc, activation of Kras, or a mutation in p53. The authors demonstrate that the evolutionary dynamics of stem cells can be well described by a simple mathematical model that assumes a ring of five to seven stem cells at the base of the crypt. A mutant stem cell replaces its neighboring wild-type stem cell with probability $P_s$, whereas the wild-type cell replaces the mutant with probability $1 - P_s$ (see the figure).

Vermeulen et al. show that Apc inactivation and Kras activation increase the probability that a stem cell carrying those alterations replaces a neighboring wild-type stem cell. It is generally believed that inactivation of one of the Apc alleles does not lead to a phenotypic change, but the authors found that Apc exhibits some degree of haploinsufficiency (9). Even a single inactivated Apc copy ($Apc^{–/+}$) leads to some selective advantage over wild-type stem cells. The probability that an $Apc^{–/+}$ mutant cell replaces an $Apc^{+/+}$ wild-type cell is estimated to be $P_s = 0.62$. Stem cells with both copies of Apc inactivated, $Apc^{–/–}$, replaced neighboring $Apc^{+/–}$ cells with probability $P_s = 0.69$. Activation of Kras had

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**Crypt dynamics.** Stem cells reside at the bottom of crypts in the intestine. (A) The number of mutant stem cells (red) increases by 1 with probability $P_s$ and decreases by 1 with probability $1 - P_s$. A single mutant stem cell can be lost by random drift or can take over the compartment with probability $P_s$ (see the equation). (B) The genetic composition of the stem cell compartment (at the base of the crypt) determines the composition of cells (mutant cells shown in red; wild-type cells in blue) in the entire intestinal crypt.
the most pronounced effect, with a 78% chance of replacing an adjacent wild-type stem cell. The authors also found that $p53$ mutations provide a selective advantage, but only in gut affected by inflammation ($P_R = 0.58$) and not in normal conditions ($P_R = 0.48$).

Although mutant stem cells are more likely to replace wild-type stem cells than vice versa, they can nevertheless be lost by random drift (when the frequency of mutant cell in a population decreases until it disappears). The probability that a single mutant stem cell reaches fixation in a crypt (takes over the entire stem cell compartment of the crypt) can be calculated as

$$P_{fix} = \frac{1 - (1 - P_R)^N}{1 - (1 - P_R)}$$

in which $N$ is the number of stem cells per crypt. Assuming $N = 5$ stem cells in a crypt and using the experimentally derived values for $P_R$, the probability for a single $Apc^{-/−}$ stem cell to take over the crypt is 0.42. Similarly, the probability that a single $Apc^{-/−}$ cell reaches fixation in an $Apc^{+/+}$ background is 0.56. The authors report similar fixation probabilities based on direct measurement.

This detailed quantitative information about the initial steps in the somatic evolution of a solid cancer makes an important connection between theory and experiment. The values for the selective advantage of frequently occurring mutations can provide precise guidelines for future studies of tumor initiation and progression (10–15). As Vermeulen et al. point out, a similar approach can be used to investigate the role of chromosomal instability in tumor initiation. Their findings also raise new questions about the events that follow the fixation of a mutation in a single crypt, including quantification of a field carcinization effect and the change in dynamics and tissue morphology caused by the subsequent mutations. Most important, this exciting study opens the door for a quantitative understanding of cancer initiation. One can also envisage the possibility of using the devised experimental framework for the development and testing of drugs that attack mutated stem cells, thereby preventing the process of cancer initiation.

**References**


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### MATERIALS SCIENCE

**Metamaterials Beyond Optics**

**Martin Wegener**

So far, the field of metamaterials has largely dealt with negative refractive indices in optics and invisibility cloaks (1–3). However, the underlying idea of designing material properties is not that narrow. More broadly, one rationally designs a subwavelength unit cell from existing constituent materials and (periodically) arranges it into an artificial solid. The properties of that solid are then determined by structure rather than chemistry and can be tailored, extreme, or even qualitatively unprecedented. Rational design is the key and makes metamaterials a rather particular class of composite materials.

In terms of application, some aspects of metamaterials such as negative phase velocities of light (1, 2), invisibility cloaking (4, 5), or unusual optical nonlinearities (6) are fascinating but are not likely to soon appear in products, because optical absorption (loss) is too high and in part fundamentally unavoidable. Moreover, the inexpensive manufacturing of complex large-volume three-dimensional optical metamaterials is still a formidable challenge in itself (2).

Why, then, don’t we go beyond optics and also consider other material aspects (7) such as thermal, acoustic, elastic, or irreversible nonlinear mechanical properties? The corresponding wavelengths and length scales range from tens of micrometers to centimeters, rather than nanometers in optics. Hence, fabrication limitations are relaxed, thus easing real-world applications. Moreover, one lesson learned from electromagnetism is that off-resonant constituents enable low losses. In the visible spectrum, however, this results in a contrast in refractive index of no more than 3 for available nonabsorbing dielectrics. Some theoretical blueprints demand constituent-material contrasts in the range of tens or hundreds. Such values are accessible in mechanics and thermodynamics (see the figure).

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The area of metamaterials may expand to find application in mechanics, acoustics, and thermodynamics.

Consider simple mechanical waves in elastic solids. They exhibit three orthogonal polarizations, one longitudinal (like sound waves in air or water) and two transverse (like electromagnetic waves). This complex...