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# The Immortal Strand Hypothesis: Segregation and Reconstruction

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**The immortal strand hypothesis posits that the propensity of stem cell compartments to give rise to cancer in later life can be minimized if stem cells, during the process of self-renewal, retain those DNA strands with the fewest mutations acquired during DNA replication. In this Essay, I explore evidence in support of the hypothesis, the biological implications, and the key questions that remain to be answered experimentally to address the fundamental tenets of the hypothesis.**

## Introduction

Stem cells are responsible for tissue maintenance and repair throughout the life of the organism. Particularly in tissues that undergo continuous and rapid turnover, such as cellular components of blood and the epithelial cells of gut and skin, there is a constant demand for stem cells to proliferate to generate differentiated cells and, in the process, to self-renew. Given the high frequency of DNA replication errors resulting in genomic mutations, the chance of any stem cell or its persistent progeny acquiring a sufficient number of critical mutations throughout a human life span that would result in cancer is exceedingly high were it not for two mechanisms: cell-cycle checkpoints and the ability to detect and repair such mutations (Hanahan and Weinberg, 2000). Yet, paradoxically, stem cells appear to have a reduced, rather than an enhanced, DNA repair capacity compared with other somatic cells in the few populations in which it has been examined (Cairns, 2002). This suggests that stem cells have the ability to limit the accumulation of spontaneous mutations or that, having acquired deleterious mutations, they are more prone to undergo senescence or apoptosis so as to reduce the risk of generating a malignant clone. The extent to which stem cell functions (or loss thereof) are causally related to the aging process or are determinants of the maximal life span of a species is a matter of debate (Rando, 2006), but the potential for stem cells or their progeny to acquire a malignant phenotype and thereby shorten an individual's life span is undisputed.

*These paired Essays provide different perspectives on the immortal strand hypothesis first proposed thirty years ago.*

## The Immortal Strand Hypothesis

In considering mechanisms by which a stem cell population might limit the accumulation of replication-induced mutations, John Cairns put forth the "immortal strand hypothesis" in 1975 (Cairns, 1975). The hypothesis is based on the fact that each newly formed chromosome consists of the older ("grandparent") template strand and a newly synthesized ("parent") strand that is likely to contain replication-related errors (see also the Essay by P.M. Lansdorp on page 1244 of this issue). Upon a subsequent round of replication, when both the grandparent and parent strands serve as templates for DNA replication, the resulting sister chromatids could, in theory, be distinguishable based on the age of the template. The hypothesis is that when the cell then divides, there exists a mechanism to sort all of the chromatids containing the grandparent (older) templates to one daughter cell and all of the chromatids containing the parent (younger) templates to the other daughter cell (Figure 1). This would be an asymmetric cell division based solely on chromatid cosegregation according to template age. The hypothesis further suggests that this asymmetric cell division is associated with stem cell self-renewal and that the new stem cell would inherit all of the oldest templates and the other daughter, destined to differentiate, would inherit all of the younger templates with replication-induced mutations. In the extreme, a stem cell pool would retain, throughout the life of the organism, its original (i.e., "immortal") DNA strands that were generated when the cell population first arose during development, and these immortal strands would continue to serve as templates indefinitely. Thus, in theory, the original genetic code would be optimally preserved in the stem cells and replication-related mutations would be kept to a minimum.

## Implications of the Immortal Strand Hypothesis

One of the most profound implications of this hypothesis is the suggestion that the complementary DNA strands are in fact not identical but are distin-

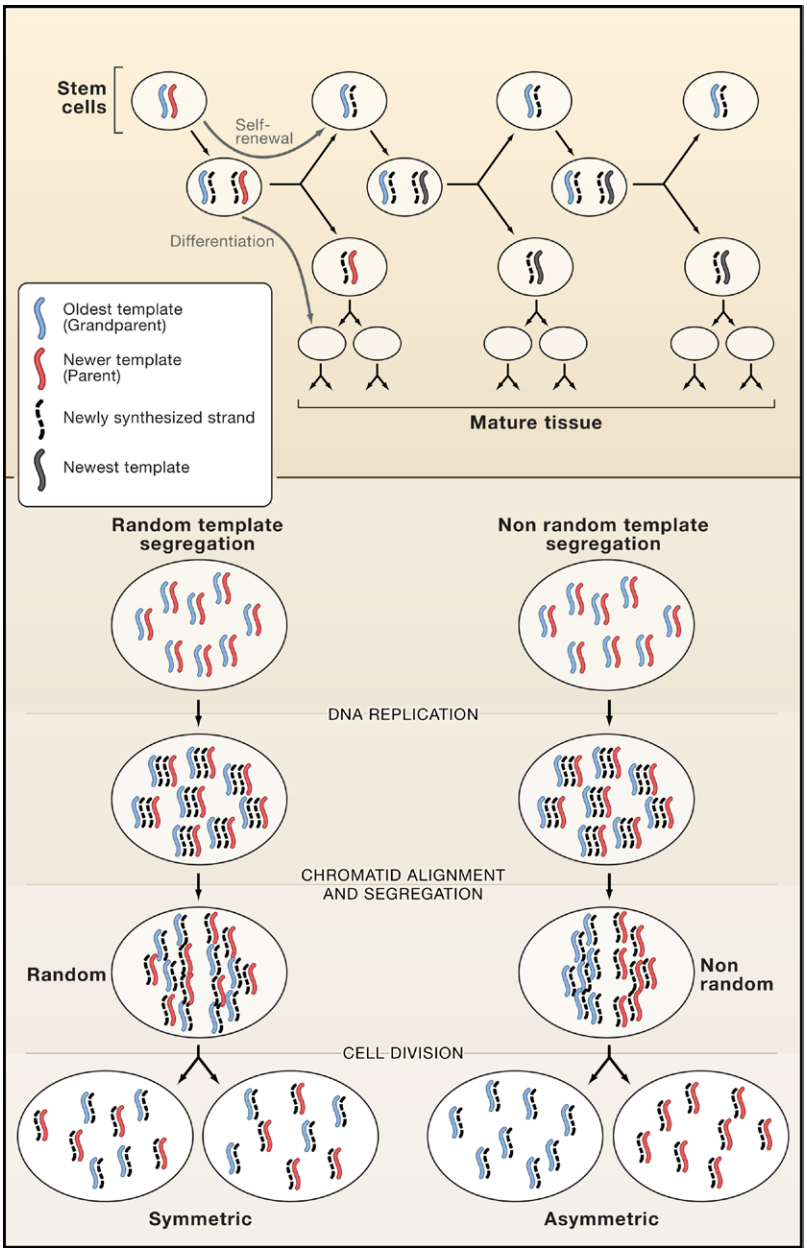


**Figure 1. The Immortal Strand Hypothesis and Template Strand Segregation**

(Top) Retention of an immortal strand during stem cell self-renewal. This panel shows a stem cell, on the far left, containing a single chromosome consisting of an older ("grandparent") template (blue) and a younger ("parent") template (red). Following DNA replication, an asymmetric cell division results in two daughters, one of which gives rise to a new stem cell by the process of self-renewal, and the other of which gives rise to the differentiated mature cells of the tissue. According to the immortal strand hypothesis (Cairns, 1975), the sister chromatid containing the older ("immortal") strand (blue) remains with the continually renewing stem cell through repeated asymmetric divisions for the life of the organism.

(Bottom) Random and nonrandom template strand segregation. Shown are the differences in distribution of multiple chromatids (in this case, in a theoretical cell with 4 pairs of chromosomes giving rise to 16 sister chromatids following DNA replication) if the segregation is either random or nonrandom. According to the immortal strand hypothesis, either as initially formulated for stem cell self-renewal (Cairns, 1975) or generalized to include asymmetric cell divisions associated with divergent cell-fate decisions in stem cell progeny (Conboy et al., 2007), there is nonrandom chromatid segregation requiring the recognition and alignment of all chromatids containing the older templates separate from those containing the newer templates. Following cell division, the daughter cell acquiring the older templates would be the cell with the more undifferentiated phenotype.

guishable with regard to their roles as templates for DNA replication. A corollary of the hypothesis is that stem cells capable of retaining immortal strands would not be subject to telomere shortening associated with DNA replication since progressive telomere shortening arises as newly synthesized strands become templates in successive generations (Watson, 1972). That is not to say that stem cells would not experience other aspects of chronological aging that can lead to telomere shortening (von Zglinicki, 2002). The validity of the immortal strand hypothesis also depends on the assumption that sister chromatid exchange and mitotic recombination are exceedingly low in stem cell populations. Otherwise, the underlying premise would be undermined because high levels of either process would diminish the ability of the stem cell to retain the sequences with the fewest replication-induced errors. Finally, one of the most important, practical implications of the immortal strand hypothesis in the context of current stem cell biology relates to the identification of stem cell populations as "long-term label-retaining cells" (Bickenbach, 1981), a designation that alludes to the presumed very long cell cycle times of stem cells compared with their proliferative progeny. The "label-retaining" concept is that the administration of a label, such as bromodeoxyuridine (BrdU), that can be incorporated into the DNA of dividing cells will be quickly diluted by cell divisions but will be retained for much longer periods in slowly dividing stem cells. However, this designation is based on the assumption of random segregation of sister chromatids into stem cell daughters. If the segregation is not random, and if the stem cell retains the older unlabeled template strands, then the stem cell will lose all label by the second division after administration of the label as a pulse. By contrast, if a label is given at the time when a stem cell is generated and is incorporated into the immortal strand, then the stem cell will indeed be label retaining, theoretically indefinitely, but the retention of the label would not relate in any way to the length of the cell cycle. Whether the

stem cell is slowly dividing or rapidly dividing, the label would be retained in the stem cell's immortal strands.

## Evidence to Support the Immortal Strand Hypothesis

Cairns' immortal strand hypothesis was not purely theoretical. Evidence of template strand segregation, based on DNA template age, in embryonic fibroblasts had been published in 1966 (Lark et al., 1966). In those studies, $^{3}$H-thymidine ($^{3}$H-Td) was administered to embryonic cells in culture followed by growth in radiolabel-free medium, and the distribution of label was assessed in successive generations. Based on quantitative analysis of the distribution of label, the authors concluded that there was nonrandom segregation of the older template strands that had incorporated the label and the newer templates that had not; the labeled strands tended to segregate together.

The possibility of DNA strands in mammalian cells cosegregating based upon the cycle in which they were synthesized was also based upon evidence of similar processes occurring in simpler prokaryotic and eukaryotic organisms such as bacteria, plants, and fungi. In bacteria, the homologs of sister chromatids in eukaryotes are segregated into daughter cells in a manner that distinguishes the template strand synthesized in the most recent round of replication from that synthesized during the previous round (Lark and Bird, 1965; Lark, 1966; Cuzin and Jacob, 1965). It was hypothesized that the distinction between template strands of different ages might be related to a physical association of the oldest template to a membrane segregation apparatus that is permanent. In studies of growing root tips of the plant *Vicia faba* (a species of bean), analysis of cells in anaphase or telophase after growth in $^{3}$H-Td for one generation followed by growth in nonradioactive medium for one or two generations revealed a tendency for radioactive chromatids to cosegregate separately from nonradioactive chromatids; a diploid strain of *Triticum boeoticum* (a species of wheat) showed a similar pattern (Lark, 1967). Likewise, DNA strands synthesized during the same cell cycle tend to cosegregate during subsequent nuclear divisions during hyphae development in a filamentous fungus, the ascomycete *Aspergillus nidulans* (Rosenberger and Kessel, 1968). In this organism, mononucleated conidiospores give rise to multinucleated hyphae by synchronous rounds of nuclear division. Consistent with a model of nonrandom segregation of DNA strands, nearly all of the label administered during the earliest division remained with the two oldest nuclei rather than being passed on to progeny. Intriguingly, unrelated to strand segregation but relevant to models of stem cell self-renewal and stem cell niches, these oldest nuclei tended to remain closest to the hyphal tip, as if this spatial localization were somehow related to the sister chromatid segregation (Rosenberger and Kessel, 1968). An initial report of template strand segregation by age in budding yeast using whole-cell autoradiography (Williamson and Fennell, 1981) was not confirmed by subsequent studies using immunofluorescence analysis of BrdU incorporation (Neff and Burke, 1991), although different strains were used in the two studies.

The work of Potten and colleagues was the first rigorous test of nonrandom template strand segregation in a mammalian stem cell population (Potten et al., 1978, 2002). Focusing primarily on the small intestinal epithelium in the mouse, a tissue with a very high turnover rate and with an anatomically well-defined stem cell compartment, the segregation of label was followed in short-term and long-term studies of stem cell progeny. The investigators attempted to label the immortal strands by administering label at the time of stem cell formation (during development or following irradiation treatment). Results of these studies clearly showed long-term retention of label, consistent with segregation of labeled strands to the self-renewing stem cells (Potten et al., 1978, 2002). Perhaps most convincing were studies in which the oldest strands were labeled with $^{3}$H-Td, and BrdU was later administered to label newly synthesized strands (and thus younger templates). Analysis revealed two important observations. First, soon after administration of BrdU, nearly all $^{3}$H-Td-labeled cells were also labeled with BrdU, demonstrating that the cells that had retained the labeled templates were indeed proliferating (that is, they had not retained label simply because they were not cycling) (Potten et al., 2002). Second, over the next several days, the BrdU label was rapidly cleared from the cells that continued to retain the $^{3}$H-Td label. This is consistent with and highly supportive of nonrandom template strand segregation based on template age. It should be noted, however, that even in this well-defined niche, the absence of a definitive in situ marker of the stem cell means that template strand cosegregation cannot be unequivocally attributed to the adult stem cell.

Subsequent studies have used similar approaches to label either younger or older templates and then follow the retention or disappearance of label over successive generations in mammalian stem and progenitor cell populations in vivo and in vitro (reviewed in Cairns, 2006). These studies have largely confirmed the observations of Potten and colleagues, demonstrating nonrandom template strand segregation associated with the rare event of stem cell self-renewal. A noteworthy finding has been the work by Sherley and colleagues linking template strand cosegregation with asymmetric stem cell kinetics and demonstrating a potential role of the tumor suppressor protein p53 as a regulatory switch influencing whether the cells would segregate templates randomly or nonrandomly (Merok et al., 2002; Rambhatla et al., 2005). These studies were done in vitro using an immortalized cell line with an inducible *p53* gene, so the relevance to stem cells in vivo remains to be demonstrated.

In the process of studying self-renewal of stem cells in skeletal muscle, we have recently found evidence that not only supports nonrandom template strand segregation but also expands the scope of the immor-

tal strand hypothesis (Conboy et al., 2007). Our data suggest that template strand segregation occurs along successive divisions of the proliferative expansion of stem cell progeny and is not limited to a single set of immortal strands or the asymmetric division involved in self-renewal. Rather, our data suggest that template strand segregation occurs in multiple subsequent divisions in which the daughters adopt different fates, always with the older strands segregating to the daughter retaining the more undifferentiated phenotype. These findings were possible because of the use of sequential DNA labeling using different markers (in addition to BrdU, other halogenated thymidine analogs, iododeoxyuridine and chlorodeoxyuridine) during successive cell division (Conboy et al., 2007). In addition, the frequency of asymmetric divisions associated with template strand segregation was not rare, as in stem cell self-renewal studies, but was high, approaching 50% when the proliferating progenitor cells were labeled in vivo. The simplicity of the technical approach will allow for similar studies on other stem cell compartments, either during normal turnover or during tissue repair.

## Contrary Evidence

Disproving the immortal strand hypothesis in any general sense is impossible, but clearly there have been failures to demonstrate template strand segregation in specific cell populations under specific conditions. However, most of the negative reports come from studies of cellular populations that are not considered stem cells. For example, Comings demonstrated random segregation of sister chromatids in synchronized Chinese hamster cells in vitro (Comings, 1970). This was followed by a series of reports also showing random segregation of sister chromatids in various eukaryotic cells undergoing division in vitro or in vivo (Geard, 1973; Fernandez-Gomez et al., 1975; Morris, 1977). These studies suggest that nonrandom segregation of template strands does not occur as a general rule. Ito and McGhee tested template strand segregation during development in the worm *Caenorhabditis elegans*. When either sperm or oocyte DNA was labeled with BrdU, distribution of the label in developing embryos was consistent with random segregation of parental strands in developing embryos (Ito and McGhee, 1987). However, there was considerable statistical variation from random segregation, and the studies test for strand segregation during specific early stages of development. Kuroki and Murakami examined distribution of $^{3}$H-Td in mouse epidermis induced to undergo successive cell divisions following the injection of cholera toxin (Kuroki and Murakami, 1989). The main observation was that basal cells exhibited retention of label up to 50 days after $^{3}$H-Td injection, and the authors concluded that this was not consistent with segregation of immortal strands to the epidermal stem cells. This conclusion was based on the assumption that treatment with the toxin did not result in the formation of new stem cells in which the label could have been incorporated into the newly forming DNA templates. If that were the case, the evidence of label-retaining cells could be explained by the existence of immortal strands.

## Mechanistic Considerations

To date, no studies have shed any light on the biochemical mechanism by which template strands, differing in replicative age by as little as one cell division, might be differentially recognized, aligned, and segregated during mitosis, with all sister chromatids harboring the older templates across the entire spectrum of chromosomes being segregated to the same daughter cell. Template strands of different ages could, in theory, differ in any number of ways, each of which could subsequently be distinguished and used as a code for segregation. The biochemical signal could be a differential covalent modification (such as methylation) of the DNA strands themselves. Although differential DNA methylation of alleles underlies epigenetic phenomena such as imprinting, there is no evidence that differential methylation of DNA strands subsequently serving as templates for DNA synthesis can distinguish sister chromatids. Alternatively, chromatids formed from templates of different ages may acquire different patterns of chromatin organization, being distinguishable by the associated histones or their modifications (such as methylation or acetylation). Nonrandom distribution of chromosomal proteins during replication has been demonstrated, and especially relevant is evidence of differential segregation of "old" histone octamers to one sister chromatid and "new" histone octamers to the other sister chromatid in dividing cells, or the mechanisms of transfer of parental histones onto newly synthesized DNA (Leffak et al., 1977; Sogo et al., 1986; Jackson, 1988). An intriguing possibility is that the coding mechanism is related to the biochemical mechanism that underlies the establishment of chromosomal territories. These are disrupted during cell division but re-established in daughter cells (Cremer and Cremer, 2001), a phenomenon heretofore associated primarily with topological models of gene regulation.

Beyond the issue of how template strands of different ages are distinguished, additional mechanistic issues need to be considered. In particular, it would be important to understand how the centrosome and associated microtubules are organized so that all kinetochores bound to the template strands of the same age are coordinately recognized and segregated to the same daughter cell. The fidelity of the process also suggests that the spindle checkpoint would have to be configured to be able to assure strand-specific segregation. The molecular mechanisms that underlie the relationship between these processes and the ability of some stem cells to retain the oldest template strands remain a complete mystery.

In the strictest interpretation of the immortal strand hypothesis, the encoding of templates would have to occur only at the time of birth of a stem cell when the immortal strand is established. To the extent that

template strand segregation occurs sequentially during lineage progression of stem cell progeny (Conboy et al., 2007), the mechanism would have to occur as a counting mechanism, analogous to telomere shortening, resulting in a small but distinguishable change with each cell division. Pharmacological treatments that could selectively disrupt template strand segregation, or the demonstration of template strand segregation in an organism such as *Drosophila* or *C. elegans*, which would allow for genetic screening, would be a major advance for the field.

## Summary

The immortal strand hypothesis can be dissected and tested on multiple levels, and studies that preceded and followed the explicit statement of the hypothesis have provided variable degrees of support at those different levels. At the base, and seemingly most well-founded, is evidence in support of the most fundamental aspect of the hypothesis, namely that template strands can segregate nonrandomly to daughters of a dividing cell, sorted by template age. Although context dependent, the cumulative data suggest mechanisms that exist in cells across a vast phylogenetic spectrum and challenge the dogma that the two strands of the double helix are identical with regard to their roles as templates during DNA replication. At an intermediate level, the premise of the hypothesis that such a nonrandom segregation would occur only during asymmetric cell division associated with stem cell self-renewal may be true but incomplete. This may need to be generalized to other contexts of tissue development and regeneration when the progeny of stem cells undergo asymmetric cell divisions that lead to divergent fates of the daughters. Finally, at the level with the least empirical evidence is the explicit premise of the immortal strand hypothesis that nonrandom segregation of template strands is a mechanism to limit the propensity of stem cells to acquire mutations resulting in tumor formation. No studies have specifically tested for an association between template strand segregation and mutation frequency or cancer incidence. In order to do this rigorously and to test causality, it would be necessary to selectively disrupt nonrandom segregation in a stem cell pool and then measure both the cumulative mutation frequency over a lifetime and the incidence of cancer in that tissue in comparison to the organism in which nonrandom template segregation persisted. The controls that would be necessary to demonstrate disruption of template strand cosegregation and not other aspects of mitosis, such as chromatid alignment and separation, would pose a major challenge, and the measurement of cumulative, random genomic mutations in a rare cell population would be daunting. However, comparing the incidence of cancer in the tissue would be straightforward. If it were possible to disrupt template strand segregation specifically and if this resulted in no increase in cancer incidence, then it would be necessary to seek another "reason" for nonrandom template strand segregation in the context of evolutionary theory and selective advantage to unicellular or multicellular organisms.

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