Supporting Information

Huh et al. 10.1073/pnas.1310323110

SI Results and Discussion

Gene Expression Evidence for the Model. As outlined in Fig. S2, experimental support for the proposed 5-hydroxymethylcytosine (5hmC) asymmetry model of immortal DNA strand specification and maintenance also is provided by microarray studies with previously described engineered cell lines that undergo either random segregation or nonrandom segregation conditionally (1). The mRNAs for two pertinent genes are respectively down-regulated (Fig. S2, A_down) and up-regulated (Fig. S2, A_up) when the cells initiate nonrandom segregation (1). The down-regulated mRNA encodes the enzyme L-2-hydroxyglutarate dehydrogenase (L2HGDH, EC 1.1.99.2), whereas the up-regulated mRNA encodes aminoadipate aminotransferase (AADAT, EC 2.6.1.39). These two enzymes share in common a biochemical potential to regulate ten-eleven translocase (TET) enzyme activity. TET enzymes are 2-oxoglutarate-Fe(II) oxygenases that require the mitochondrial tricarboxylic acid (TCA) cycle intermediate α-ketoglutarate (αKG = 2-oxoglutarate) for activity (Fig. S2) (2). In addition, a reduction product of αKG, 2-hydroxyglutarate (2HG), is an inhibitor of TETs (2). This catabolic conversion is well known from cancers associated with mutations in the TCA-cycle enzyme isocitrate dehydrogenase (IDH, EC 1.1.1.41), which normally catalyzes the production of αKG. Certain mutant forms of IDH lose this function and gain an aberrant activity in catalyzing the production of 2HG from αKG. In vivo, elevation of wild-type IDH activity increases chromosomal 5hmC content, whereas elevation of mutant forms of the enzyme decrease chromosomal 5hmC content (2). These findings illustrate the importance of the [αKG]/[2HG] fraction as a determinant of cellular TET activity levels.

As shown in Fig. S2, L2HGDH converts 2HG to αKG (3). Therefore, the down-regulation of its mRNA (0.54; $P = 0.018$) (1) is predicted to lower the [αKG]/[2HG] fraction. AADAT mRNA was detected only during asymmetric self-renewal ($P = 0.041$) (1). Although AADAT mRNA is up-regulated, the predicted effect on TET activity is the same. AADAT uses αKG to catalyze a step in lysine degradation (4). Therefore, its up-regulation is predicted to compound the effect of L2HGDH down-regulation in decreasing the [αKG]/[2HG] fraction and, consequently, TET activity. (The probability that the combined changes in L2HGDH and AADAT expression would occur by chance is <0.001).

Rationale for Guanine Ribonucleotide Dependency. A particularly attractive feature of the proposed mechanism of 5hmC asymmetry is that it also could account for the long-unexplained role of guanine ribonucleotide biosynthesis in the regulation of DSC asymmetric self-renewal and nonrandom segregation (5–8). GTP is a substrate for the AMP branch of the de novo purine nucleotide biosynthetic pathway (Fig. S2 *Right*, cytoplasm). Cytoplasmic adenylosuccinate synthetase (ADSS, EC 6.3.4.4), the rate-limiting enzyme for de novo AMP biosynthesis, uses IMP, aspartate, and GTP to produce the next pathway intermediate, succinyl-AMP (sAMP). The ADSS reaction is also the rate-limiting step for the purine nucleotide cycle (PNC), which plays an important role in cellular ammonia metabolism (9). The following adenylosuccinate lyase (ADSL, EC 4.3.2.2) step of the PNC produces AMP with the release of fumarate. Because the K_m of ADSS for GTP is in the range of the cellular GTP concentration (10), ADSS activity is predicted to be sensitive to the modest reduction in GTP concentration associated with the initiation of asymmetric self-renewal and nonrandom segregation (7, 11). Therefore, the down-regulation of inosine 5′ monophosphate dehydrogenase type 2 (IMPDH II) by p53 is predicted to reduce the concentration of cytoplasmic fumarate. In the TCA cycle, fumarate is the third metabolite after αKG (Fig. S2). Under conditions of decreased cytoplasmic fumarate caused by the decreased production of GTP, an equilibrating efflux of fumarate through passive mitochondrial transporters (12) potentially could accelerate αKG utilization, decreasing the [αKG]/[2HG] fraction.

It also is noteworthy that a recently described, highly specific gene-expression signature of label-retaining breast basal epithelial cells, which undergo nonrandom segregation, included up-regulated AADAT (2.5-fold; $P = 0.01$) and down-regulated ADSS (2.4-fold; $P = 0.015$) (13). Both changes are predicted to reduce the [αKG]/[2HG] fraction, as discussed. The signature also included the 2.3-fold up-regulation of the mRNA encoding IDH3b, the beta subunit of IDH ($P = 0.025$) and the 2.5 fold down-regulation of the gene encoding the C subunit of succinate dehydrogenase (SDHC) (Fig. S2; SDH, EC 1.3.99.1; $P = 0.005$) (13), which catalyzes the conversion of succinate to fumarate in mitochondria. Currently, it is unclear how the observed changes in the expression of these enzyme subunit mRNAs might impact the cellular concentrations of αKG and fumarate and, more importantly, TET activity. However, it is very clear from our data that xanthine (Xn) supplementation robustly increases the 5hmC chromosomal content of hair follicle distributed stem cells (DSCs) undergoing nonrandom segregation, and this change is associated with a major shift in their pattern of chromosome segregation.

From the earliest recognition of asymmetric self-renewal and nonrandom segregation in cultured cells, whether engineered or natural DSCs, integration with cellular metabolism has been implicated by the involvement of guanine ribonucleotide biosynthesis in p53-dependent growth regulation (11). Our hypotheses suggest a convergence of guanine ribonucleotide biosynthesis with energy metabolism. This implied regulatory integration of cellular energetics with the selection of crucial coordinate DSC cellular programs for tissue-cell renewal and genetic fidelity is attractive as a possible evolutionary milestone in the appearance of long-lived multicellular organisms. Such systems could serve to optimize growth and tissue development with respect to energy availability. High-energy states are inferred to promote expansive tissue growth and high mutation rates, whereas low-energy states foster homeostatic tissue growth with reduced mutation. Thus, in the future, our findings also may inform ideas about the cellular mechanisms responsible for nutritional effects on carcinogenesis and lifespan.

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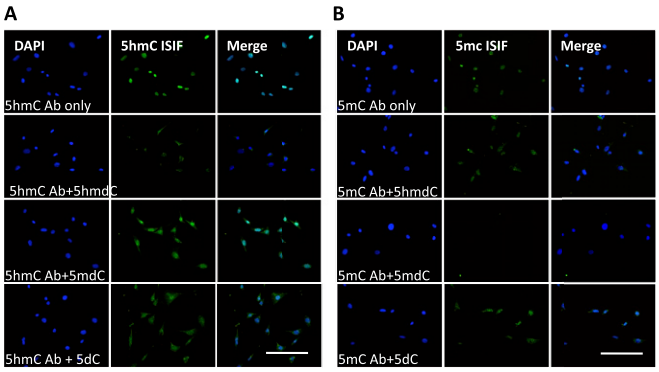

Fig. S1. Nucleoside- and nucleotide-blocking analyses to confirm the specificity of anti-5mC and anti-5hmC antibodies. Shown are examples of in situ indirect immunofluorescence micrographs from the blocking experiments described in *Materials and Methods* in the main text. (*A*) Blocking-specificity series against anti-5hmC antibodies. (*B*) Blocking-specificity series against 5mC antibodies. 5hmC, 5-hydroxymethyl-2′-deoxycytidine-5′-triphosphate; 5mdc, 2′-deoxy-5-methylcytidine; 5dc, 2′-deoxycytidine. (Scale bars, 300 microns.)

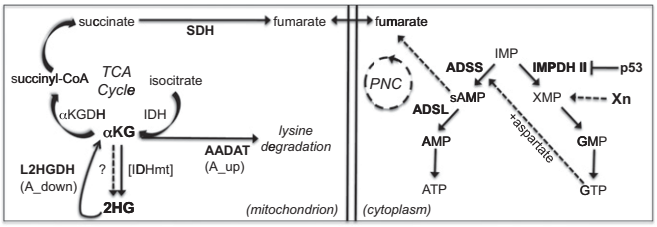

Fig. S2. Proposed biochemical pathway underlying coordinate loss to TET enzymatic activity with initiation of nonrandom segregation by asymmetrically self-renewing DSCs. The TCA cycle intermediate αKG is a cofactor required by TET enzymatic activity, and 2HG is an inhibitor of the enzymes. αKG is removed from the TCA cycle by cellular reductases of uncertain identity (indicated by the question mark). Mutant forms of IDH associated with human cancers (IDHmt) also can reduce αKG levels. Induction of asymmetric self-renewal occurs with down-regulation L2HGDH (A_down) and up-regulation of AADAT (A_up), which uses αKG for lysine degradation. These two coordinated gene-regulatory events are predicted to decrease the [αKG]/[2HG] fraction, compounding the loss of TET enzymatic activity. The described mitochondrial reactions might be influenced by the rates of guanine ribonucleotide biosynthesis occurring in the cytoplasm (*Right*) via the common metabolite fumarate, which is produced in the mitochondrion (*Left*) from succinate by SDH. GTP produced by the p53-regulated IMPDH II branch of the de novo purine nucleotide biosynthesis pathway is a substrate for the reaction of ADSS with aspartate to form sAMP. As a part of the ammonia-producing PNC, sAMP is converted by ADSL to fumarate, which is equilibrated across the mitochondrial outer membrane (parallel lines) down its concentration gradient by dicarboxylic acid transporters (double-headed arrow). Because ADSS has a K_m for GTP similar to the cellular concentration of GTP, even modest reductions in GTP concentration caused by the down-regulation of IMPDH II by p53 could lead to significant decreases in cytoplasmic fumarate concentration. A responsive outflow of this TCA-cycle metabolite from the mitochondria could result in lower levels of other TCA-cycle constituents such as αKG. Conversely, by the same pathways, Xn supplementation is projected to increase cytoplasmic fumarate by expanding GTP pools, thereby accounting for its ability to suppress 5hmC chromosomal asymmetry.