

A quantitative model for age-dependent expression of the p16^{INK4a} tumor suppressor

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Edited by Joseph F. Fraumeni, Jr., National Institutes of Health, Bethesda, MD, and approved August 6, 2009 (received for review April 21, 2009)

Recent work has shown that expression of the p16^{INK4a} tumor suppressor increases with chronological age. Expression is accelerated by gerontogenic behaviors such as tobacco use and physical inactivity, and is also influenced by allelic genotype of a polymorphic single nucleotide polymorphism (SNP) rs10757278 that is physically linked with the p16^{INK4a} ORF. To understand the relationship between p16^{INK4a} expression, chronologic age, subject characteristics and host genetics, we sought to develop a mathematical model that links p16^{INK4a} expression with aging. Using an annotated dataset of 170 healthy adults for whom p16^{INK4a} expression and subject genotypes were known, we developed two alternative stochastic models that relate p16^{INK4a} expression to age, smoking, exercise and rs10757278 genotype. Levels of p16^{INK4a} increased exponentially and then saturated at later chronologic ages. The model, which best fit the data, suggests saturation occurs because of p16^{INK4a}-dependent attrition of subjects at older chronologic ages, presumably due to death or chronic illness. An important feature of our model is that factors that contribute to death in a non p16^{INK4a}-dependent manner do not affect our analysis. Interestingly, tobacco-related increases in p16^{INK4a} expression are predicted to arise from a decrease in the rate of p16^{INK4a}-dependent death. This analysis is most consistent with the model that p16^{INK4a} expression monotonically increases with age, and higher expression is associated with increased subject attrition.

aging | gene expression | smoking | stochastic model | senescence

Aspects of mammalian aging seem to result from a progressive decline in tissue replicative capacity, which has been linked to activation of antiproliferative tumor suppressor mechanisms such as telomere shortening and p16^{INK4a} up-regulation (1–3). In particular, expression of p16^{INK4a} has been shown to sharply increase with aging in most tissues of diverse mammalian species (4–9); and caloric restriction, which retards aging in rodents, attenuates this age-induced increase in p16^{INK4a} (4, 5). Increasing p16^{INK4a} expression in mice seems to play a causal role in the age-associated decline of the proliferative capacity of hematopoietic stem cells (10), pancreatic beta-cells (11) and neural stem cells (12), and germ-line p16^{INK4a} inactivation ameliorates several aspects of aging in a progeroid mouse model (13). Therefore, p16^{INK4a} seems to be a biomarker and effector of mammalian aging.

An emerging view of p16^{INK4a} regulation with aging is that loss of Polycomb group silencing leads to a heritable epigenetic derepression with aging. The PcG proteins BMI-1 (14–18) and EZH2 (19–21) have been shown to act as epigenetic repressors of p16^{INK4a} expression. Loss of EZH2 and BMI-1 binding to the *INK4a/ARF* locus, which encodes p16^{INK4a}, has been reported with prolonged fibroblast culture with attendant increased expression of the locus in senescent cells (20). Loss of EZH2 expression has also been reported in pancreatic β -cells in vivo with aging in humans and mice (21), and inactivation of Bmi-1 or Ezh2 in this tissue causes *Ink4a/Arf* overexpression, a relative failure of insulin secretion and hyperglycemia; phenotypes which are attenuated by p16^{INK4a} deficiency (17, 21). Accordingly, little

if any increase in p16^{INK4a} expression with aging is seen in true hematopoietic stem cells (HSC) (22), which are highly dependent on PcG activity for in vivo persistence (16, 18), whereas dramatic and functionally significant increases of p16^{INK4a} expression are noted in the immediate progeny (e.g., lymphoid progenitors) of HSC (23). These results suggest that a stochastic, heritable loss of PcG repression in individual cells with aging contributes to the observed increase in expression of p16^{INK4a} noted in specific cell types of most mammalian tissues.

In accord with these observations, we have recently shown that expression of p16^{INK4a} in peripheral blood T lymphocytes (PBTL) has the properties of a faithful biomarker of chronological age in two independent, unselected, and prospectively collected cohorts of healthy human subjects (24). Importantly, expression of p16^{INK4a} increases exponentially early in life, well before other overt stigmata of aging are evident. Expression of p16^{INK4a} also correlated with plasma IL-6 level, a marker of aging and frailty. Likewise, p16^{INK4a} expression positively correlated with cigarette smoking and inversely correlated with exercise, both in a dosage-dependent manner. These data suggest a model whereby age-promoting stimuli induce p16^{INK4a} expression starting in young adulthood or earlier, and that frailty, tobacco use and physical inactivity are additionally associated with increased p16^{INK4a} expression.

Of interest, we also noted that expression of all 4 *INK4a/ARF*-associated transcripts (p16^{INK4a} as well as p15^{INK4b}, ARF, and ANRIL; reviewed in ref. 3) is strongly associated with a subject's genotype of a SNP (rs10757278) 120 kb centromeric to the *INK4/ARF* tumor suppressor locus (25). This SNP is highly polymorphic in individuals of Asian and European ancestry, with minor allele frequencies approaching 50% in these populations. Interest in this SNP came from its strong association with human atherosclerotic diseases [coronary artery disease (26–30), ischemic stroke (31, 32) and abdominal aortic aneurysm (31)] in large, unbiased genome-wide association studies. Although individuals with SNP genotypes associated with increased risk of atherosclerotic disease also exhibit decreased expression of *INK4/ARF*-associated transcripts, the mechanism whereby altered expression of the *INK4/ARF* locus might contribute to vascular disease is unknown.

Against this background, we sought to develop a predictive mathematical model of p16^{INK4a} expression with aging in heterogeneous human populations. Using straightforward assumptions based on the initial dataset and the known biology of human aging, we developed two types of stochastic models relating p16^{INK4a} expression to subject age and other clinical features. Both models assume that the rate of increase in

Author contributions: D.T., N.E.S., and T.C.E. designed research; D.T., Y.L., and H.K.S. performed research; D.T. analyzed data; and D.T., Y.L., N.E.S., and T.C.E. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0904405106/DCSupplemental.

$p16^{INK4a}$ expression with age is influenced by expression of $p16^{INK4a}$ itself, in accord with the observed exponential growth of $p16^{INK4a}$ with chronologic age. Additionally, to capture the observed saturation of $p16^{INK4a}$ expression with age, we investigated two mechanisms that would attenuate the rate of $p16^{INK4a}$ expression increase with aging. In model I (the “down-regulation” model), we assume $p16^{INK4a}$ expression can stochastically decrease at a rate that depends on $p16^{INK4a}$ expression levels. In model II (the “attrition” model), we assume that cells or subjects are stochastically removed from the study sample at a rate that has $p16^{INK4a}$ -dependent and independent components. The attrition model would encompass the possibility of selective loss from the cohort of either cells (e.g., by apoptosis or immune clearance) or individual donors (e.g., to death or comorbid illness). In general, it is possible that mechanisms reflected by both models could contribute to the saturation of $p16^{INK4a}$ expression with age. However, splitting down-regulation and attrition into distinct classes of models allows us to determine which mechanism is necessary to capture the observed behavior and minimizes the number of free model parameters that need to be estimated from the data.

Our work shows that the attrition model (model II) is more consistent with the observed data. Surprisingly, model II also suggests that whereas cigarette use is associated with increased $p16^{INK4a}$ expression, this behavior produces a more marked effect on the rate of $p16^{INK4a}$ -dependent attrition rather than on the rate of $p16^{INK4a}$ increase with aging. This modeling of the observed expression of $p16^{INK4a}$ with chronologic age in a heterogeneous human population supports the view that PBTL $p16^{INK4a}$ expression monotonically increases with chronologic age and is positively associated with the rate of age-related cellular or donor attrition due to cell clearance and/or donor death or illness.

Methods

Subjects. The study design and subject characteristics are described elsewhere (24, 25). All participants in this study were recruited from a central site on the campus of the University of North Carolina Hospitals. For inclusion, participants had to state they were in good health, had to be able to perform routine physical activities, and had to be able to provide informed written consent. Participants answered questionnaires regarding overall health, tobacco use and exercise; and were compensated for participation. Peripheral blood T-lymphocytes (PBTL) were harvested and processed as described (24). Potential acute and chronic conditions and their possible effect on $p16^{INK4a}$ expression were controlled by excluding subjects demonstrating peripheral T cell activation as described (24). All human studies were approved by the University of North Carolina Institutional Review Board.

Mathematical Models. The collected data (24, 25) demonstrate that $p16^{INK4a}$ expression increases with age and saturates at older ages. These data also show a considerable amount of variability in expression levels of different individuals at the same age. Therefore, we chose to model the dynamics of $p16^{INK4a}$ expression as a stochastic process (Fig. 1). Let the random variable $N(t)$ denote the transcript level of an individual at time t . We assume that $N(t)$ changes by a unit amount randomly in time, and let $p_k(t) = \text{Prob}[N(t) = k]$, where k is nonnegative integer. At birth ($t = 0$) each individual has an initial $p16^{INK4a}$ level $N(0) \equiv n_0$, where n_0 also is a random number with mean μ_0 and a standard deviation σ_0 .

We investigated two models for how $p16^{INK4a}$ levels could change with age. In both scenarios, increases in $p16^{INK4a}$ expression occur at a rate $u_k = uk + u_0$. That is, $p16^{INK4a}$ positively affects its rate of expression. If u is zero, the average expression level grows linearly in time. Positive values of u lead to exponential growth of $p16^{INK4a}$. In model I, we include transitions that decrease the $p16^{INK4a}$ level by one unit. These transitions occur at a rate $w_k = wk + w_0$. The average transcript level for model I is given by (see *SI Appendix*)

$$\langle \bar{N}(t) \rangle = \mu_0 e^{-(w-u)t} + \frac{u_0 - w_0}{w - u} [1 - e^{-(w-u)t}]. \quad [1]$$

The linear dependence of u_k and w_k on k is not a necessary condition but represents the simplest example of a more general class of down-regulation

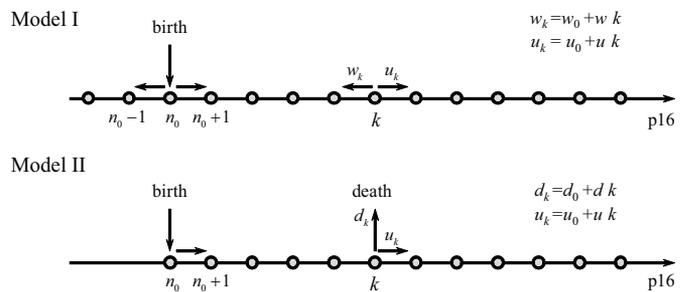


Fig. 1. Schematic diagram for the models of stochastic $p16^{INK4a}$ expression. The gray dots represent $p16^{INK4a}$ expression levels. In both models, the $p16^{INK4a}$ level increases at a rate u_k . Model I includes transitions that decrease the $p16^{INK4a}$ level. These backward transitions occur at a rate w_k . In contrast, model II includes transitions that terminate the stochastic process. These transitions occur at a rate d_k .

models in which saturation emerges due to the balance of production and degradation at a given level of $p16^{INK4a}$. We show in *SI Appendix* that the main conclusions from model I remain valid for models in which the rates u_k and w_k depend nonlinearly on k . It also should be noted that model parameters, such as the rate constants for $p16^{INK4a}$ production and degradation, should be interpreted as effective rates that capture the relative time scales for these complex processes and are not meant to represent a single biochemical step.

In model II, expression levels of $p16^{INK4a}$ saturate because individuals or cells expressing high levels of the gene have a higher probability of being removed from the sample pool (e.g., by cell clearance, illness or death). The recruiting criteria excluded individuals with acute illness or chronic comorbidity and subjects exhibiting peripheral T cell activation were excluded. This, in addition to natural factors, such as death, may account for the saturation of $p16^{INK4a}$ expression observed at older ages. Alternatively attrition might occur at the cellular level through immune clearance or apoptosis. Mathematically, both mechanisms are equivalent and removal from the sample population means that at any time there is finite probability of the stochastic process terminating. To model this situation we define the “death rate” as $d_k = dk + d_0(t)$, where the term dk accounts for the dependency of the death rate on $p16^{INK4a}$, whereas the second term, $d_0(t)$, is an arbitrary function of age and includes all other factors that contribute to an individual or cell being removed from the sample pool (e.g., chronic illness or death from $p16^{INK4a}$ -independent causes).

Within model II the mean level of gene expression as a function of time is given by

$$\langle \bar{N}(t) \rangle \equiv \frac{1}{1 - g(t)} \left[\mu_0 + g(t) \frac{u_0}{u} \right] \quad [2]$$

and the standard deviation is

$$\sigma(t) \equiv \frac{1}{1 - g(t)} \sqrt{\sigma_0^2 + g(t) \left(\mu_0 + \frac{u_0}{u} \right)}, \quad [3]$$

where

$$g(t) = \frac{u}{u + d} [1 - e^{-(u+d)t}]. \quad [4]$$

The details of the derivations of these expressions and full analyses of the models are given in *SI Appendix* and *Figs. S1–S12*.

Results

As an initial test of both models, we first fit all of the data simultaneously (Fig. 2). To better establish trends in the data we computed a moving average (blue lines, Fig. 2). The moving average clearly demonstrates the tendency of $p16^{INK4a}$ levels to saturate with age. The green lines in Fig. 2 are the best results for model I and the red lines are for model II. Thick lines represent the mean behavior and thin dashed lines represent standard deviation from the mean. From Eq. 1, we see that saturation of $p16^{INK4a}$ expression levels occurs in model I only if $u < w$ and $u_0 > w_0$. When these conditions are satisfied, Eq. 1

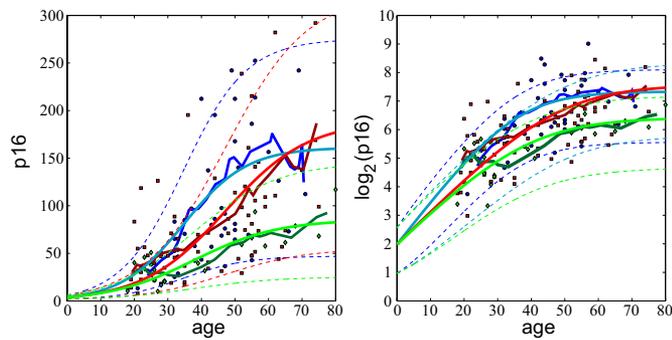


Fig. 5. Model II results for different genotypes (A/A, blue; A/G, red; G/G, green). In this figure, the initial mean expression level is fixed at $\mu_0 = 4$ and the free parameters u (growth rate) and d (death rate) are fit to the mean expression level. The parameters are $u = 0.10/\text{year}$ and $d = 0.0026/\text{year}$ for A/A, $u = 0.08/\text{year}$ and $d = 0.0017/\text{year}$ for A/G, and $u = 0.08/\text{year}$ and $d = 0.0037/\text{year}$ for G/G (see also parameters for Fig. S1). (Left) The data plotted on a linear scale. (Right) The same data plotted on \log_2 scale. The standard deviations are plotted according to Eq. 3 with $\sigma_0 = \sqrt{\mu_0} = 2$.

for A/A, 20 for A/G, and 13 for G/G. Although the precise values of the other model parameters depend on the initial transcript level, the qualitative relationship between genotype is always similar. The genotypes A/G and G/G have identical expression rates, whereas the death rate is twice as large for G/G as compared with A/G. In contrast, individuals with the A/A genotype have higher $p16^{\text{INK4a}}$ -dependent expression rates. Overall, these findings suggest that, unlike smoking, genotype affects the level of $p16^{\text{INK4a}}$ expression both through differences in the expression rates and death rates.

Finally, we considered the data grouped according to physical activity. Two groups were formed: people who reported exercising for at least 240 min (median value) per month and those who exercised less than this amount. With this grouping, the running averages clearly show that people who exercise have lower expression levels of $p16^{\text{INK4a}}$ (Fig. 6). This difference is more pronounced for younger individuals and almost completely disappears by the age of 65. This behavior is not captured by the model, unless it is assumed that initial $p16^{\text{INK4a}}$ levels are significantly different for the two groups (Fig. 6), an assumption that is hard to justify on any biological grounds. A possible explanation for the observed behavior is that the effects of exercising are not uniform over an individual's life span, so that $p16^{\text{INK4a}}$ levels in younger people are more sensitive to physical activity than in later years. Mathematically, to capture this effect

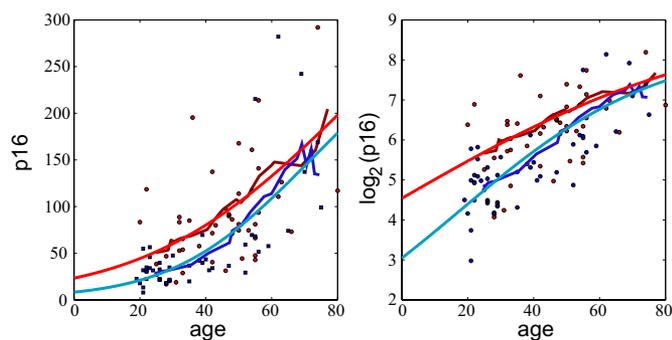


Fig. 6. Model II results for people who reported exercising >240 min per month (blue dots and curves) and <240 min per month (red dots and curves). The mean initial expression level μ_0 is taken to be a free parameter for each group. The estimate values of the parameters are $u = 0.021$, $d = 0.012$, $\mu_0 = 8$ and $u = 0.010$, $d = 0.010$, $\mu_0 = 23$, respectively. (Left) The data plotted on a linear scale. (Right) The same data plotted on \log_2 scale.

would require introducing a time dependence of the $p16^{\text{INK4a}}$ -dependent rates u and d in our model. Additionally, unlike smoking, exercise habits might change significantly over the life span of an individual making it difficult to discern long term effects of exercise on $p16^{\text{INK4a}}$ levels. Thus, grouping people based on their current exercise regime may not be a reliable representation of how sustained physical activity affects $p16^{\text{INK4a}}$ levels. Further complications also arise from possible correlations between exercise and other characteristics, such as smoking habits or gender. Indeed, males and females might respond very differently to the same average amount of exercise. Similarly, the intensity, not just the duration, of the physical activity may be important in determining $p16^{\text{INK4a}}$ levels.

Conclusions

The expression of $p16^{\text{INK4a}}$ in humans monotonically increases with age such that early in life there is exponential growth in the expression level that eventually saturates with age. We considered two stochastic models that capture this saturation effect. In model I saturation results from a balance between synthesis and degradation of the $p16^{\text{INK4a}}$ mRNA, whereas in model II saturation results from a $p16^{\text{INK4a}}$ -dependent attrition or “death” rate. Our analysis revealed that only model II accurately reproduces both changes in time-dependent mean expression level and variations about the mean.

The main parameters of model II are the $p16^{\text{INK4a}}$ -dependent production rate ($u_k = u_0 + uk$) and death rate ($d_k = d_0(t) + dk$). An interesting feature of this model is that the $p16^{\text{INK4a}}$ -independent part of the death rate, $d_0(t)$, which can vary arbitrarily in time, does not contribute to the mean expression level of $p16^{\text{INK4a}}$ or fluctuations about the mean. Therefore, the saturation of the $p16^{\text{INK4a}}$ expression level is attributed only to the $p16^{\text{INK4a}}$ -dependent part of the death rate. Note that a higher value of the parameter d in a certain subgroup of people (e.g., smokers) does not necessarily mean that these individuals have a higher probability of death, because the contribution $d_0(t)$ can be significantly different between subgroups.

It is important to note that the attrition model is robust to loss of entire individuals from the cohort and/or only cells within a given individual. As described, $p16^{\text{INK4a}}$ expression and genotype have been linked to frailty, frailty markers and specific diseases (e.g., diabetes) through human and murine genetic approaches. Therefore, we favor the model that increased $p16^{\text{INK4a}}$ expression leads to an increase in the attrition of individuals to death and comorbid illness which are strongly associated with frailty and diabetes. It is also possible, however, that there is selective attrition of cells rather than individuals with the highest levels of $p16^{\text{INK4a}}$. The model of cellular attrition is at odds with the findings that hypoproliferative $p16^{\text{INK4a}}$ -expressing cells are highly stable in vitro and in vivo. For example, cultures of senescent fibroblasts that highly express $p16^{\text{INK4a}}$ have been maintained in vitro for years without loss, and senescent lesions such as cutaneous nevi (moles), which highly express $p16^{\text{INK4a}}$ (33, 34), may persist for years or decades in humans. Nonetheless, the long-term fate of old, damaged lymphocytes that highly express $p16^{\text{INK4a}}$ is not known, and our model is not inconsistent with the possibility of enhanced clearance of PBTL which highly express $p16^{\text{INK4a}}$. In particular, attrition of cells highly expressing $p16^{\text{INK4a}}$ could explain the observed paradoxical reduction in $p16^{\text{INK4a}}$ -dependent death rate associated with tobacco use as tobacco mutagens could induce aberrant proliferation without $p16^{\text{INK4a}}$ expression, as in neoplasia, or otherwise reduce the clearance of $p16^{\text{INK4a}}$ -expressing cells.

Estimating the model parameters u and d from experimental data provides a method for quantifying differences among sample populations grouped by gender, genotype, smoking habit or physical activity. Using moving averages to establish trends in the data, we observed that gender does not affect $p16^{\text{INK4a}}$ expression levels, whereas smoking, rs10757278 genotype or

physical activity produced clear differences. Our analysis revealed that the higher level of $p16^{INK4a}$ expression observed for smokers relative to non-smokers results from a roughly two-fold smaller value of the $p16^{INK4a}$ dependent death rate d with no detectable change in the production rate u . These two parameters represent rates per unit transcript level. Thus, the mean $p16^{INK4a}$ -dependent parts of the production and death rates are $\langle u \rangle \equiv u\langle N(t) \rangle$ and $\langle d \rangle \equiv d\langle N(t) \rangle$, respectively. For older ages, we find that $\langle u \rangle_s \approx 2\langle u \rangle_n$ and $\langle d \rangle_s \approx \langle d \rangle_n$, where subscripts s and n represent smokers and non-smokers, respectively. A possible interpretation of this observation is that the elevated expression of $p16^{INK4a}$ in smokers plays some beneficial role in terms of $p16^{INK4a}$ -dependent attrition. That is, increased $p16^{INK4a}$ expression in response to the negative effects of tobacco seems to be associated with a reduced rate of attrition (e.g., by reducing patient loss to cancer, atherosclerosis, etc) for any given level of $p16^{INK4a}$ expression. In accord with this view, $p16^{INK4a}$ has a well-documented anti-cancer role in smoking-associated malignancies, and we have recently provided evidence that $p16^{INK4a}$ may play an important role in limiting human atherosclerosis (25). Again, however, it is important to stress that $p16^{INK4a}$ -independent increases in the death rate due to smoking are expected to greatly exceed any “positive” effect of the smoking on the $p16^{INK4a}$ -dependent death rate.

Analyzing the data grouped by genotype, we conclude that A/G and G/G genotypes have qualitatively similar $p16^{INK4a}$ age dependence with identical production rates u but different death rates d . The A/A genotype is significantly different from both A/G and G/G due mainly to a differences in the production rate u . The association of the rs10757278 genotype with *INK4a/ARF* transcript expression is most parsimoniously explained by the existence of a transcriptional *cis*-regulatory element in linkage disequilibrium with this SNP. One would predict that the effects of such an enhancer or repressor of transcription would primarily control the $p16^{INK4a}$ production rate, u . Alternatively, however, because SNP genotype is also associated with the expression of two other potent cell cycle regulators, $p15^{INK4b}$ and *ARF*, it is possible that these effects could influence the $p16^{INK4a}$ -dependent death rate d . For example, Serrano and colleagues have suggested that *ARF* expression exerts a general anti-aging

effect in mammals (35). Because expression of *ARF* and $p16^{INK4a}$ strongly cocorrelate in human PBTL (24), our results would be consistent with such an anti-aging effect of *ARF* leading to an apparent decrease in the $p16^{INK4a}$ -dependent death rate.

We were not able to obtain satisfactory fits to the data when the sample population was grouped according to exercise routine. Many factors may contribute to the failure of the model for this case. For example, the population was divided into two groups: individuals who exercise >240 min per month and those that exercised less. No consideration was given to how long the exercise regime had been followed, the type of exercise or the intensity of the training or whether exercise behavior may change with chronologic aging. Interpretation of these data also may be confounded by correlations with other factors, such as gender or race. We believe a more complex model that allows for an effect of exercise on $p16^{INK4a}$ production that decreases with advancing chronologic age would capture the observed data, and would be biologically rational.

In model II the saturation of $p16^{INK4a}$ levels occurs because of a $p16^{INK4a}$ -dependent attrition rate. However, the model does not definitively establish a mechanism for this effect. A clearer interpretation of the attrition rate and more quantitative analysis of its dependence on $p16^{INK4a}$ levels will be the subject of future work using an expanded dataset we are presently generating. Despite its relative simplicity, our current model quantitatively captures the mean behavior and variability of data for $p16^{INK4a}$ expression as a function age. Because the model contains a minimal number of parameters, differences among subgroups of the sample population are readily interpreted and the effects of genetic and environmental factors easily quantified. We believe the work presented here clearly illustrates the power of using mathematical modeling to investigate the role of $p16^{INK4a}$ expression in aging and points to clear avenues for future research.

ACKNOWLEDGMENTS. This work was supported by National Institutes of Health (NIH) Grants R01GM079271 (to T.C.E. and D.T.), P01ES014635 (to T.C.E.), RR023248 (to H.K.S.), and AG024379 (to N.E.S.); the Ellison Medical Foundation (to N.E.S.); the Burroughs Wellcome Fund (to N.E.S.); and NIH Training Grant CA009156-34 (to Y.L.).

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