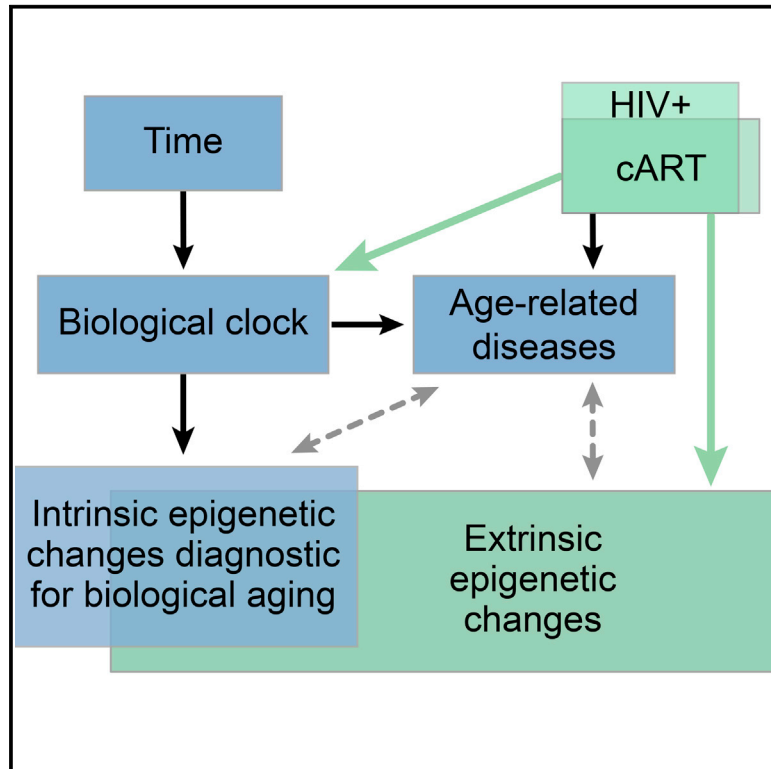# Molecular Cell

Methylome-wide Analysis of Chronic HIV Infection Reveals Five-Year Increase in Biological Age and Epigenetic Targeting of HLA

Graphical Abstract

Authors

Andrew M. Gross, Philipp A. Jaeger, Jason F. Kreisberg, ..., Kang Zhang, Howard S. Fox, Trey Ideker

Correspondence

hfox@unmc.edu (H.S.F.), tideker@ucsd.edu (T.I.)

In Brief

Gross et al. investigate the impact of chronic HIV infection by profiling the DNA methylomes of HIV+ individuals and matched HIV− controls. Using epigenetic models of aging, they observe that HIV+ individuals show an age advancement of 4.9 years in whole blood and validate these results in pure cell samples.

Highlights

- Methylome-wide analysis of HIV chronically infected, cART treated individuals

- HIV+ individuals have an epigenetic age 4.9 years older than healthy controls

- HLA locus is hypomethylated in HIV+ individuals

- HIV methylation aging signature is validated in purified cells

Accession Numbers

GSE67705

CellPress

Methylome-wide Analysis of Chronic HIV Infection Reveals Five-Year Increase in Biological Age and Epigenetic Targeting of HLA

Andrew M. Gross,[1,2] Philipp A. Jaeger,[2] Jason F. Kreisberg,[2] Katherine Licon,[2] Kristen L. Jepsen,[3]
Mahdieh Khosroheidari,[3] Brenda M. Morsey,[4] Susan Swindells,[10] Hui Shen,[5] Cherie T. Ng,[9] Ken Flagg,[3,6,11] Daniel Chen,[3,6]
Kang Zhang,[3,6,7,8] Howard S. Fox,[4,*] and Trey Ideker[1,2,3,8,*]
[1]Bioinformatics and Systems Biology Program
[2]Department of Medicine
[3]Institute for Genomic Medicine
University of California, San Diego, La Jolla, CA 92093, USA
[4]Department of Pharmacology and Experimental Neuroscience, University of Nebraska Medical Center, Omaha, NE 68198, USA
[5]Van Andel Research Institute, Grand Rapids, MI 49503, USA
[6]Department of Ophthalmology and Biomaterials and Tissue Engineering Center, Institute for Engineering in Medicine, University of California,
San Diego, La Jolla, CA 92093, USA
[7]Veterans Administration Healthcare System, San Diego, CA 92093, USA
[8]Moores Cancer Center, University of California, San Diego, La Jolla, CA 92093, USA
[9]aTyr Pharmaceuticals, San Diego, CA 92121, USA
[10]Department of Internal Medicine, University of Nebraska Medical Center, Omaha, NE 68198, USA
[11]Guangzhou Kang Rui Biological Pharmaceutical Technology Company Ltd., Guangzhou 510005, China
*Correspondence: hfox@unmc.edu (H.S.F.), tideker@ucsd.edu (T.I.)
http://dx.doi.org/10.1016/j.molcel.2016.03.019
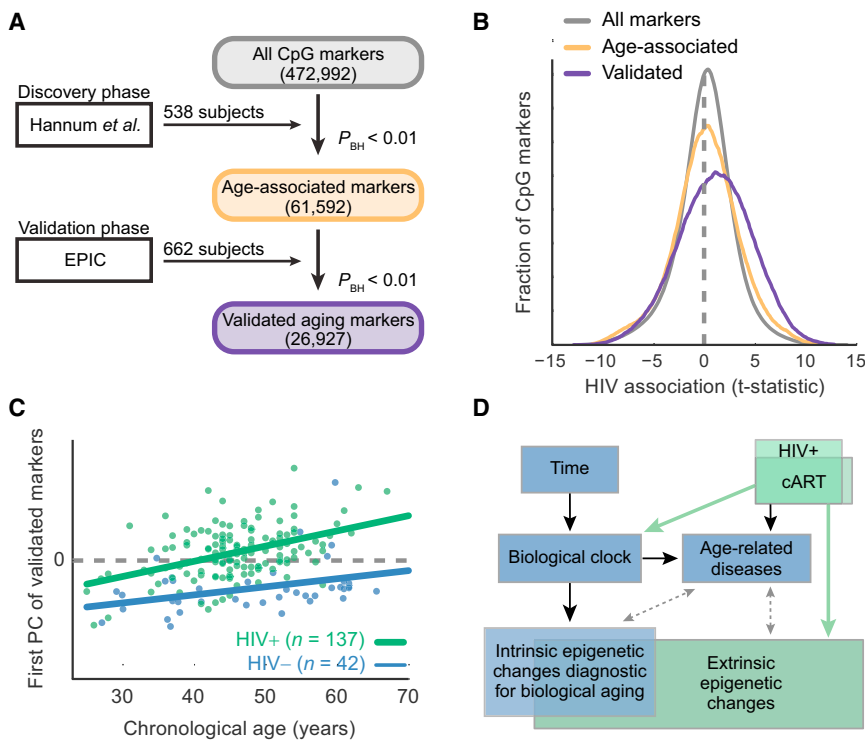
SUMMARY

HIV-infected individuals are living longer on antiretroviral therapy, but many patients display signs that in some ways resemble premature aging. To investigate and quantify the impact of chronic HIV infection on aging, we report a global analysis of the whole-blood DNA methylomes of 137 HIV+ individuals under sustained therapy along with 44 matched HIV− individuals. First, we develop and validate epigenetic models of aging that are independent of blood cell composition. Using these models, we find that both chronic and recent HIV infection lead to an average aging advancement of 4.9 years, increasing expected mortality risk by 19%. In addition, sustained infection results in global deregulation of the methylome across >80,000 CpGs and specific hypomethylation of the region encoding the human leukocyte antigen locus (HLA). We find that decreased HLA methylation is predictive of lower CD4 / CD8 T cell ratio, linking molecular aging, epigenetic regulation, and disease progression.

INTRODUCTION

It is an open question why some people show early or delayed onset of aging-associated disorders (Kennedy et al., 2014). Recent studies have found that aging is associated with epigenetic changes (Christensen et al., 2009; Day et al., 2013; Heyn et al., 2012; Numata et al., 2012; West et al., 2013), and based on this work we (Hannum et al., 2012) and others (Horvath,

2013; Weidner et al., 2014) have built models capable of predicting a person's age using DNA methylation patterns across a large number of CpG sites. Although these models are fairly accurate, errors of prediction—differences between the chronological and predicted age—serve as a quantitative readout of the relative advancement or retardation of the "biological age" of an individual. Biological age advancement has been correlated with factors such as gender, genetic polymorphisms, and diseases including cancer and diabetes, and it may influence the onset of other age-associated disorders (Day et al., 2013; Hannum et al., 2012). A recent longitudinal study validated the clinical utility of these models by demonstrating a link between biological age advancement and increased mortality rates (Marioni et al., 2015).

Biological aging has become of particular interest in treatment of HIV, in which the development of combination Anti-Retroviral Therapy (cART) now enables infected individuals to live many decades (Deeks, 2011; Deeks et al., 2013; Maartens et al., 2014). Several studies have suggested links between chronic HIV infection and early onset of neurodegeneration (Nightingale et al., 2014), liver or kidney failure (Joshi et al., 2011; Kovari et al., 2013), cancer (Dubrow et al., 2012), cardiovascular disease (Freiberg et al., 2013), and telomere shortening (Leeansyah et al., 2013; Pathai et al., 2013), leading to the hypothesis that HIV+ patients might experience advanced or accelerated aging (Appay and Rowland-Jones, 2002; Guaraldi et al., 2011; Smith et al., 2012). While these studies report rough estimates of HIV-mediated age advancement in the range of 0–20 years, it has been difficult to accurately quantify this number due to sampling effects, co-morbidities, and relatively low incidence rates of any single age-associated disease. To this effect, the existence, extent, and molecular basis of a bona-fide increase in aging have been unclear (Althoff et al., 2014; Solomon et al., 2014), in part due to lack of an objective biological clock or aging biomarker.

Figure 1. Shared Epigenetic Signature of HIV Infection and Aging

(A) Discovery and validation of CpG methylation markers associated with age.

(B) Distribution of t-statistics measuring association of each methylation marker with HIV status. Colors indicate groups of markers identified in (A): Gray, all markers; yellow, age-associated markers from discovery phase; violet, subset of age-associated markers confirmed in validation.

(C) Principal component (PC) analysis of the validated age-associated markers, in which the first PC (y axis) is positively associated with both age (x axis) and disease status (HIV+, green; HIV−, blue).

(D) Potential relationships among HIV infection, epigenetics, disease, and aging. Black: known; Dashed gray: potential; Green: connections explored in this study. See also Tables S2 and S3.

RESULTS

Genome-wide DNA Methylation Profiling

To determine whether HIV is associated with signs of aberrant biological aging, samples of whole-blood DNA were obtained from 137 HIV-infected, cART-treated but otherwise healthy non-Hispanic white males (no hepatitis C co-infection, no diabetes, and high adherence to therapy) and 44 healthy non-Hispanic white male controls (Table S1; Figure S1). Genome-wide methylation profiles of each sample were determined using the Illumina Infinium HumanMethylation450 BeadChip array. Data were normalized and controlled for quality using standard techniques, resulting in removal of two control patients due to poor signal (Experimental Procedures).
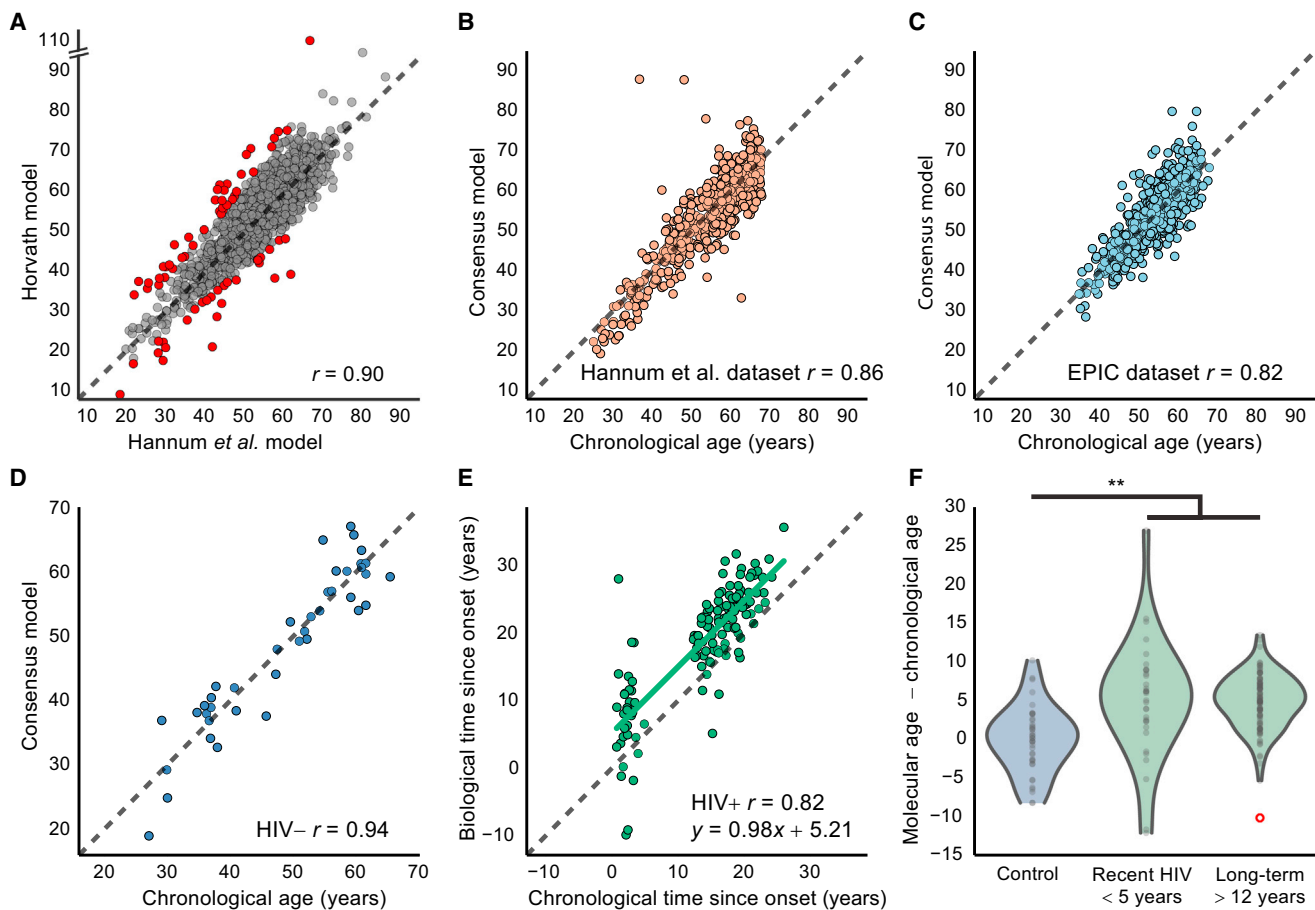
Unsupervised Analysis Shows Shared Phenotypes of HIV and Age

As a preliminary exploration of this dataset, we ran an unsupervised analysis to identify age-associated methylation sites and their relation to HIV infection. Analysis of a previous methylome-wide screen of 538 healthy subjects (Hannum et al., 2012) identified as many as 61,592 methylation sites associated with age at a 1% false-discovery rate (FDR) (likelihood ratio test in multivariate regression model with Benjamini-Hochberg correction). Validation of these sites in whole blood from a second control cohort from the European Prospective Investigation into Cancer and Nutrition (Riboli et al., 2002) (EPIC, N = 662) confirmed 26,927 of these sites as strongly associated with age (Figure 1A; Table S2).

Among these validated age-associated sites, we found a striking association with methylation in the HIV+ patients relative to healthy controls (p < 10^{-100}, Figure 1B). Further analysis of these sites found a positive association of the first principal component with both age and HIV status (Figure 1C; Table S3, association by multivariate linear model p < 10^{-8}). These findings support a link between HIV infection and aging (Rickabaugh et al., 2015), as quantitatively measured by epigenomic profiling (Figure 1D).

In parallel with such epidemiological observations, a number of studies report age effects using blood-based biomarkers. Analysis of cell surface markers in T cells has shown HIV+ subjects to show phenotypes of older cells (Cao et al., 2009). Other studies have observed shortened telomeres in certain cell populations (Rickabaugh et al., 2011) as well as whole blood (Zanet et al., 2014), indirectly linking HIV to aging via the well-studied connection between telomere length and age (Lindsey et al., 1991; Cawthon et al., 2003). Furthermore, a recent analysis of untreated HIV+ individuals found DNA methylation sites that are associated with both HIV infection and age (Rickabaugh et al., 2015). Together, these results raise the possibility that HIV infection results in an increase in biological age. Many questions remain, however: Are the epigenetic changes associated with HIV the same as those previously identified (Hannum et al., 2012; Horvath, 2013) in normal individuals as markers of "biological age," and how complete is the correspondence between these two responses? What is the quantitative effect on aging in years, and is it fixed age advancement or continuous acceleration? What is the impact on aging of chronic HIV infection and sustained cART treatment? Are there other impacts of HIV on the methylome that are unrelated to aging?

Here we begin to address these questions by analyzing the methylomes of HIV-infected, cART-treated subjects, in which we observe a strong shared phenotype of HIV and age. To understand this signal, we develop models of biological age that allow us to establish a clear quantitative link between HIV infection and aging as observed in the general population. We identify both global and targeted epigenomic effects of HIV, including specific hypomethylation of the HLA locus. Together, these results shed light on the epigenetic consequences and/or gerontological aspects of chronic HIV infection.

Figure 2. Epigenetic Models Accurately Predict Age and Indicate Advanced Aging for HIV-Infected Individuals

(A) Scatter plot comparing the ages predicted using the Hannum et al. and Horvath models on healthy controls (n = 1,242 from HIV−, Hannum et al. and EPIC datasets). Red points indicate patients that were discarded due to disagreement between the two aging models (n = 68).

(B and C) Accuracy of the consensus model (y axis) to predict true chronological age (x axis) in datasets from Hannum et al. (n = 497, [B]) or EPIC (n = 637, [C]).

(A–C) Patients between 25 and 68 years old.

(D) Scatter plot of predicted biological age (consensus aging model) versus chronological age for HIV− healthy controls (n = 40).

(E) Scatter plot of biological time versus chronological time since HIV onset for infected subjects (n = 134).

(F) Violin plots showing the distribution of residuals from regression of biological versus chronological age. Three groups are shown: HIV− controls, short-term HIV+ infected individuals, and long-term HIV+ infected individuals. Note that the red circle indicates an outlier, which is not used to fit the violin profile, but is used in all statistical assessments.

(A–E) Black dashed lines indicate diagonal (y = x). r, Pearson's correlation coefficient. ** indicates $p < 10^{-5}$. See also Table S4.

Benchmarking and Refinement of Epigenetic Aging Models

Given the shared effects of HIV and aging, we sought to determine whether HIV causes the same biological aging signature as previously found in cohorts of uninfected individuals (Hannum et al., 2012; Horvath, 2013; Marioni et al., 2015). We tested aging models from both our group (Hannum et al., 2012) and Horvath (Horvath, 2013) in independent datasets derived from whole blood samples (Hannum et al., 2012; Riboli et al., 2002; Table S4). Although the Hannum and Horvath modeling efforts were based on different methodologies and training data, we found they made very similar predictions (r = 0.9, Pearson's correlation, Figure 2A) and furthermore that a consensus of the two models outperformed either model individually (Figures 2B and 2C;

Table S4). For this reason, we used this consensus model for all remaining analyses.

A potential issue with these models arises in the fact that methylation profiles from whole blood are influenced by cell composition, and different cell types have different methylation states (Jaffe and Irizarry, 2014). These differences might be particularly pronounced in HIV-infected patients, some of whom have low CD4+ T cell counts (Trono et al., 2010). To understand the sensitivity of epigenetic aging models to cell type composition, we downloaded two datasets profiling sorted cells across shared sets of individuals (Absher et al., 2013, GEO: GSE59250; Reynolds et al., 2014, GEO: GSE56046). Among these sorted cell datasets, we saw good concordance of epigenetic age predictions with chronological age (Figures S2A–S2F).

Table 1. Multivariate Linear Models of Biological Age Based on Chronological Age, HIV, and Cellular Composition

Independent Variable		Effect	StdErr	t	P
Model 1. Dependent variable: Biological age					
–	HIV	3.76	1.14	3.3	0.003
–	Chronological age	−0.12	0.04	−3.0	0.001
Cell composition (%)	NK cell	0.21	0.08	2.6	0.011
Cell composition (%)	CD4 T cell	−0.07	0.07	−1.1	0.293
Cell composition (%)	CD8 T cell	0.13	0.06	0.2	0.812
Cell composition (%)	B cell	−0.17	0.13	−1.4	0.174
Cell composition (%)	Monocyte	−0.09	0.14	−0.6	0.521
Model 2. Dependent variable: Biological age					
–	Chronological age	−0.22	0.07	−3.1	0.002
Cell composition (%)	NK cell	0.15	0.07	2.1	0.041
Cell composition (%)	CD4 T cell	−0.23	0.08	−2.9	0.004
Cell composition (%)	CD8 T cell	0.15	0.08	1.8	0.076
Cell composition (%)	B cell	−0.10	0.07	−1.3	0.190
Cell composition (%)	Monocyte	−0.05	0.07	−0.7	0.492
Model 3. Dependent variable: Model 2 residuals					
–	HIV	0.18	0.08	2.4	0.019

In Model 3, residuals from model 2 are carried over to a second regression against HIV status.

Epigenetic age was reproducible across different cell types profiled from the same patients, with high agreement of age estimates (r >0.77–0.88) and moderate but very significant agreement of age advancement (Pearson's r > 0.45–0.68; p < 0.0001 for all associations, Figures S2G–S2J).

While we therefore expect the contribution of cell composition to be minimal, we nonetheless developed an algorithm to individually normalize each methylation profile using methylation-derived cell type information. In brief, we used a previously reported method (Jaffe and Irizarry, 2014) to reliably predict blood composition (Figure S3) and adjust out the expected contribution of cell-type-specific effects. This procedure greatly limited the effects of age- and HIV-induced blood composition changes in downstream analyses (Experimental Procedures; Figure S4).

HIV+ Individuals Have Advanced DNA Methylation Age

We next used this consensus aging model to calculate the "biological age" of each individual in our cohort (Table S4). For uninfected controls, the calculated biological age had a very high concordance with chronological age (Figure 2D, Pearson's r = 0.94). In contrast, the HIV+ patients had a biological age advancement of 4.9 years on average (p < 10^{-8} by Student's t test, 95% confidence interval 3.4–7.1 years, Figures 2E and 2F). These results were consistent with our previous unsupervised analysis (Figures 1B and 1C) in suggesting that HIV infection leads to advanced aging. Furthermore, we found that the age advancement of HIV+ individuals was negatively correlated with the ratio of $CD4^+$ / $CD8^+$ T lymphocytes (Spearman's rho = −0.2, p < 0.02). $CD4^+$ T cells are a major indicator of immune

integrity (Leung et al., 2013; Serrano-Villar et al., 2014) and are inversely associated with morbidity and mortality, including from non-AIDS defining diseases (El-Sadr et al., 2006); similarly, the CD4/CD8 ratio predicts non-AIDS morbidity (Leung et al., 2013; Serrano-Villar et al., 2014). This finding links biological aging of HIV-infected individuals to a clinical measure of disease progression, and it raises the possibility that patients with stable immune responses may be less affected by the advanced aging phenotype. Taking into account a recently estimated 4.2% increase in mortality risk per year of biological age advancement using the Hannum model (Marioni et al., 2015), the changes observed in HIV+ patients result in an expected total mortality risk increase of 19%.
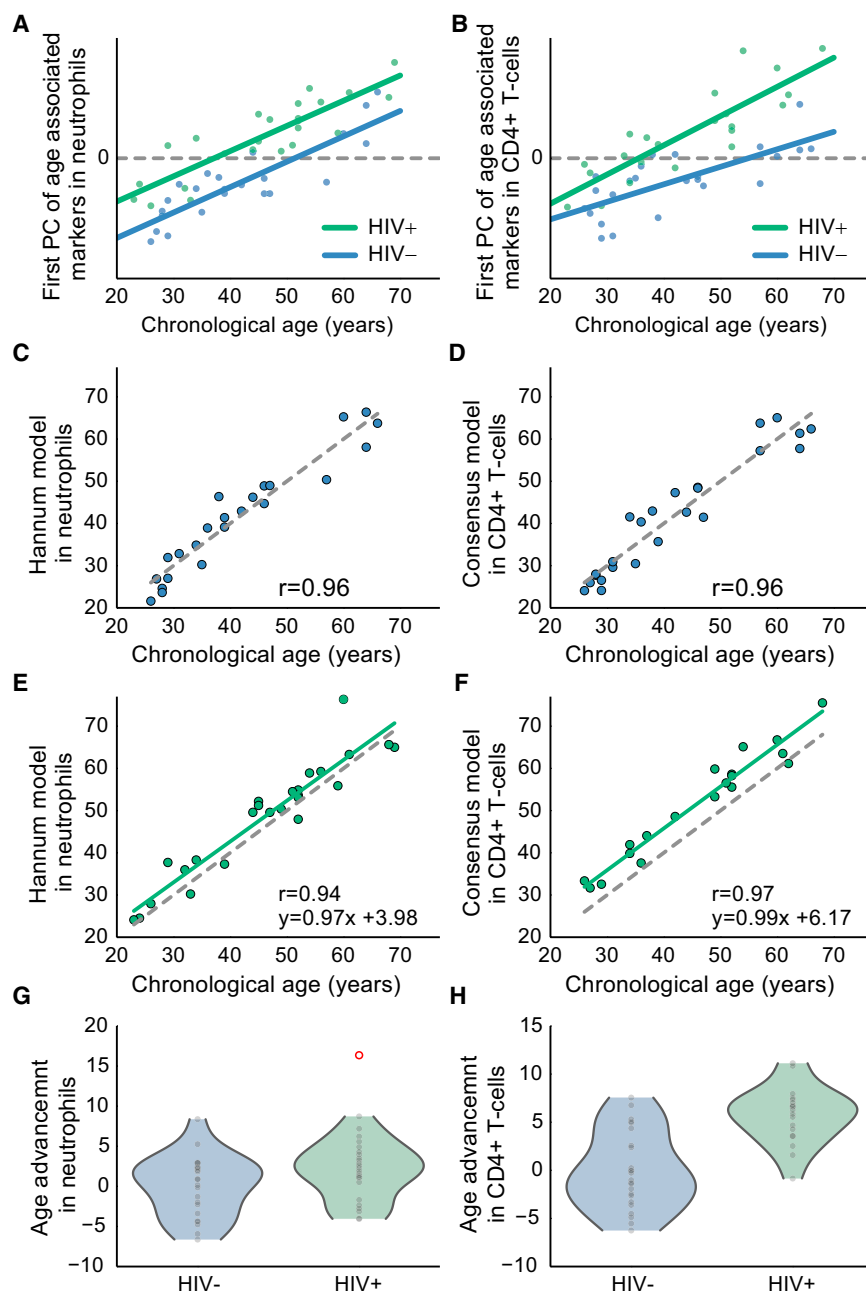
Age Advancement Is Independent of HIV Duration

Notably, patients more recently infected with HIV (<5 years) had no significant difference in age advancement from those patients with chronic (>12 years) infection (p > 0.5, Mann-Whitney U Test; Figure 2F). Similar findings emerged from a regression analysis of the chronological versus biological time since infection: the slope did not differ from one (0.98 ± 0.06, SE) whereas the y-intersect was significantly positive (5.2 ± 0.9; Figures 2E and 2F). These findings lend support to the theory that age advancement occurs early in the course of disease as a consequence of acute infection or reaction to drug treatment (Guaraldi et al., 2011; Smith et al., 2012). The lack of an increase of age advancement with disease duration seems to contradict alternative views that HIV-mediated aging occurs through cumulative effects of latent virus (Appay and Rowland-Jones, 2002) or chronic therapeutic intervention (Torres and Lewis, 2014). We did however observe less variation in age advancement within the chronically infected HIV+ individuals (Figure 2F, p < 0.002, Bartlett's test relative to recently infected group), perhaps reflecting the comparative stability of infection and immune response on long-term cART therapy (Luz et al., 2014; Rosenblatt et al., 2005).

Age Advancement Is Independent of Cellular Composition

While the direct effects of cell type composition on the whole-blood methylome were corrected by the adjustment described above (also see Experimental Procedures), we considered that it was still possible that changes in cell type composition could lead to downstream, indirect changes in the epigenomes of all blood cells. If this were the case, cell-type-associated changes could be responsible for the observed increase in biological age in the HIV+ cases. To assess this possibility, we constructed a multivariate linear model in which cell type composition variables and HIV status were used to predict biological age as measured by the methylome (Table 1). In this model, the presence of HIV was associated with an age advancement of 3.8 ± 1.1 years, while the presence of natural killer cells accounted for additional increases in biological age. In an even more conservative test, we modeled age advancement with cell type composition variables alone and found that the unexplained variation in this model still had a significant association with HIV infection (p = 0.02, Likelihood Ratio Test, Table 1). Thus, even in a very conservative analysis, HIV infection has association with advanced aging that is independent of cell composition.

Figure 3. Age Advancement in Validation Cohorts of Purified Cells

(A and B) Unsupervised principal component (PC) analysis of methylation patterns in purified blood cell types, in which the first PC is positively associated with both age (x axis) and disease status (HIV+, green; HIV−, blue).

(A) New CD4+ T cell cohort across 5,999 markers that are age-associated in CD4+ T cells (GEO: GSE59250).

(B) New neutrophil cohort across markers probes that are age-associated in neutrophils (GEO: GSE65097).

(C–F) Control ([C] and [D]) and HIV+ ([E] and [F]) subjects for sorted cell validation datasets comparing chronological age to the Hannum et al. epigenetic aging model in neutrophils ([C] and [E]) and consensus aging model in CD4+ T cells ([D] and [F]).

(G and H) Violin plots showing age advancement in the two sorted cell datasets. For (B), in initial analysis the first PC heavily reflected an outlier point, which was removed after which the PC was recalculated. See also Figure S2.
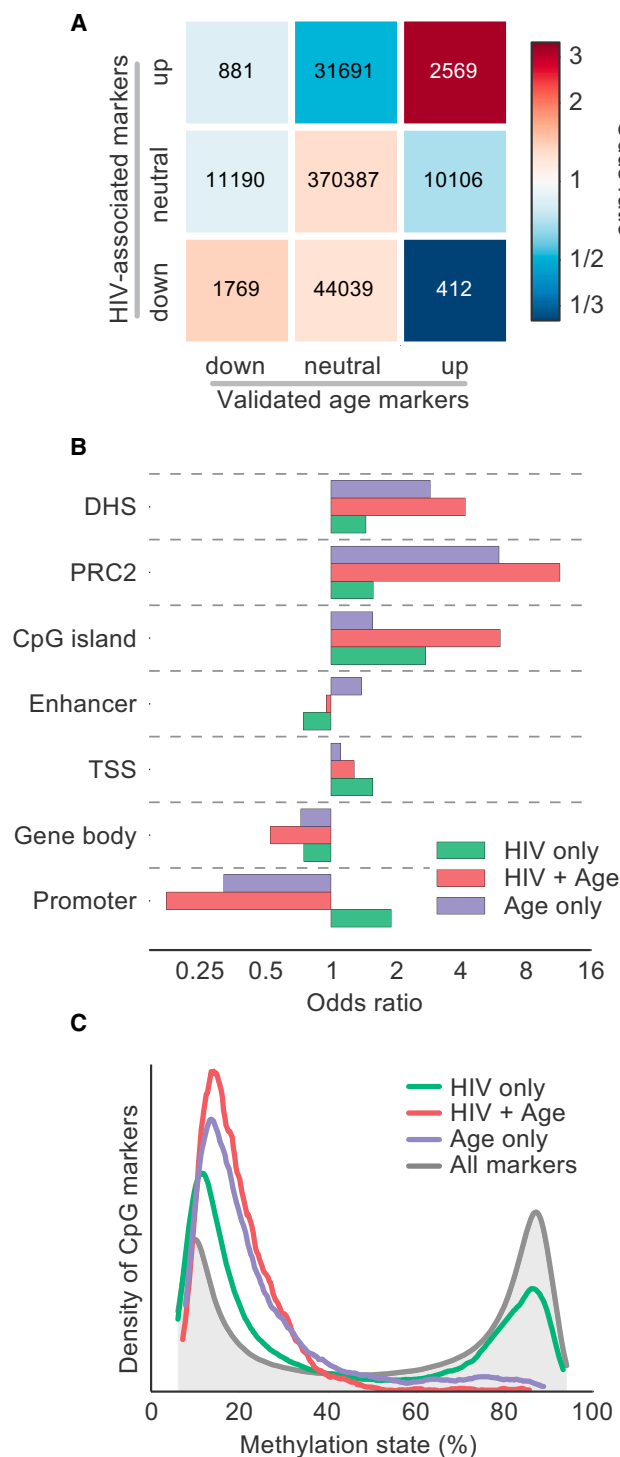
As in whole blood, unsupervised analysis showed a clear effect of HIV in age-associated methylation markers (Figures 3A and 3B). Application of epigenetic models of aging in these pure-cell datasets showed good concordance of predicted age with chronological age in both cell types (Figures 3C–3F). In neutrophils, the Hannum model predicted a 2.5 year increase in age due to HIV infection (p < 0.03, 95% CI 0.6–5.0 years, Figure 3E) whereas the Horvath model showed a smaller effect of 0.4 year (p > 0.05). In contrast, CD4+ T cells had a much stronger and more consistent HIV response in both models, with the consensus aging model showing an increase of 5.7 years in the HIV+ subjects (p < 10^{-5}, 95% CI 3.4–7.9 years, Figure 3F). These data indicate that the effect of epigenetic age advancement is not merely an artifact of changing blood composition, but likely reflects true aging signals. The stronger effect size within CD4+ T cells (Figures 3G and 3H) suggests that these cells may be exposed to more age-like stress than neutrophils, although further work is needed to understand how disease may affect aging rates across different cell types and tissues.

HIV and Aging Have Shared and Distinct Methylation Patterns

Having identified a large effect of HIV infection on age associated methylation signals, we then sought to better understand the wider changes instigated by HIV. We identified 81,361 CpG

We also sought to experimentally assess if the observed age advancement due to HIV infection was observed in purified cell populations. Using standard calculations of statistical power, we estimated that a sample of 48 patients, balanced approximately between cases and controls, would have 81% power to detect the same aging advancement effect as our primary screen at p < 0.01. Accordingly, this number of subjects was prospectively recruited from the University of Nebraska Medical Center under an approved IRB protocol (501-15-EP), and blood obtained following informed consent (Experimental Procedures; Table S5). Whole blood was separated immunomagnetically to isolate pure populations of neutrophils and CD4+ T cells.

Figure 4. HIV and Aging Have Shared and Distinct Methylation Patterns.

(A) Overlap table comparing the set of CpG markers associated with HIV and the set of validated age-associated markers (see Figure 1A). Numbers indicate probe counts in each overlap, colors correspond to odds ratio of overlap compared to background.

(B) Odds ratios of enrichment for a panel of genomic features, evaluated in sets of markers associated with age, HIV, or both. PRC2, polycomb repressive

markers associated with HIV infection (Benjamini-Hochberg corrected p < 0.01; likelihood ratio test using a multivariate linear model, Table S2). Of these, 2,569 upregulated markers and 1,769 downregulated markers were also associated with aging, a 3.2 and 1.4-fold enrichment over random expectation, respectively (Figure 4A, Fisher's Exact Test $p < 10^{-30}$, Table S6). We found that markers associated with both HIV and aging were enriched in DNase hypersensitivity sites and CpG islands, suggesting methylation changes in DNA regions under active regulation. These CpG markers were also enriched in binding sites for polycomb repressive complex (PRC2) (Figure 4B), a switch that tightly regulates genes required for differentiation and renewal, and in *Drosophilia* is linked to longevity (Siebold et al., 2010). These findings reinforce previous reports that PRC2 targets are irreversibly repressed by methylation during the aging process (Beerman et al., 2013; Deaton and Bird, 2011; Teschendorff et al., 2010). Interestingly, markers associated with HIV but not aging had a very different functional enrichment profile (Figure 4B), indicating an additional mechanism(s) for epigenetic alteration associated with HIV.
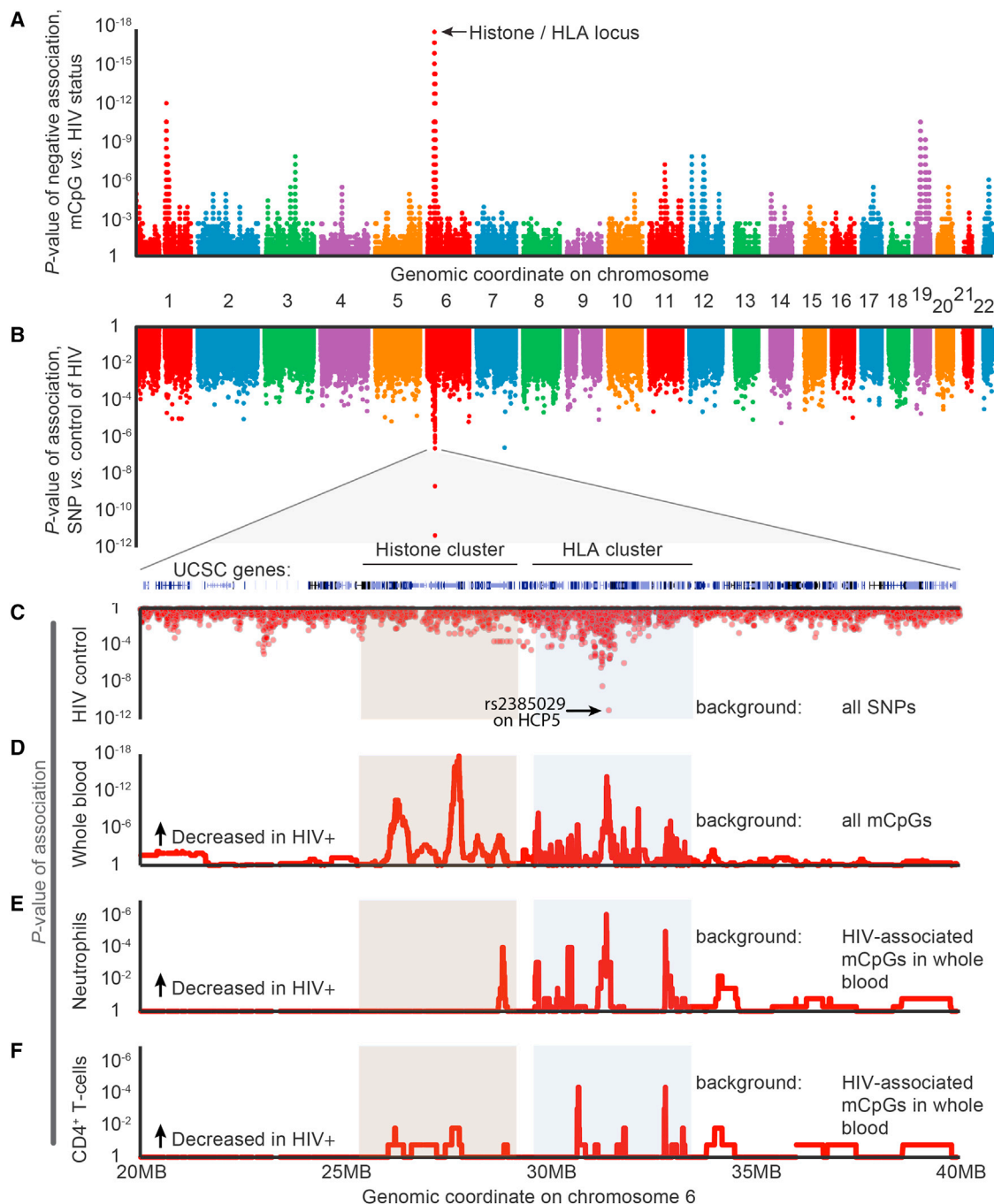
We have previously reported that age-associated markers in older subjects tend away from a fully methylated or unmethylated state and instead move toward disorder (with a methylation fraction of 50% representing complete disorder) (Hannum et al., 2012). We found that HIV-infected patients displayed a similar trait: among markers associated with HIV, 66% tended toward disorder, compared with 70% of age-associated markers (Experimental Procedures, Figure S5). Furthermore, whereas age-associated markers tended to have a low methylation fraction that increased with age, HIV-associated markers were more equally balanced between low and high methylation states (Figure 4C).

HIV Is Associated with Hypomethylation of the HLA Locus, Independent of Aging

Thus far, we had observed multiple effects of HIV on the methylome, including changes in cellular composition, age advancement, and a general increase in methylome disorder. We next sought to determine whether there are specific genomic regions for which the methylation state is particularly associated with HIV infection, independent of aging or other factors (Experimental Procedures). Toward this aim, we conducted an analysis of HIV-associated CpG markers independent of disorder or age, controlling for the effects of cellular composition. Analysis of the whole-blood data identified a single genomic region that was enriched in CpG markers associated with HIV; this region, consisting of 10 Mb on chromosome 6 including histone gene cluster 1 and the entire HLA locus, had particularly reduced methylation levels in HIV+ cases as compared to HIV− controls ($p < 10^{-10}$, Figure 5A; Experimental Procedures). HLA genes encode the Major Histocompatibility Complexes (MHCs), the key antigen-presenting molecules that govern the acquired immune response and impact innate immunity (Figure 5B) (Goulder

complex 2 binding sites; DHS, DNase hypersensitivity sites; TSS, transcription start sites.

(C) Distribution of methylation states for the CpG marker sets defined in (A). See also Tables S2 and S6.

Figure 5. Methylome Remodeling under Sustained HIV Infection Targets HLA

(A) Epigenome-wide association of CpG methylation (mCpG) with HIV status (presence or absence). Each point represents the P-value of enrichment for differentially methylated CpG within a bin of ± 100 consecutive markers along the genome.

(B and C) p values of genome-wide association of SNPs with host control of HIV, reproduced from Fellay et al. (2007).

(D) Epigenome-wide association of mCpG with HIV status (presence or absence), zoomed in to target histone/HLA locus.

(E and F) Validation screen of HIV-downregulated markers in purified populations of neutrophils (E) and CD4+ T cells (F). See also Table S2 and Figure S6.

and Walker, 2012). We found that the differentially methylated markers surround the rs2395029 variant, for which common genetic variation has been repeatedly implicated in HIV host control (Figures 5C and 5D) (Fellay et al., 2007; International HIV Control-lers Study et al., 2010). Examination of this locus in the validation samples of purified neutrophils and CD4+ T cells identified the *HCP5* gene body as particularly differentially methylated in neutrophils (Figures 5E, 5F, and S6). As further evidence that the

observed changes are functional, we found that the amount of methylation at this gene was correlated with a patient's CD4$^+$/CD8$^+$ T cell ratio (Figure S6). Taken together, these results indicate that the HLA locus is likely differentially methylated across blood cell types and also changes within individual cell types in response to HIV. An intriguing interpretation of our results is that some of the previously reported changes in HLA expression and corresponding HIV control (Apps et al., 2013) are attributable to methylation dynamics.

DISCUSSION

We have shown that methylome-wide changes previously ascribed to aging are also induced by HIV (Figures 1 and 3). By using highly accurate, externally trained and validated models of biological aging, our study provides a robust estimate of a 5-year age advancement in HIV/cART individuals (Figure 2). These results, in combination with the link between molecular age advancement and increased mortality risk (Marioni et al., 2015), support the idea that chronic HIV infection is accompanied by a tangible gerontological phenotype. In addition to an aggregate estimate of HIV age advancement, the methylation aging model allows for patient-by-patient estimates. Patients deemed more likely to suffer from HIV-mediated aging effects might be placed on alterative schedules for preventative care, including early screening and further testing if warranted.

While epidemiological studies have attempted to measure age acceleration and increased mortality rates in HIV+ individuals (Appay and Rowland-Jones, 2002; Guaraldi et al., 2011; Smith et al., 2012), such measurements are made difficult by the myriad co-factors associated with HIV infection. For instance, metabolic disorders such as diabetes, HCV infection, and medication adherence are important factors of HIV infection that are also suspected to significantly affect mortality rates. Most previous studies have not attempted to control for these factors; in contrast, our study has focused specifically on well-characterized subjects. Nonetheless, our estimate of HIV age advancement of 4.9 years, calculated from a quantitative analysis of the methylome, falls within the range of the previous epidemiological studies. Further work will be needed to understand if the observed epigenetic age advancement is generalizable to broader slices of the HIV+ population (i.e., patients with complex co-morbidities such as drug use or additional viral infections).

This study is based on the same epigenetic model of biological aging as many others, including recent reports associating epigenetic aging with Down's Syndrome (Horvath et al., 2015), traumatic stress (Boks et al., 2015), and even all-cause mortality (Marioni et al., 2015). Here, we implement key data processing and analysis steps to improve the application of these models, which should aid in future applications. By minimizing the effects of cell type composition, we find better calibration of our control samples (Figures 2B–2D; Table S2), and the model is less affected by confounding associations such as the changing blood composition that occurs in HIV+ individuals (Figure S4). Furthermore, integration of both the Hannum et al. (2012) and Horvath models of epigenetic aging serves to limit biases in model training and allows us to filter samples that are of low quality or ill-suited for use in aging studies (Experimental Procedures; Table S4).

Our finding of a 5-year age advancement in cART-treated subjects (Figure 2E) is similar to one recent report (Horvath and Levine, 2015) but contrasts with another study in untreated patients, in which shared effects of age and HIV on the methylome were used to report an age advancement of 14 years (Rickabaugh et al., 2015). Although this discrepancy could be due to a beneficial effect of cART, we believe it is more likely due to differing statistical approaches. The previous number is based on comparison of the effects of HIV and age in a single cohort, rather than an epigenetic model of aging built for normal individuals, as performed here. Moreover, the authors derive their estimate from the ratio of linear coefficients for HIV and age, which are themselves highly correlated; such co-linearity is a well-known cause of instability in such estimates (Farrar and Glauber, 1967).

The discovery of HLA hypomethylation as a targeted consequence of HIV infection (Figure 5) has compelling synergy with the earlier discoveries of HLA genotype and expression level as major determinants of HIV control. Common genetic variation in HLA has been identified as the major contributing factor to host control of HIV infection (Fellay et al., 2007; International HIV Controllers Study et al., 2010), and HLA has been reported as a hotspot for integration of HIV provirus (Ambrosi et al., 2011). HIV infection has also been associated with decreased expression of some HLA genes but not others (Bonaparte and Barker, 2004; Cohen et al., 1999), and higher HLA-C expression is associated with HIV control (Apps et al., 2013; Kulkarni et al., 2011; Thomas et al., 2009). Our result suggests an epigenetic component to the regulation of HLA expression in this region. It also raises the possibility that the ability to control HIV infection could be acquired through epigenetic modification, as well as inherited through genotype.

In summary, we have shown that an extrinsic perturbation to a human population, driven by HIV infection and cART, is capable of inducing changes in the epigenomic state of affected individuals. This perturbation may influence regulation of HLA gene expression and also encompasses signatures of aging. Our findings help address a long-standing debate regarding the effects of HIV infection on biological aging in cART-treated individuals, in a manner that can be assessed numerically using an epigenome-based readout. Taken together, our findings show that the epigenome adds a quantitative means of assessing the interaction of HIV with normal and pathogenic processes associated with aging, and they shed light on the underlying mechanisms by which acute and chronic viral infection impact the host.

EXPERIMENTAL PROCEDURES

Reproduction of Computation Procedures
All data retrieval and processing steps are documented in a series of Jupyter notebooks at https://www.github.com/theandygross/HIV_Methylation.

Selection Criteria and Subject Recruitment
HIV+ subject samples were obtained from CHARTER as a Resource (http://www.charterresource.ucsd.edu). The CHARTER study was comprised of HIV-infected participants at varying stages of disease and with differing histories of antiretroviral treatment, with a focus on neuromedical and neurobehavioral assessments (Heaton et al., 2010). We requested information on subjects for which DNA had been obtained. Demographic and clinical data were filtered for non-Hispanic white males (to match the control group) who were free of Hepatitis C virus, not diabetic, on cART, and adherent to therapy.

Two groups were selected for study, those more recently infected by HIV (but after the acute infection stage, 0.8–5.0 years of infection) and those chronically infected (>12.0 years). As a control, 44 non-Hispanic white males without HIV were recruited from the San Diego area.

For validation samples, 35 HIV+ subjects along with 25 healthy controls were recruited prospectively for the purpose of this study to match the characteristics of the primary cohort. Cells were purified using immunomagnetic separation, and DNA was extracted from purified cell populations. While most subjects used had both neutrophils and CD4+ T cells profiled, differing DNA yield for some subjects prohibited profiling of both cell types for some patients.

Clinical and demographic data are presented in Tables S1 and S5.

Sample Collection and Methylation Analysis

DNA was purified from whole-blood samples using PaxGene collection tubes (QIAGENe) and FlexiGene DNA extraction kits (QIAGEN). Methylation analysis was performed using Infinium HumanMethylation450 BeadChip Kits (Illumina). 500 ng of DNA was bisulfite converted using EZ DNA Methylation Kits (Zymo Research) and subsequently processed for HumanMethylation450 BeadChips following manufacturer's instructions. Following hybridization, BeadChips were scanned using the Illumina HiScan System.

Data Pre-Processing

All methylation data for HIV+ and HIV− subjects were deposited in the Gene Expression Omnibus (GEO) under GEO: GSE67705. For the Hannum et al. (2012) and EPIC (Riboli et al., 2002) studies, raw data were obtained from GEO: GSE40279 and GSE51032. All data were processed through the Minfi R processing pipeline (Aryee et al., 2014). Cell counts were estimated by the estimateCellCounts function in Minfi using flow sorted cell populations made available by Houseman et al. (2012). To limit variability in methylation levels due to differing cell type composition in the whole-blood samples, methylation levels were adjusted for each CpG marker as follows:

- Average methylation levels for each cell type were obtained from the Houseman et al. (2012) flow sorted blood dataset.
- A theoretical methylation level was assessed for each patient by assuming their blood to be a mixture of these pure cell populations at the estimated cell type proportions.
- The difference of each patient's methylation level from the average was assessed.
- This difference was subtracted from the original raw dataset.

We followed the protocol established to be optimal by Marabita et al. (2013) first quantile normalizing the data and then performing beta-mixture quantile (BMIQ) normalization (Teschendorff et al., 2013). To limit batch effects, all arrays across the three studies were normalized together. For use in the Horvath methylation age model, raw data were normalized to a gold standard reference distribution following the protocol provided in the manuscript (Horvath, 2013). The sole deviation from the Horvath protocol was an additional cell composition adjustment performed in a similar manner as described above, after BMIQ normalization. While the cell composition adjustment was not part of either the Horvath or Hannum et al. (2012) processing pipeline, recent work (Jaffe and Irizarry, 2014) has shown cell type composition to be a key confounding factor in methylation analysis.

Benchmarking the Aging Models

Aging models were assessed using the Hannum et al. (2012) and EPIC (Riboli et al., 2002) datasets. While the Hannum et al. dataset was used to train both epigenetic aging models, the EPIC data were made available after the time of construction of both models and thus provide an independent assessment of performance. We limited analysis to patients between the ages of 25 and 68 years of age for better comparison to the HIV cohort. Among the HIV and EPIC cohorts, we saw slightly better performance of the Hannum model (which was trained using only whole blood data) than the Horvath model (trained in a variety of tissues), but when a simple average of these two models was taken (the "consensus model"), we found better performance than either separately (Table S2).

Epigenetic Model Concordance Filter

One key drawback of current models of molecular age is the lack of a confidence measure in model prediction for any particular individual. To address, this we utilized the concordance between the two models as an additional filter of data quality. Despite general agreement between the models, in a number of subjects biological age predictions varied by more than 20%. This analysis resulted in the filtering of three HIV+ cases and two HIV− controls in our primary cohort (Table S1).

Linear Scaling of Epigenetic Age

For both models and across all datasets a linear scaling factor existed when comparing chronological verses biological age. In order to properly compare the performance of the models and to best calibrate them to our dataset, we performed a linear adjustment to all model fits for the control data to a unit slope with a zero intercept. Note that this affected the model error when compared in an absolute sense but did not affect the correlation between biological and chronological age. In HIV+ patients, we adjusted this particular cohort to the regression fit of the matched controls.

Screening for Differentially Methylated Markers in Response to HIV Infection

For the results described in Figures 4 and 5, we ran a multivariate linear model to test for differentially methylated markers in response to HIV infection. This model used predicted cell type composition and age as covariates. Significance was assessed via a likelihood-ratio test for the improvement of a model fit with HIV as the variable of interest.

Disorder of Methylation in Response to HIV and Aging

We observed increasing disorder of the methylome by both aging and HIV infection. To assess the possibility that the increasing age advancement might be explained by increasing disorder, we conducted a principal component analysis on 7,967 age-associated markers that trended away from <50% of sites methylated with age (Figure S5A). This analysis produced a similar result to that shown in Figure 1C, in which the first principal component of the cohort was still associated with both with age and HIV infection. Furthermore, we observed that only 231 of 436 (53%) markers used in the two aging models tended toward methylation values of 50% of sites methylated (i.e., disorder, binomial p = 0.2) and found that while there is a general entropy increase in HIV+ patients across the entire methylome (Figure S5B), there is no such effect in the markers used in the biological age models (Figure S5C).

Identification of Differentially Methylated Regions

We identified 25,491 markers that were associated with HIV, trended away from disorder, and were not associated with age. For the discovery and visualization of differentially methylated regions (Figure 5A), we calculated a rolling statistic on the density of "hits" in 200 marker windows. From this analysis it was clear that a genomic region encompassing the HLA and histone gene clusters was enriched for markers in our query set, and post hoc analysis confirmed a strong enrichment in the genomic interval traditionally assigned to the HLA region ($\sim$29 MB–33 MB on chromosome 6, odds ratio = 1.3, $p < 10^{-10}$). For further refinement and visualization of this signal, we conducted a similar scan statistic on a section of chromosome 6 in Figure 5D. In this targeted analysis, we relaxed our criteria and looked for regions of consistent increases or decreases in methylation in HIV+ verses HIV− subjects. This analysis showed a number of "peaks" of hypomethylation both in the histone gene region as well as near the HLA genes.

Accounting for the Probe Density of the HLA Region

One potential confounding factor of this analysis is the high density of markers in the HLA region due to the design of the Illumina chip. Taking the non-uniform density of the chip into account, the scan statistic searched for regions across a fixed number of markers as opposed to a fixed-width genomic interval. Despite this, it is possible that the tight clustering of markers in this region gave us more power to detect short differentially methylated regions within this genetic locus. The presence of two peaks in the histone cluster region directly upstream the HLA locus gives strong support to this being a specific effect. The density of probes in the histone region was typical compared to the rest

of the genome, and the coincidence of these two signals being close to each other solely by chance is minimal.

ACCESSION NUMBERS

SUPPLEMENTAL INFORMATION

AUTHOR CONTRIBUTIONS

H.S.F. and T.I. conceived of the study. H.S.F., S.S., B.M.M., and K.Z. organized the HIV+ and control patient cohorts. D.C., K.F., K.L., K.L.J., and M.K. performed the sample preparation and methylation profiling experiments. A.M.G., P.A.J., H.S., C.T.N., and T.I. conducted study and analysis design. A.M.G. performed all statistical and bioinformatics analyses. A.M.G., P.A.J., J.F.K., H.S.F., and T.I. wrote the manuscript.

ACKNOWLEDGMENTS

REFERENCES

Absher, D.M., Li, X., Waite, L.L., Gibson, A., et al. (2013). Genome-wide DNA methylation analysis of systemic lupus erythematosus reveals persistent hypomethylation of interferon genes and compositional changes to CD4+ T-cell populations. PLoS Genet. 9, http://dx.doi.org/10.1371/journal.pgen.1003678.

Althoff, K.N., McGinnis, K.A., Wyatt, C.M., Freiberg, M.S., Gilbert, C., Oursler, K.K., Rimland, D., Rodriguez-Barradas, M.C., Dubrow, R., Park, L.S., et al. (2014). Comparison of Risk and Age at Diagnosis of Myocardial Infarction, End-Stage Renal Disease, and Non-AIDS-Defining Cancer in HIV-Infected Versus Uninfected Adults. Clin. Infect. Dis. 60, 627–638.

Ambrosi, A., Glad, I.K., Pellin, D., Cattoglio, C., Mavilio, F., Di Serio, C., and Frigessi, A. (2011). Estimated comparative integration hotspots identify different behaviors of retroviral gene transfer vectors. PLoS Comput. Biol. 7, e1002292.

Appay, V., and Rowland-Jones, S.L. (2002). Premature ageing of the immune system: the cause of AIDS? Trends Immunol. 23, 580–585.

Apps, R., Qi, Y., Carlson, J.M., Chen, H., Gao, X., Thomas, R., Yuki, Y., Del Prete, G.Q., Goulder, P., Brumme, Z.L., et al. (2013). Influence of HLA-C expression level on HIV control. Science 340, 87–91.

Aryee, M.J., Jaffe, A.E., Corrada-Bravo, H., Ladd-Acosta, C., Feinberg, A.P., Hansen, K.D., and Irizarry, R.A. (2014). Minfi: a flexible and comprehensive Bioconductor package for the analysis of Infinium DNA methylation microarrays. Bioinformatics 30, 1363–1369.

Beerman, I., Bock, C., Garrison, B.S., Smith, Z.D., Gu, H., Meissner, A., and Rossi, D.J. (2013). Proliferation-dependent alterations of the DNA methyl-

ation landscape underlie hematopoietic stem cell aging. Cell Stem Cell 12, 413–425.

Boks, M.P., van Mierlo, H.C., Rutten, B.P.F., Radstake, T.R.D.J., De Witte, L., Geuze, E., Horvath, S., Schalkwyk, L.C., Vinkers, C.H., Broen, J.C.A., and Vermetten, E. (2015). Longitudinal changes of telomere length and epigenetic age related to traumatic stress and post-traumatic stress disorder. Psychoneuroendocrinology 51, 506–512.

Bonaparte, M.I., and Barker, E. (2004). Killing of human immunodeficiency virus-infected primary T-cell blasts by autologous natural killer cells is dependent on the ability of the virus to alter the expression of major histocompatibility complex class I molecules. Blood 104, 2087–2094.

Cao, W., Jamieson, B.D., Hultin, L.E., Hultin, P.M., Effros, R.B., and Detels, R. (2009). Premature aging of T cells is associated with faster HIV-1 disease progression. J. Acquir. Immune Defic. Syndr. 50, 137–147.

Cawthon, R.M., Smith, K.R., O'Brien, E., Sivatchenko, A., and Kerber, R.A. (2003). Association between telomere length in blood and mortality in people aged 60 years or older. Lancet 361, 393–395.

Christensen, B.C., Houseman, E.A., Marsit, C.J., Zheng, S., Wrensch, M.R., Wiemels, J.L., Nelson, H.H., Karagas, M.R., Padbury, J.F., Bueno, R., et al. (2009). Aging and environmental exposures alter tissue-specific DNA methylation dependent upon CpG island context. PLoS Genet. 5, e1000602.

Cohen, G.B., Gandhi, R.T., Davis, D.M., Mandelboim, O., Chen, B.K., Strominger, J.L., and Baltimore, D. (1999). The selective downregulation of class I major histocompatibility complex proteins by HIV-1 protects HIV-infected cells from NK cells. Immunity 10, 661–671.

Day, K., Waite, L.L., Thalacker-Mercer, A., West, A., Bamman, M.M., Brooks, J.D., Myers, R.M., and Absher, D. (2013). Differential DNA methylation with age displays both common and dynamic features across human tissues that are influenced by CpG landscape. Genome Biol. 14, R102.

Deaton, A.M., and Bird, A. (2011). CpG islands and the regulation of transcription. Genes Dev. 25, 1010–1022.

Deeks, S.G. (2011). HIV infection, inflammation, immunosenescence, and aging. Annu. Rev. Med. 62, 141–155.

Deeks, S.G., Tracy, R., and Douek, D.C. (2013). Systemic effects of inflammation on health during chronic HIV infection. Immunity 39, 633–645.

Dubrow, R., Silverberg, M.J., Park, L.S., Crothers, K., and Justice, A.C. (2012). HIV infection, aging, and immune function: implications for cancer risk and prevention. Curr. Opin. Oncol. 24, 506–516.

El-Sadr, W.M., Lundgren, J., Neaton, J.D., Gordin, F., Abrams, D., Arduino, R.C., Babiker, A., Burman, W., Clumeck, N., Cohen, C.J., et al.; Strategies for Management of Antiretroviral Therapy (SMART) Study Group (2006). CD4+ count-guided interruption of antiretroviral treatment. N. Engl. J. Med. 355, 2283–2296.

Farrar, D.E., and Glauber, R.R. (1967). Multicollinearity in Regression Analysis: The Problem Revisited. Rev. Econ. Stat. 49, 92–107.

Fellay, J., Shianna, K.V., Ge, D., Colombo, S., Ledergerber, B., Weale, M., Zhang, K., Gumbs, C., Castagna, A., Cossarizza, A., et al. (2007). A whole-genome association study of major determinants for host control of HIV-1. Science 317, 944–947.

Freiberg, M.S., Chang, C.-C.H., Kuller, L.H., Skanderson, M., Lowy, E., Kraemer, K.L., Butt, A.A., Bidwell Goetz, M., Leaf, D., Oursler, K.A., et al. (2013). HIV infection and the risk of acute myocardial infarction. JAMA Intern. Med. 173, 614–622.

Goulder, P.J.R., and Walker, B.D. (2012). HIV and HLA class I: an evolving relationship. Immunity 37, 426–440.

Guaraldi, G., Orlando, G., Zona, S., Menozzi, M., Carli, F., Garlassi, E., Berti, A., Rossi, E., Roverato, A., and Palella, F. (2011). Premature age-related comorbidities among HIV-infected persons compared with the general population. Clin. Infect. Dis. 53, 1120–1126.

Hannum, G., Guinney, J., Zhao, L., Zhang, L., Hughes, G., Sadda, S., Klotzle, B., Bibikova, M., Fan, J.-B., Gao, Y., et al. (2012). Genome-wide Methylation Profiles Reveal Quantitative Views of Human Aging Rates. Mol. Cell 49, 359–367.

Heaton, R.K., Clifford, D.B., Franklin, D.R., Jr., Woods, S.P., Ake, C., Vaida, F., Ellis, R.J., Letendre, S.L., Marcotte, T.D., Atkinson, J.H., et al.; CHARTER Group (2010). HIV-associated neurocognitive disorders persist in the era of potent antiretroviral therapy: CHARTER Study. Neurology 75, 2087–2096.

Heyn, H., Li, N., Ferreira, H.J., Moran, S., Pisano, D.G., Gomez, A., Diez, J., Sanchez-Mut, J.V., Setien, F., Carmona, F.J., et al. (2012). Distinct DNA methylomes of newborns and centenarians. Proc. Natl. Acad. Sci. USA 109, 10522–10527.

Horvath, S. (2013). DNA methylation age of human tissues and cell types. Genome Biol. 14, R115.

Horvath, S., and Levine, A.J. (2015). HIV-1 Infection Accelerates Age According to the Epigenetic Clock. J. Infect. Dis. 212, 1563–1573.

Horvath, S., Garagnani, P., Bacalini, M.G., Pirazzini, C., Salvioli, S., Gentilini, D., Di Blasio, A.M., Giuliani, C., Tung, S., Vinters, H.V., and Franceschi, C. (2015). Accelerated epigenetic aging in Down syndrome. Aging Cell 14, 491–495.

Houseman, E.A., Accomando, W.P., Koestler, D.C., Christensen, B.C., Marsit, C.J., Nelson, H.H., Wiencke, J.K., and Kelsey, K.T. (2012). DNA methylation arrays as surrogate measures of cell mixture distribution. BMC Bioinformatics 13, 86.

International HIV Controllers Study, Pereyra, F., Jia, X., McLaren, P.J., Telenti, A., de Bakker, P.I.W., Walker, B.D., Ripke, S., Brumme, C.J., Pulit, S.L., et al. (2010). The major genetic determinants of HIV-1 control affect HLA class I peptide presentation. Science 330, 1551–1557.

Jaffe, A.E., and Irizarry, R.A. (2014). Accounting for cellular heterogeneity is critical in epigenome-wide association studies. Genome Biol. 15, R31.

Joshi, D., O'Grady, J., Dieterich, D., Gazzard, B., and Agarwal, K. (2011). Increasing burden of liver disease in patients with HIV infection. Lancet 377, 1198–1209.

Kennedy, B.K., Berger, S.L., Brunet, A., Campisi, J., Cuervo, A.M., Epel, E.S., Franceschi, C., Lithgow, G.J., Morimoto, R.I., Pessin, J.E., et al. (2014). Geroscience: linking aging to chronic disease. Cell 159, 709–713.

Kovari, H., Sabin, C.A., Ledergerber, B., Ryom, L., Worm, S.W., Smith, C., Phillips, A., Reiss, P., Fontas, E., Petoumenos, K., et al. (2013). Antiretroviral drug-related liver mortality among HIV-positive persons in the absence of hepatitis B or C virus coinfection: the data collection on adverse events of anti-HIV drugs study. Clin. Infect. Dis. 56, 870–879.

Kulkarni, S., Savan, R., Qi, Y., Gao, X., Yuki, Y., Bass, S.E., Martin, M.P., Hunt, P., Deeks, S.G., Telenti, A., et al. (2011). Differential microRNA regulation of HLA-C expression and its association with HIV control. Nature 472, 495–498.

Leeansyah, E., Cameron, P.U., Solomon, A., Tennakoon, S., Velayudham, P., Gouillou, M., Spelman, T., Hearps, A., Fairley, C., Smit, V., et al. (2013). Inhibition of telomerase activity by human immunodeficiency virus (HIV) nucleos(t)ide reverse transcriptase inhibitors: a potential factor contributing to HIV-associated accelerated aging. J. Infect. Dis. 207, 1157–1165.

Leung, V., Gillis, J., Raboud, J., Cooper, C., Hogg, R.S., Loutfy, M.R., Machouf, N., Montaner, J.S.G., Rourke, S.B., Tsoukas, C., and Klein, M.B.; CANOC Collaboration (2013). Predictors of CD4:CD8 ratio normalization and its effect on health outcomes in the era of combination antiretroviral therapy. PLoS ONE 8, e77665.

Lindsey, J., McGill, N.I., Lindsey, L.A., Green, D.K., and Cooke, H.J. (1991). In vivo loss of telomeric repeats with age in humans. Mutat. Res. 256, 45–48.

Luz, P.M., Grinsztejn, B., Velasque, L., Pacheco, A.G., Veloso, V.G., Moore, R.D., and Struchiner, C.J. (2014). Long-term CD4+ cell count in response to combination antiretroviral therapy. PLoS ONE 9, e93039.

Maartens, G., Celum, C., and Lewin, S.R. (2014). HIV infection: epidemiology, pathogenesis, treatment, and prevention. Lancet 384, 258–271.

Marabita, F., Almgren, M., Lindholm, M.E., Ruhrmann, S., Fagerström-Billai, F., Jagodic, M., Sundberg, C.J., Ekström, T.J., Teschendorff, A.E., Tegnér, J., and Gomez-Cabrero, D. (2013). An evaluation of analysis pipelines for DNA methylation profiling using the Illumina HumanMethylation450 BeadChip platform. Epigenetics 8, 333–346.

Marioni, R.E., Shah, S., McRae, A.F., Chen, B.H., Colicino, E., Harris, S.E., Gibson, J., Henders, A.K., Redmond, P., Cox, S.R., et al. (2015). DNA methylation age of blood predicts all-cause mortality in later life. Genome Biol. 16, 25.

Nightingale, S., Winston, A., Letendre, S., Michael, B.D., McArthur, J.C., Khoo, S., and Solomon, T. (2014). Controversies in HIV-associated neurocognitive disorders. Lancet Neurol. 13, 1139–1151.

Numata, S., Ye, T., Hyde, T.M., Guitart-Navarro, X., Tao, R., Wininger, M., Colantuoni, C., Weinberger, D.R., Kleinman, J.E., and Lipska, B.K. (2012). DNA methylation signatures in development and aging of the human prefrontal cortex. Am. J. Hum. Genet. 90, 260–272.

Pathai, S., Lawn, S.D., Gilbert, C.E., McGuinness, D., McGlynn, L., Weiss, H.A., Port, J., Christ, T., Barclay, K., Wood, R., et al. (2013). Accelerated biological ageing in HIV-infected individuals in South Africa: a case-control study. AIDS 27, 2375–2384.

Reynolds, L.M., Taylor, J.R., Ding, J., Lohman, K., Johnson, C., Siscovick, D., Burke, G., Post, W., Shea, S., Jacobs, D.R., Jr., et al. (2014). Age-related variations in the methylome associated with gene expression in human monocytes and T cells. Nat. Commun. 5, http://dx.doi.org/10.1038/ncomms6366.

Riboli, E., Hunt, K.J., Slimani, N., Ferrari, P., Norat, T., Fahey, M., Charrondière, U.R., Hémon, B., Casagrande, C., Vignat, J., et al. (2002). European Prospective Investigation into Cancer and Nutrition (EPIC): study populations and data collection. Public Health Nutr. 5 (6B), 1113–1124.

Rickabaugh, T.M., Kilpatrick, R.D., Hultin, L.E., Hultin, P.M., Hausner, M.A., Sugar, C.A., Althoff, K.N., Margolick, J.B., Rinaldo, C.R., Detels, R., et al. (2011). The dual impact of HIV-1 infection and aging on naïve CD4 T-cells: additive and distinct patterns of impairment. PLoS ONE 6, e16459.

Rickabaugh, T.M., Baxter, R.M., Sehl, M., Sinsheimer, J.S., Hultin, P.M., Hultin, L.E., Quach, A., Martínez-Maza, O., Horvath, S., Vilain, E., and Jamieson, B.D. (2015). Acceleration of age-associated methylation patterns in HIV-1-infected adults. PLoS ONE 10, e0119201.

Rosenblatt, H.M., Stanley, K.E., Song, L.Y., Johnson, G.M., Wiznia, A.A., Nachman, S.A., and Krogstad, P.A.; Pediatric AIDS Clinical Trials Group 377 Study Team (2005). Immunological response to highly active antiretroviral therapy in children with clinically stable HIV-1 infection. J. Infect. Dis. 192, 445–455.

Serrano-Villar, S., Sainz, T., Lee, S.A., Hunt, P.W., Sinclair, E., Shacklett, B.L., Ferre, A.L., Hayes, T.L., Somsouk, M., Hsue, P.Y., et al. (2014). HIV-infected individuals with low CD4/CD8 ratio despite effective antiretroviral therapy exhibit altered T cell subsets, heightened CD8+ T cell activation, and increased risk of non-AIDS morbidity and mortality. PLoS Pathog. 10, e1004078.

Siebold, A.P., Banerjee, R., Tie, F., Kiss, D.L., Moskowitz, J., and Harte, P.J. (2010). Polycomb Repressive Complex 2 and Trithorax modulate Drosophila longevity and stress resistance. Proc. Natl. Acad. Sci. USA 107, 169–174.

Smith, R.L., de Boer, R., Brul, S., Budovskaya, Y., and van Spek, H. (2012). Premature and accelerated aging: HIV or HAART? Front. Genet. 3, 328.

Solomon, A., Tennakoon, S., Leeansyah, E., Arribas, J., Hill, A., Van Delft, Y., Moecklinghoff, C., and Lewin, S.R. (2014). No difference in the rate of change in telomere length or telomerase activity in HIV-infected patients after three years of darunavir/ritonavir with and without nucleoside analogues in the MONET trial. PLoS ONE 9, e109718.

Teschendorff, A.E., Menon, U., Gentry-Maharaj, A., Ramus, S.J., Weisenberger, D.J., Shen, H., Campan, M., Noushmehr, H., Bell, C.G., Maxwell, A.P., et al. (2010). Age-dependent DNA methylation of genes that are suppressed in stem cells is a hallmark of cancer. Genome Res. 20, 440–446.

Teschendorff, A.E., Marabita, F., Lechner, M., Bartlett, T., Tegner, J., Gomez-Cabrero, D., and Beck, S. (2013). A beta-mixture quantile normalization method for correcting probe design bias in Illumina Infinium 450 k DNA methylation data. Bioinformatics 29, 189–196.

Thomas, R., Apps, R., Qi, Y., Gao, X., Male, V., O'hUigin, C., O'Connor, G., Ge, D., Fellay, J., Martin, J.N., et al. (2009). HLA-C cell surface expression and control of HIV/AIDS correlate with a variant upstream of HLA-C. Nat. Genet. 41, 1290–1294.

Torres, R.A., and Lewis, W. (2014). Aging and HIV/AIDS: pathogenetic role of therapeutic side effects. Lab. Invest. *94*, 120–128.

Trono, D., Van Lint, C., Rouzioux, C., Verdin, E., Barré-Sinoussi, F., Chun, T.-W., and Chomont, N. (2010). HIV persistence and the prospect of long-term drug-free remissions for HIV-infected individuals. Science *329*, 174–180.

Weidner, C.I., Lin, Q., Koch, C.M., Eisele, L., Beier, F., Ziegler, P., Bauerschlag, D.O., Jöckel, K.-H., Erbel, R., Mühleisen, T.W., et al. (2014). Aging of blood can be tracked by DNA methylation changes at just three CpG sites. Genome Biol. *15*, R24.

West, J., Beck, S., Wang, X., and Teschendorff, A.E. (2013). An integrative network algorithm identifies age-associated differential methylation interactome hotspots targeting stem-cell differentiation pathways. Sci. Rep. *3*, 1630.

Zanet, D.L., Thorne, A., Singer, J., Maan, E.J., Sattha, B., Le Campion, A., Soudeyns, H., Pick, N., Murray, M., Money, D.M., and Côté, H.C.; CIHR Emerging Team Grant on HIV Therapy and Aging: CARMA (2014). Association between short leukocyte telomere length and HIV infection in a cohort study: No evidence of a relationship with antiretroviral therapy. Clin. Infect. Dis. *58*, 1322–1332.