HHS Public Access

Author manuscript

J Steroid Biochem Mol Biol. Author manuscript; available in PMC 2017 October 16.

Published in final edited form as:

J Steroid Biochem Mol Biol. 2017 April ; 168: 49–59. doi:10.1016/j.jsbmb.2017.02.001.

Colonic transcriptional response to 1α,25(OH)$_2$ vitamin D$_3$ in African- and European-Americans

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Abstract

Colorectal cancer (CRC) is a significant health burden especially among African Americans (AA). Epidemiological studies have correlated low serum vitamin D with CRC risk, and, while hypovitaminosis D is more common and more severe in AA, the mechanisms by which vitamin D modulates CRC risk and how these differ by race are not well understood. Active vitamin D (1α, 25(OH)$_2$D$_3$) has chemoprotective effects primarily through transcriptional regulation of target genes in the colon. We hypothesized that transcriptional response to 1α,25(OH)$_2$D$_3$ differs between AA and European Americans (EA) irrespective of serum vitamin D and that regulatory variants could impact transcriptional response. We treated *ex vivo* colon cultures from 34 healthy subjects (16 AA and 18 EA) with 0.1 μM 1α,25(OH)$_2$D$_3$ or vehicle control for 6 hours and performed genome-wide transcriptional profiling. We found 8 genes with significant differences in transcriptional response to 1α,25(OH)$_2$D$_3$ between AA and EA with definitive replication of inter-ethnic differences for uridine phosphorylase 1 (*UPP1*) and zinc finger-SWIM containing 4 (*ZSWIM4*). We performed expression quantitative trait loci (eQTL) mapping and identified response *cis*-eQTLs for *ZSWIM4* as well as for histone deacetylase 3 (*HDAC3*), the latter of which showed a trend toward significant inter-ethnic differences in transcriptional response. Allele frequency differences of eQTLs for *ZSWIM4* and *HDAC3* accounted for observed transcriptional differences between populations. Taken together, our results demonstrate that transcriptional response to 1α,25(OH)$_2$D$_3$ differs between AA and EA independent of serum 25(OH)D levels . We provide evidence in support of a genetic regulatory mechanism underlying transcriptional differences between populations for *ZSWIM4* and *HDAC3*. Further work is needed to elucidate how response eQTLs modify vitamin D response and whether genotype and/or transcriptional response correlate with chemopreventive effects. Relevant biomarkers, such as tissue-specific 1α, 25(OH)$_2$D$_3$ transcriptional response, could identify individuals likely to benefit from vitamin D for CRC prevention as well as elucidate basic mechanisms underlying CRC disparities.

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Keywords

vitamin D; gene transcription; expression quantitative trait loci; colorectal cancer; health disparities

1. Introduction

Colorectal cancer (CRC) is a significant health burden worldwide but especially among African Americans (AA) who have the highest CRC incidence and mortality of all US populations (1–3). Multiple lines of evidence show that vitamin D protects against CRC (4–6). Active vitamin D ($1\alpha,25(OH)_2D_3$) is a steroid hormone with direct transcriptional effects mediated through the vitamin D receptor (VDR) (7). Transcriptome-wide studies have identified thousands of differentially expressed (DE) genes of which many are primary targets of the VDR and show cell type-specificity (7, 8). $1\alpha,25(OH)_2D_3$ is thought to exert its chemoprotective effects in the colon through inhibition of proliferation and induction of differentiation and apoptosis (9–13), although additional mechanisms are likely and remain understudied. Inactive 25-hydroxyvitamin D (25(OH)D), which is measured in the serum, is converted to $1\alpha,25(OH)_2D_3$ locally in the colonic epithelium (7, 8), and also likely contributes to anti-tumor effects. Epidemiological studies have found an inverse correlation between serum hypovitaminosis D with CRC risk (6, 14), and this effect was attenuated in AA due to lower 25(OH)D levels due to dark skin pigmentation, which inhibits the local synthesis of vitamin D in the skin (15, 16). Higher 25(OH)D levels have not only been associated with reduced risk of developing CRC, but was also shown to improve survival in patients with CRC (17). While hypovitaminosis D is more common and more severe in AA (18), the mechanisms by which vitamin D modulates CRC risk and how these differ by race are not well understood. Answering these questions is likely to have an important impact on understanding and addressing CRC disparities.

Despite strong epidemiological data of an inverse relationship between vitamin D status and CRC, reanalysis of the Women's Health Initiative of calcium and vitamin D supplementation (19) and a recent intervention trial did not support a protective role for vitamin D supplementation on CRC incidence or recurrence of colorectal neoplasia (adenomas or CRC), respectively (20). In the intervention trial, even when stratifying by serum 25(OH)D levels before and after treatment, there was no benefit of vitamin D supplementation. There are several potential explanations for these unexpected findings (21). The dose administered was low (1000 IU) and higher doses are likely required for chemoprevention in the colon (14, 22). Subjects participating in this study had a mean baseline 25(OH)D levels of 24 ng/ml that could have been too high to observe a beneficial effect for supplementation (23). There were relatively few AA included in the study, so it is not possible to generalize the results for this high-risk population. Another explanation is that there could be inter-individual and inter-ethnic differences in responses to the effects of active vitamin D irrespective of vitamin D serum levels which could impact treatment response. Identification of more biologically relevant endpoints of treatment response, such as tissue-specific transcriptional response, could help personalize chemoprevention and identify individuals most likely to benefit from treatment.

Previous studies of responses to glucocorticoids and 1α,25(OH)$_2$D$_3$ in peripheral blood (19, 20) and monocytes (21) have demonstrated inter-individual and inter-ethnic differences in transcriptional and cellular responses and have characterized the genetic architecture of treatment-specific effect(24–26)(23–25)(22–24)(21–23)(20–22). Taken together, these findings provide rationale for testing the hypothesis that there are inter-individual and inter-ethnic differences in responses to 1α,25(OH)$_2$D$_3$ in human colon and that genetic variants contribute to these differences. To do this, we utilized an *ex vivo* system in which colon biopsies were maintained in short-term culture and treated with 1α,25(OH)$_2$D$_3$ or vehicle in parallel (27). Use of primary tissue is advantageous because it is not transformed, comes from normal colon, the target tissue for chemoprevention, and can be obtained from diverse subjects. In addition, treatment of biopsies from the same individual with vitamin D and vehicle in parallel controls for confounding variables that could impact transcriptional response. In this study, we identified genes with differences in transcriptional response to 1α,25(OH)$_2$D$_3$ between AA and EA in normal colon. eQTL mapping of transcriptional response yielded insights into the contribution of genetic variants to inter-individual and inter-ethnic response to active vitamin D treatment.

2. Materials and methods

2.1 Human Subjects

Healthy individuals undergoing outpatient screening colonoscopy at the University of Chicago Medical Center were recruited. Two cohorts were recruited (hereafter referred to as “discovery” and “validation” cohorts). For the discovery cohort, a total of 34 subjects (16 AA and 18 EA) were recruited between May 2012 and February 2013. For the validation cohort, a total of 27 subjects (12 AA and 15 EA) were recruited between September 2014 and January 2015. Demographic data including self-identification as AA or EA, age and gender were collected. Peripheral blood was obtained at consent and serum 25(OH)D$_3$ levels were measured in the Clinical Chemistry laboratory at the University of Chicago. During colonoscopy, 4 biopsies were obtained using standard forceps (Boston Scientific; Natick, MA) in the recto-sigmoid colon approximately 20cm from the anal verge and immediately placed in transport media containing antibiotics. Biopsies were transported to the laboratory for further processing as previously described (27). This study was approved by the Institutional Review Board at the University of Chicago, and all subjects signed informed consent prior to data and sample collection.

2.2 Organ culture and treatment

We used an *ex vivo* organ culture protocol previously described (27). Briefly, colon biopsies were washed and cut into 1–2 mm pieces in cold phosphate-buffered saline (PBS). The pieces from each biopsy were placed on a cell strainer and placed into a 6-well culture dish, containing culture media that partially submerged the tissue. Two biopsies each were treated in parallel with 0.1 μM 1α,25(OH)$_2$D$_3$ or vehicle (ethanol) for 6 hours. This dose was selected based on a number of previous studies of vitamin D response (8, 27, 28). The 6 hour treatment point had the greatest number of DE genes in response to vitamin D treatment in a pilot study. The dish was incubated at 37°C in 5% CO_2 and 95% air. After treatment,

biopsies pieces were washed in cold PBS and immediately submerged in RNAlater (Ambion Inc; Austin, TX) and placed at 4 °C.

2.3 Transcriptional response profiling

Genome-wide transcriptional profiling was performed on samples from the discovery cohort after 6 hours of treatment with vitamin D or vehicle. Profiling was done in 2 batches from 24 and 12 subjects, respectively. The second batch included 2 subjects from batch 1 and concordance in gene expression across batches was high (Supplementary Figure 1). Total RNA was extracted from each sample using the QIAgen RNeasy Plus mini kit (Qiagen; Cat. no 74134), and RNA from replicate treatments was pooled. Total RNA was reverse transcribed into cDNA, labeled, hybridized to Human HT-12 v3 Expression Beadchips (Illumina; San Diego, CA) and scanned at the University of Chicago Functional Genomics Core facility. Low-level microarray analyses were performed using the Bioconductor R package, LUMI (29). Probes were annotated by mapping RNA sequences to RefSeq (GRCh37) using BLAT. Probes mapped to multiple genes or containing one or more SNPs identified by the 1000 Genomes Project were discarded. Further, we performed variance stabilizing transformations and probes indistinguishable from background fluorescence levels were also discarded and quantile normalization was done across all arrays. 12,175 probes remained after quality control for downstream analysis.

The Bioconductor R package LIMMA (30) was used to perform linear regression at each gene, with vitamin D treatment as the variable of interest. Covariates including ancestry, age, gender, serum $25(OH)D_3$ levels and principal components (PCs) 1&2 of the expression data (to account for unmeasured variation in the expression data) were included in the regression model. FDR was estimated using the Q-value function in R (31). Gene set enrichment of all DE genes was performed using DAVID (32). To assess the difference in genome-wide transcriptional response between populations, we used LIMMA to fit a linear regression model at each gene with LFC regressed on race including the covariates age, gender, serum $25(OH)D_3$ levels and 2 PCs of the expression data. An adjusted p-value from LIMMA was used to assess significance after correction for multiple testing.

2.4 Validation of inter-ethnic candidate genes

To validate inter-ethnic candidate genes identified in our genome-wide transcriptome study, biopsy samples from the validation cohort were collected and treated with $1\alpha,25(OH)_2D_3$ or vehicle in the same manner as the discovery cohort. Total RNA was extracted and reverse transcribed to cDNA, after which real-time quantitative PCR (RT-PCR) was performed to measure transcript levels of candidate genes relative to treatment with vitamin D. Primers for each gene were obtained from Integrated DNA Technologies (Coralville, IA) and their sequences are listed in Supplementary Table 1. RT-PCR was performed using Power SYBR Green PCR Master Mix (Life Technologies). 18S ribosomal RNA transcript levels were used as the endogenous control. For each gene, reactions were loaded in triplicate for each treatment condition and assayed on an Applied Biosystems 7900HT Fast Real Time PCR system. Expression data was exported, and LFC for each gene was calculated using the $2^{-\Delta\Delta Ct}$ method. A one-sided Wilcoxon rank-sum test was utilized to test the significance of fold change differences between AA and EA. Since *a priori* fold change data was available

from the discovery cohort, a sign test was used in R to determine concordance of differences in response in both cohorts.

2.5 VDR/RXR ChIP-seq meta-analysis

We reanalyzed published data of a VDR ChIP-seq experiment obtained from LS180 CRC cell line treated with 0.1 μM 1α,25(OH)$_2$D$_3$ for 24 hours (8). Sequence reads were aligned to the human reference genome (GRCh37) using BWA backtrack 0.7.5 (33) and samtools v1.1 (34) was used to select sequence reads with a phred-scaled mapping quality ≥ 30. PCR duplicates were removed with picard tools v1.130 (http://broadinstitute.github.io/picard/). The quality of this dataset was confirmed by strand cross-correlation (SCC) analysis (35) implemented in the R script packaged in phantompeakqualtools (https://code.google.com/p/phantompeakqualtools/). Statistically significant peaks were identified using MACS version 2 (MACS2) (36), using the essential command line arguments: macs2 callpeak – bw X – g hs – qvalue = 0.001 – m 5 50, where X is a length of the bandwidth that was defined as a fragment length calculated by SCC analysis. We then further annotated peaks called from MACS2 using HOMER (37) to find the closest gene to each peak and vitamin D response elements within each peak.

2.6 *cis*-eQTL mapping of transcriptional response to 1α,25(OH)$_2$D$_3$

To test the hypothesis that genetic variation underlies differences in transcriptional response to active vitamin D, we performed *cis*-eQTL mapping using Matrix eQTL. Genomic DNA was extracted from PBMCs of 30 subjects (15 AA; 15 EA) from the discovery cohort and was genotyped on the Human Omni2–5Exome microarray (Illumina; San Diego, CA) which included 2.6 million SNPs. Missing genotypes were imputed with IMPUTE2 (38) using data from the 1000 Genomes Project (39). SNPs were then filtered by minor allele frequency (>0.05) and missingness (<0.10), leaving 6.9 million SNPs for analysis. This analysis was restricted to SNPs within 150kb of the gene start and end sites including the gene to identify *cis*-effects. We also performed mapping using 2,070 SNPs in VDR binding peaks and 6,998 SNPs within 150 kb of and including the 8 candidate genes that showed differential responses between AA and EA. Proportions of West African ancestry, based on Yoruban genomes from the 1000 Genomes Project were estimated for each subject using ADMIXTURE (40) (Supplementary Figure 2). Covariates used in the eQTL analysis included age, gender, and 4 PCs of gene expression data to control for unmeasured variation using a method described previously (41). eQTL mapping was performed using models with and without ancestry and vitamin D serum levels (which are correlated with ancestry) in order to dissect the effect of ancestry on genotype associations with LFC using the following models:

Full: LFC ~ geno + age + sex + ancestry + serum vitD + 4PCs

Ancestry only: LFC ~ age + sex + ancestry + serum vitD + 4PCs

No ancestry: LFC ~ geno + age + sex + 4PCs

In silico analyses were performed using publicly available data from the ENCODE (42) and GTEx projects (43) available in HaploReg (44).

2.7 Population differences in transcriptional response

To test whether allele frequency accounted for differences in transcriptional response between populations, we utilized a statistical method described by Maranville et al (24), to compare the predicted phenotypic values to observed phenotypic values. Using the genotypic effect size for each candidate eQTL and the difference in allele frequency between populations from our genotyped data, we calculated a *predicted* phenotypic value using the formula: $2^{*}\beta(p_{AA}\text{-}p_{EA})$ where β was the genotypic effect and p was the minor allele frequency in each population. The *observed* phenotypic value was calculated as the difference in average LFC between populations. The ratio of observed to predicted values was then calculated, and in cases where this ratio was >1, the reciprocal was used to enable direct comparison across all eQTLs. To generate a null distribution for this ratio, a set of genome-wide eQTLs was identified by selecting one eQTL per gene defined by the lowest p-value from Matrix eQTL. The predicted phenotypic values were calculated for 11,265 eQTLs using the formula described above and compared to the observed phenotypic values. Using this null distribution of observed-predicted ratios genome-wide, we calculated empirical p-values for candidate eQTLs.

3. Results

3.1 Characterizing the transcriptional response to 1α,25(OH)$_2$D$_3$ in primary *ex vivo* colon culture

In the discovery cohort, there were 882 genes that were DE in response to 6 hours of 1α, 25(OH)$_2$D$_3$ treatment at a false discovery rate (FDR) < 1% (Figure 1; Table 1). Of these, 465 genes were up-regulated, while 417 were down-regulated. The DE genes were enriched for toll-like receptor (TLR) signaling, transcription regulation, biological rhythms, insulin resistance and metal-binding categories (Benjamini-Hochberg p-value<0.05) (Supplementary Table 2). Among the DE genes were a number of previously validated VDR targets such as *CD14*, *CYP24A1* and *TRPV6* (8, 45) (Figure 2a, b & c). In addition, we identified several significantly DE genes implicated in CRC that were previously found to be responsive to vitamin D (8) including *c-MYC, c-FOS, AXIN2* and *TGFβR2* (Figure 2d, e, f & g). With the exception of *c-MYC*, the direction of the effect on expression was the same as that reported previously (8). In addition, we found that *MUC13*, a gene shown to be over-expressed in CRC, but not previously known to be a target of 1α,25(OH)$_2$D$_3$, was significantly down-regulated by 6 hours of treatment (Figure 2h).

In a previous study by Meyer et al (8), the LS180 CRC cell line was used for transcriptional profiling and VDR/RXR ChIP-seq studies in response to 0.1 μM 1α,25(OH)$_2$D$_3$ treatment for 24 hours. We compared DE genes identified in the LS180 study with those identified in our study and found a significant excess of overlapping genes in the top 5% of DE genes (10.2% overlap, $p = 3.5\times10^{-9}$). This overlap included known vitamin D-responsive genes (e.g *CYP24A1, TRPV6, CA2* and *ABCB1*). We then reanalyzed the raw data from LS180 VDR/RXR ChIP-seq experiment to identify regions with VDR/RXR binding peaks with or without the canonical DR3 motif. At an FDR < 0.1%, 1,271 peaks were shared between VDR and RXR in vitamin D and vehicle treated samples, whereas 920 peaks were found in vitamin D treatment alone. A number of known vitamin D targets such as *CYP24A1, CD14*

and *TRPV6* had associated VDR binding peaks (full list in Supplementary Table 3). Annotation of the VDR-specific peaks that overlapped with DE genes from our study showed that 80% of binding sites were in intergenic or intronic regions. Moreover, the majority of VDR binding peaks were located within 100 kb from the TSS of DE genes in response to vitamin D (Supplementary Figure 3); suggesting that vitamin D might play a role in gene regulation by binding to non-coding genomic elements.

3.2 Inter-ethnic differences in vitamin D transcriptional responses

Given our interest in differences in transcriptional response between AA and EA, we asked whether there were genes that, on average, respond differently to vitamin D treatment between populations. For this analysis, we included all genes that were DE in either population at FDR < 0.1%. We used these criteria because we wanted to capture genes that were DE in both populations, but also genes that were DE in one population but not the other. A linear model was used including age, gender, serum 25(OH)D levels, and two PCs of the expression data as covariates. *VDR* expression in control-treated samples did not differ by race and was not included as a covariate. There were 8 genes with significant differences in LFC by population (Table 2). For all but one (*CLRN3*) of the significant genes, the LFC was significantly greater in EA compared with AA. Four genes (*UPP1, UCKL1, EPHA2* and *CLRN3*) had a nearby VDR binding peak, suggesting these could be direct targets of the VDR (Supplementary Table 4).

These 8 candidate genes with inter-ethnic differences in vitamin D response were then tested in an independent validation cohort with similar clinical characteristics (Supplementary Table 5). Of the 8 candidates, *UPP1* and *ZSWIM4* were replicated (Wilcoxon rank-sum p-value = 0.03 and 0.02, respectively) (Figure 3). Overall, for 7 of 8 (88%) genes, the direction of the inter-ethnic difference in transcriptional response was the same as in the discovery cohort, and a sign test was significant (p=0.0008) suggesting concordance in direction of gene effect between the discovery and validation cohorts.

3.3 *cis*-eQTL mapping

To evaluate whether transcriptional response to vitamin D has a genetic basis, we first asked whether SNPs were associated with transcriptional response in a treatment-specific manner, namely in the presence and absence of vitamin D. For this analysis, we used the full model (see section 2.6) that included the following covariates: age, sex, ancestry, vitamin D serum levels and 4 PCs (to account for unmeasured variation in gene expression). We restricted eQTL mapping to SNPs located 150kb upstream and downstream of the gene start site in order to identify *cis* effects. In total, we identified 4,530 and 4,452 eQTLs in vitamin D treatment alone and vehicle treatment alone, respectively, at an FDR <10%. To identify treatment-specific eQTLs, we filtered SNPs that were significant in one condition (FDR <1%) and not in the other (FDR>30%) and identified 17 SNPs (corresponding to 3 genes when accounting for LD) in vitamin D treated samples and 38 SNPs (corresponding to 8 genes) in vehicle treated samples. We also identified eQTLs in both treatment conditions which can be thought of as “baseline” eQTLs as they were replicated in 2 experiments but did not show a treatment-specific response. To do this, we filtered SNPs that were significant at an FDR<1% in both treatment conditions and identified 233 SNPs corresponding to 11

genes. Examples of treatment-specific eQTLs are shown in Figure 4; full list in Table 3). For vitamin D and vehicle control eQTLs identified in this analysis, we noted that many had potential regulatory functions in the colon and 3 variants were predicted to alter VDR binding (Table 3). We did not find significant eQTLs when restricting the analysis to SNPs located in VDR peaks.

The advantage of our paired experimental design was that we treated biopsies from the same individual with vitamin D and vehicle control in order to control for potential confounders. The power of a paired design is that we map LFC to vitamin D treatment in each individual. Because we were interested in identifying eQTLs that might have allele frequency differences between AA and EA to explain transcriptional response differences, we performed mapping using a linear model that included age, sex and 4 PCs as covariates. We did not include ancestry and vitamin D serum levels (which are correlated with ancestry) here because these might mask genotypic effects due to allele frequency differences between populations thus reducing power to detect genome-wide significant eQTLs. Using this "no ancestry" model (see section 2.6), we identified 10 response eQTLs at an FDR< 10%: one variant associated with *ZSWIM4* and 9 variants associated with *HDAC3* (Table 4). As evidence supporting these variants as eQTLs, we noted that the association of genotype of the top eQTLs and LFC was significant in AA and EA separately for both genes (Figure 5). To ensure that we captured genotypic effects that were present in both populations and not due to population stratification, we performed a likelihood ratio test of the full model (which includes genotype and ancestry) compared to the ancestry only model which was significant for the top eQTL associations with *ZSWIM4* and *HDAC3* (p-values 2.8×10^{-6} and 2.5×10^{-9}, respectively).

Because the eQTL for *ZSWIM4* was located near the end of the 150KB window, we extended mapping in this region to include a 500KB window around *ZSWIM4*. In so doing, we identified 6 additional significant eQTLs located 192KB downstream of *ZSWIM4* with more significant p-values with rs73519631 being the most significant of these. The two most significant SNPs in the *ZSWIM4* region, rs897785 and rs73519631, had correlated genotypes (r=0.75) in our data suggesting they tag a common signal in this region. Indeed, when we controlled for the effect of rs73519631, none of the associations were significant (Figure 6a). Three variants in LD with rs897785 (rs13345162, rs149056637 and rs7252801) were predicted to alter VDR binding. rs897785 was noted to have open chromatin and enhancer marks in rectal mucosa, and a variant in high LD with rs73519631 (rs6511905) was found to have enhancer marks in rectal mucosa. For the *HDAC3* region, we identified a total of 9 significant eQTLs whose genotypes were all correlated (r=0.8) in our data (Figure 6b). A variant in LD with rs9324855 was predicted to alter the VDR binding motif, and rs9324855 itself has DNAse and enhancer marks in colon and rectal mucosa.

3.4 Allele frequency differences and transcriptional response

Finally, we tested whether allele frequency differences could account for differences in transcriptional response between populations. Applying an approach described previously (24), we compared *predicted* phenotypic values with *observed* phenotypic values and determined significance for each eQTL using a null distribution of the ratio of observed to

predicted values (Table 5). Using this approach, we found that eQTLs in *ZSWIM4* and *HDAC3* had significant empirical p-values (p=0.04 and 0.02, respectively) suggesting that allele frequency differences of these variants account for transcriptional differences in response to vitamin D. Among the other 7 candidate genes with transcriptional response differences between populations, we did not have evidence for significant eQTLs associated with response to vitamin D.

4. Discussion

Multiple lines of evidence show that vitamin D has protective actions against malignant transformation (9, 11–13, 46) and epidemiological studies correlated serum vitamin D with CRC risk (4–6). However, it is not known how individuals differ in their tissue-specific responses to a fixed dose of 1α,25$(OH)_2D_3$, which, in turn, could impact disease risk irrespective of differences in serum 25(OH)D levels. Specifically, understanding how AAs and EAs differ in vitamin D responses could elucidate pathways underlying cancer disparities as well as identify potential biomarkers of response independent of serum 25(OH)D levels. In this study, we used an *ex vivo* culture system to demonstrate, for the first time, that there are inter-ethnic differences in colonic transcriptional response to a fixed dose of 1α,25$(OH)_2D_3$ and that genetic variants underlie vitamin D transcriptional responses at *ZSWIM4* and *HDAC3*.

A key finding in this study is inter-ethnic differences in transcriptional response to 1α, 25$(OH)_2D_3$ treatment in the human colon irrespective of serum 25(OH)D levels. In particular, we identified 8 genes with significantly differences in transcriptional response to vitamin D treatment between AA and EA. These genes encode proteins involved in pyrimidine salvage and uridine homeostasis (*UPP1* and *UCKL1*), a zinc-finger protein (*ZSWIM4*), a nucleotide excision repair protein (*ERCC1*), a receptor tyrosine kinase that is overexpressed in CRC (47) (*EPHA2*), a cellular transport protein (*MFSD2A*) and integral transmembrane proteins (*KIAA1324L* and *CLRN3*). For all but *CLRN3*, response to vitamin D treatment was weaker among AA. Four of these genes (*UPP1, UCKL1, EPHA2* and *CLRN3*) had overlap with VDR binding peaks suggesting that these are direct targets of VDR. In an independent cohort of subjects, we found that gene effect sizes overall showed concordance with those in the original discovery cohort, with definitive replication of *UPP1* and *ZSWIM4* providing strong support for these 2 genes as having population-specific vitamin D transcriptional responses.

Among the replicated candidate genes, *UPP1* deserves special mention. This gene encodes uridine phosphorylase 1, a key enzyme involved in uridine homeostasis, that was previously reported to be induced by vitamin D (37) and is a direct VDR target (8). Importantly, abrogated uridine phosphorylase activity in an $UPP1^{-/-}$ mouse model led to spontaneous tumor development, including in the colon (48). These mice have increased levels of tissue uridine and dUTP, notably in the colon, and increased DNA damage with uridine treatment due to misincorporation of dUTP. Uridine-induced DNA damage was also confirmed in multiple cell lines (48). Interestingly, tissue uridine levels have been implicated as being protective of 5-FU-induced damage in the colon (49), and AA experience fewer gastrointestinal side effects in response to 5-FU, leading to the hypothesis that AA might

have higher colonic uridine levels. Taken together, these findings provide rationale for further investigation of inter-ethnic differences in 1α,25(OH)$_2$D$_3$ regulation of *UPP1*, uridine homeostasis and DNA damage in the colon.

In addition to identifying genes with inter-ethnic differences in response to vitamin D, our study extends findings from previous studies of vitamin D transcriptional response in CRC (8, 50, 51). Our enrichment analysis was significant for TLR signaling which overlaps with enriched pathways from a 4-week 1α,25(OH)$_2$D$_3$ human intervention trial (52) underscoring vitamin D's impact on innate immune pathways in the colonic epithelium. As proof-of-principle, the two most significant genes in the present study were *CD14* and *CYP24A1*, which are well-established vitamin D targets (45, 53). Moreover, we found that genes implicated in colon carcinogenesis such as *c-FOS, AXIN2* and *TGFβR2* were responsive to vitamin D at 6 hours and the effect was in the same direction as in a CRC cell line at 24 hours (8). The relatively early time point of 6 hours could explain why other cancer-related genes were not found to be DE in the present study. Another notable finding is that *MUC13* was down-regulated in response to 1α,25(OH)$_2$D$_3$. Expression of *MUC13* was increased in CRC and over-expression was associated with poorly differentiated tumors (54, 55). *MUC13* protein expression is increased by IL-6 through JAK2/STAT5 signaling which leads to increased growth and invasion in CRC cell lines (56). Our finding that 1α,25(OH)$_2$D$_3$ significantly modulates expression of *MUC13* suggests a possible new mechanism of prevention for cancer and inflammation in the colon and warrants further study.

A second key finding in this study is that eQTLs were identified for response to 1α, 25(OH)$_2$D$_3$ in human colon. *Cis*-acting eQTLs have been associated previously with response in peripheral blood and immune cells to vitamin D (26), glucocorticoids (24), infectious agents (57) and other immune stimulants (58) and could underlie differences in transcriptional response between populations as was demonstrated for glucocorticoids (24) and response to infectious agents (59). Our study identified eQTLs associated with colonic response of *ZSWIM4* and *HDAC3* to 1α,25(OH)$_2$D$_3$ treatment. We did not replicate previously reported eQTLs for vitamin D in monocytes (21) but this could be explained by tissue-specific effects of vitamin D. In the *ZSWIM4* region, we identified significant eQTLs located approximately 90KB and 190KB from the 3' end of the gene, and conditioning on the most significant eQTL in the more distal region accounted for all associations. In the *HDAC3* region, we identified 2 association peaks located 57KB and 71KB upstream of the gene, and conditioning on the top eQTL in this region also accounted for associations in both regions. Given that the top eQTL (or SNPs in LD) have enhancer marks in relevant GI tissues or alter the VDR motif, it is likely that these eQTLs have a regulatory function and possibly alter VDR binding; additional work is needed to test these hypotheses and fine-map the region for causative SNPs.

Our results also provide evidence that allele frequency differences of *ZSWIM4* and *HDAC3* eQTLs contribute to transcriptional differences in vitamin D response between AA and EA, thus supporting the hypothesis that genetic variation accounts for some of the inter-ethnic differences in transcriptional response. While we did not observe inter-ethnic differences in transcriptional response of *HDAC3* after correction for multiple testing, as we did with *ZSWIM4*, there was a trend toward a significant difference between AA and EA for *HDAC3*

(p=0.11). Thus, we believe this gene likely exhibits differences in transcriptional response to vitamin D between AA and EA and that allele frequency differences in eQTLs account for the transcriptional differences, but we were limited by inadequate power given a small sample size.

Little is currently known about the function of the *ZSWIM4* protein. There is some evidence that SWIM-type zinc finger proteins could be involved in ubiquitination (60) but this has not been established for *ZSWIM4*. *HDAC3* encodes a histone deacetylase enzyme that represses transcription and has been shown to interact with p53 (61) as well as to coordinate microbiome-dependent intestinal homeostasis (62). In the colon, HDAC3 was found to be localized to the proliferative zone of the colonic crypt suggesting a role in colon cell maturation and proliferation (63), and is overexpressed in CRC (63). While *HDAC3* has not been previously described as a vitamin D responsive gene, other enzymes that modify chromatin have been identified as targets of the VDR including *HDAC4, HDAC6* and *KDM6B* (64). Our results are the first to demonstrate eQTLs associated with *HDAC3* response to $1\alpha,25(OH)_2D_3$ treatment which could have implications for CRC development. Taken together, our results provide strong evidence that *ZSWIM4* and *HDAC3* response to vitamin D in the colon differs between AA and EA and that a *cis*-regulatory genetic mechanism underlies these transcriptional differences. Further work is needed to elucidate the impact of this regulation on colonic homeostasis and cancer risk.

In summary, our work provides evidence of differences in transcriptional responses to a fixed dose of $1\alpha,25(OH)_2D_3$ between AA and EA and we replicated these differences definitively for *UPP1* and *ZSWIM4* in an independent cohort. These inter-ethnic response differences are irrespective of serum 25(OH)D levels suggesting that even if equivalent serum levels are achieved between populations, there could still be differences in response at the tissue level. We provide evidence supporting a genetic mechanism underlying inter-ethnic differences in vitamin D response for *ZSWIM4* and *HDAC3* adding to growing literature about inter-individual and inter-ethnic variation in gene by treatment responses (24, 26, 57). We did not identify eQTLs for *UPP1* for which we validated inter-ethnic transcriptional response differences, and it is possible that our sample size was not adequate to detect eQTL associations or, alternatively, that transcriptional response differences are due to non-genetic mechanisms. We favor the first explanation because we found 13 significant eQTLs for *UPP1* in colon tissue in GTEx (39) of which several SNPs were predicted to alter the RXR motif (the transcription factor that complexes with VDR) and have allele frequency differences that could potentially contribute to *UPP1* inter-ethnic differences. Additional genotyping in a larger sample is ongoing to test for response eQTLs for *UPP1*. Further work is needed to elucidate the mechanisms by which response eQTLs modify vitamin D response in the colon and to test whether eQTL genotype and/or transcriptional response is predictive of cellular or clinical responses for diseases of the colon especially CRC. In an era where the need for precision medicine at the individual and population level is recognized, understanding of inter-individual and -ethnic differences in $1\alpha,25(OH)_2D_3$ response might eventually lead to personalized vitamin D-based interventions for cancer prevention especially in high risk groups.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are grateful to Silvia Kariuki and Joseph Maranville for their helpful comments on this manuscript.

Funding sources

This work was supported by a pilot and feasibility grant from the University of Chicago Digestive Disease Research Core Center [NIDDK P30DK42086] and by the Gastrointestinal Research Foundation (GIRF).

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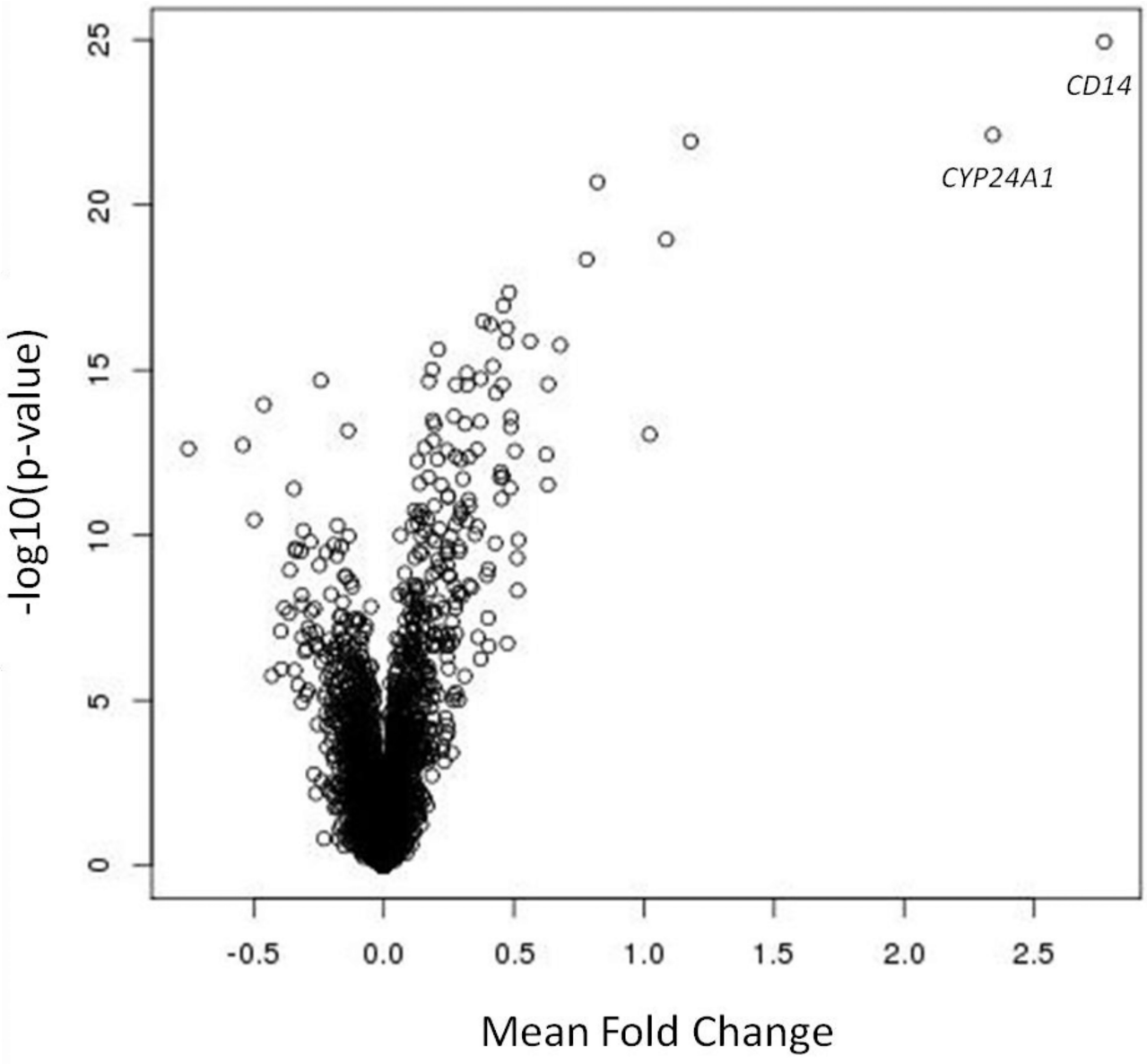

Figure 1. Transcriptional response to 1α,25(OH)$_2$D$_3$ at 6 hours in *ex vivo* human primary colon culture

This volcano plot summarizes mean fold change by -log(p-value). There are 883 genes that were significantly (FDR < 1%) differentially expressed in response to 1α,25(OH)$_2$D$_3$. Of these, 465 were up-regulated and 418 were down-regulated. As a proof-of-concept, the top responding up-regulated genes were *CD14* and *CYP24A1*, both of which are known vitamin D responsive genes.

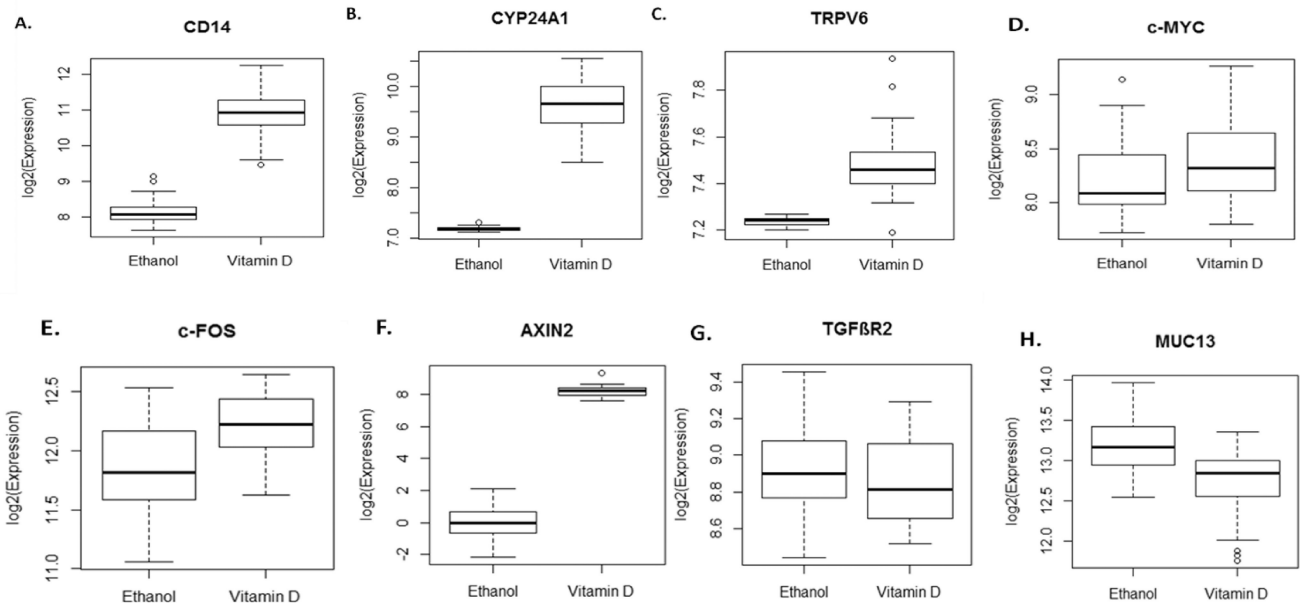

Figure 2. Box-plots showing differentially expressed genes in response to 1α,25(OH)$_2$D$_3$ (FDR ≤ 1%)

(A) *CD14*, (B) *CYP24A1*, (C) *TRPV6*, (D) *c-MYC*, (E) *c-FOS*, (F) *AXIN2*, (G) *TGFβR2*, and (H) *MUC13*

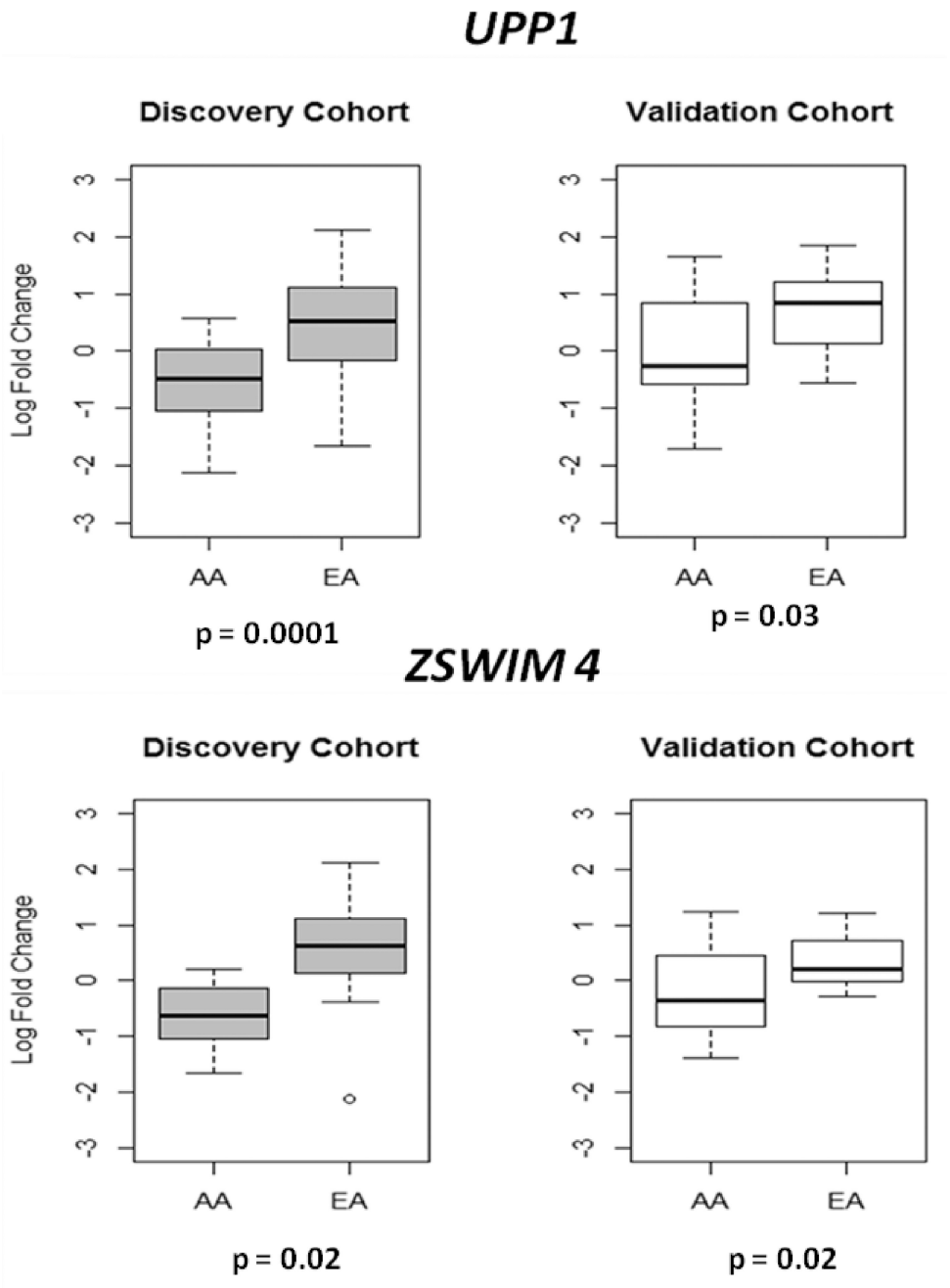

Figure 3. Box-plots showing log fold change expression of replicated inter-ethnic DE genes in African- and European-Americans

*UPP1 - Validation Cohort (*Wilcoxon rank-sum p-value = 0.03) and *ZSWIM4 - Validation Cohort* (Wilcoxon rank-sum p-value = 0.02)

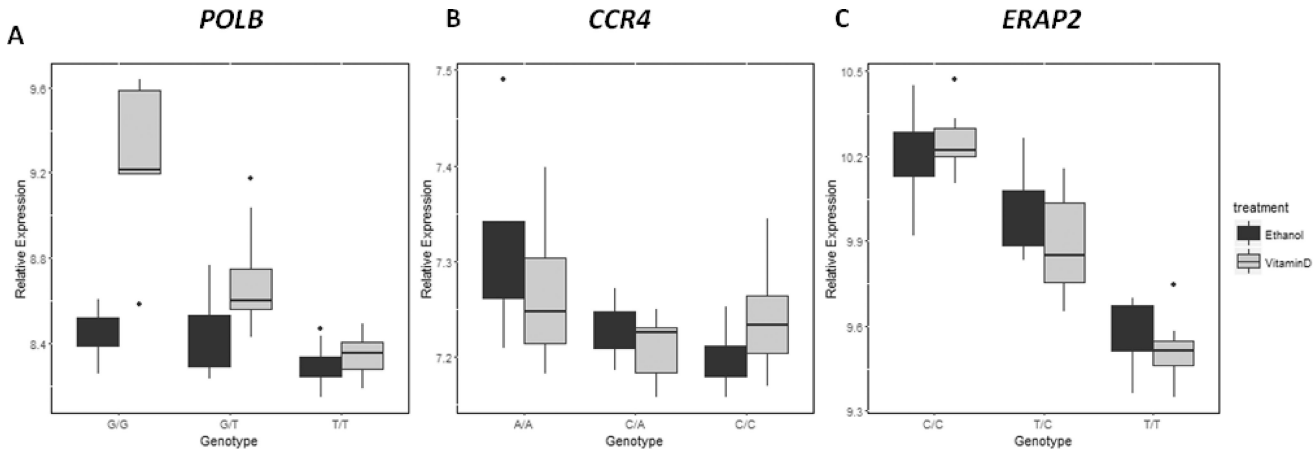

Figure 4. Treatment-specific *cis*-regulatory variants associated with transcriptional response to 1α,25(OH)$_2$D$_3$

(A) *POLB* (Vitamin D), (B) *CCR4* (Ethanol) and (C) *ERAP2* (both conditions)

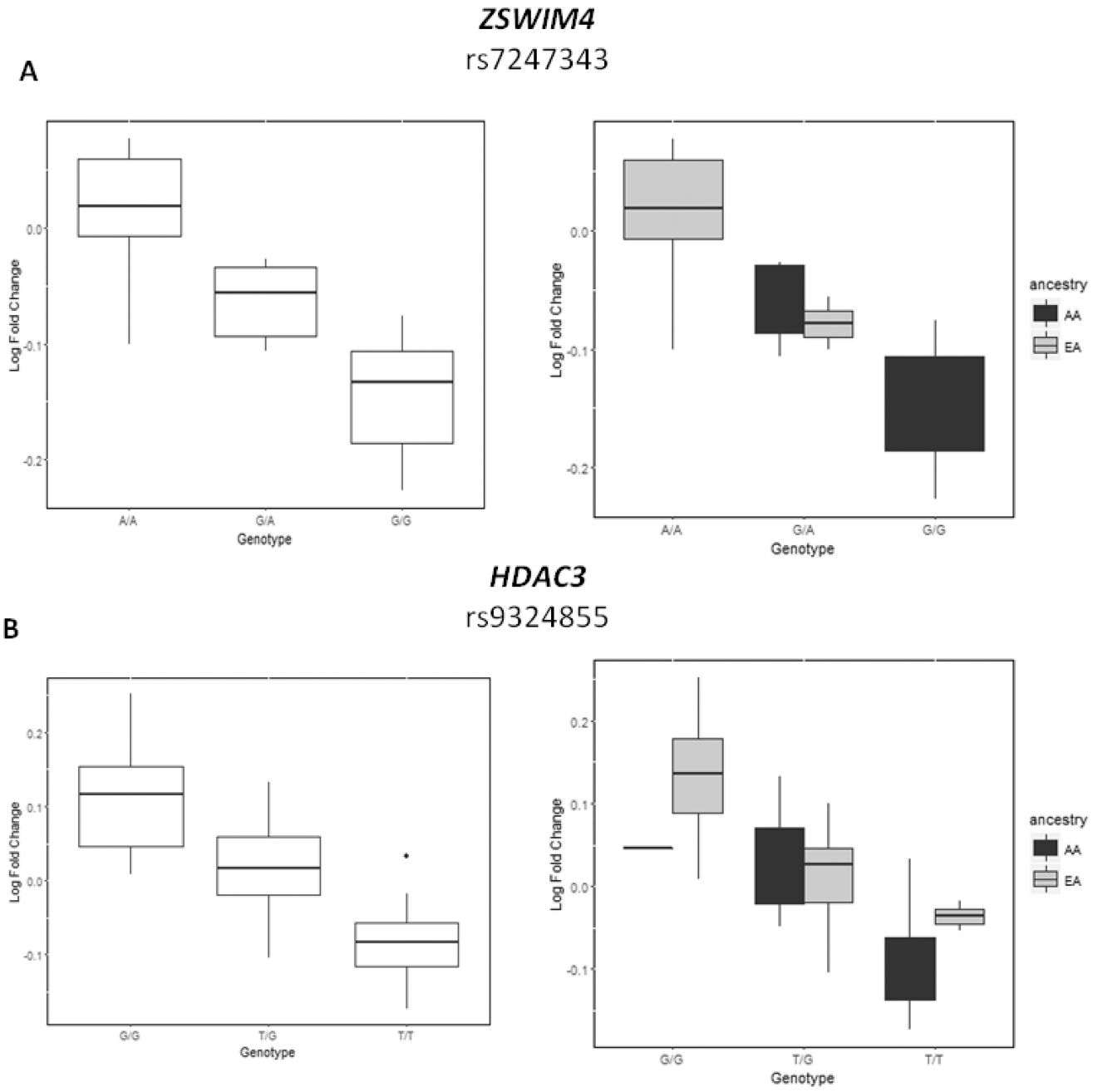

Figure 5. *Cis*-regulatory variants associated with transcriptional response to 1α,25(OH)$_2$D$_3$

Box-plots showing effect of genotype on log fold change in *ZSWIM4* and *HDAC3*, overall as well as by race.

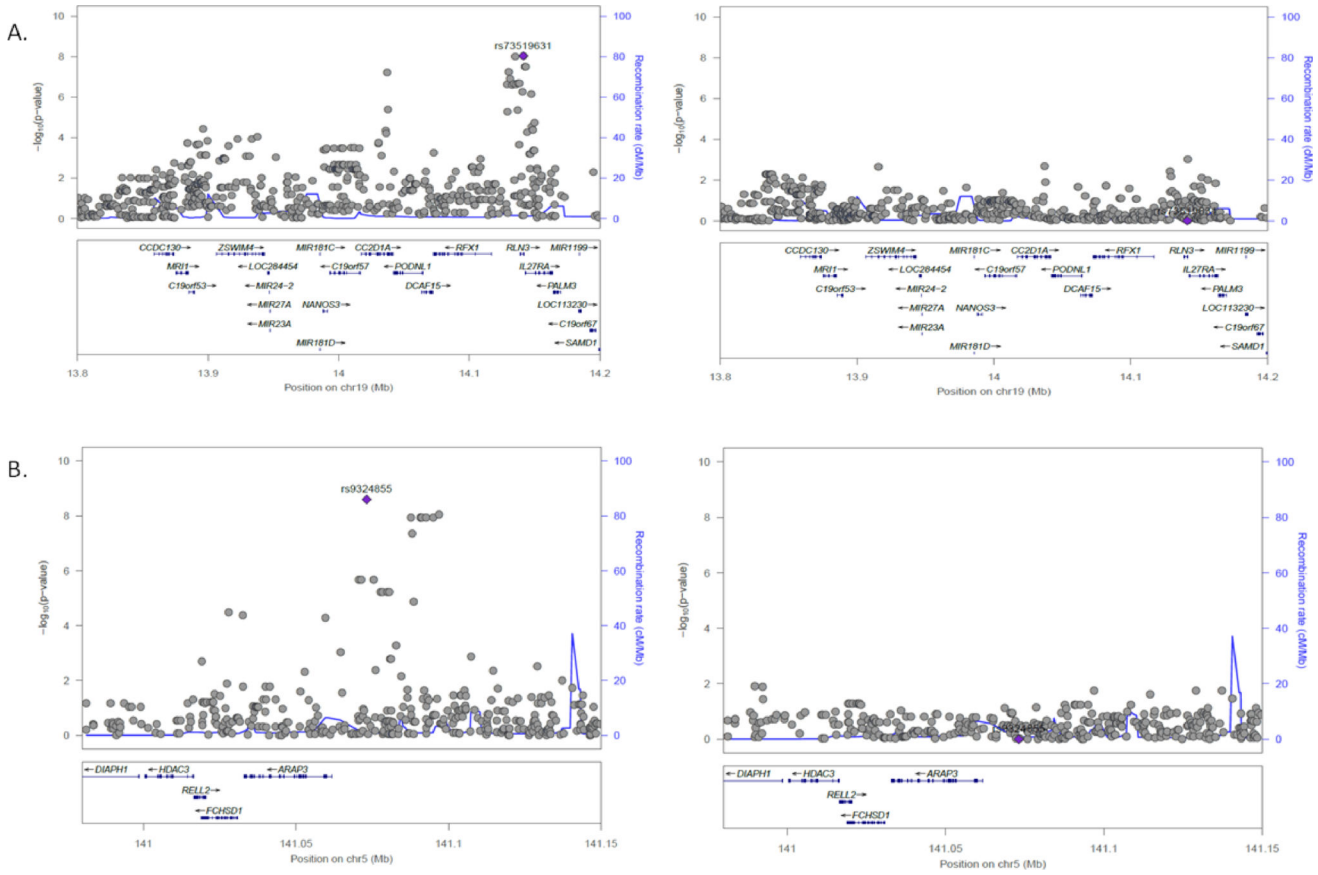

Figure 6. Locus-zoom plots show location of *cis*-regulatory variants relative to the genes they are associated with as well as their significance conditioned on the most significant SNP

(A) *ZSWIM4* and (B) *HDAC3*

Table 1

Top differentially expressed genes in response to treatment with $1\alpha,25(OH)_2D_3$ at 6 hours.

	Gene	Log Fold Change	p-value	q-value
Up-regulated	*CD14*	2.77	1.15E-25	9.95E-22
	CYP24A1	2.34	7.65E-23	3.31E-19
	MAT2A	1.18	1.19E-22	3.44E-19
	EFTUD1	0.82	2.04E-21	4.41E-18
	TSKU	1.09	1.12E-19	1.94E-16
	NET1	0.78	4.45E-19	6.42E-16
	SLC9A1	0.48	4.49E-18	5.55E-15
	TRIM38	0.46	1.12E-17	1.21E-14
	EID3	0.38	3.32E-17	3.20E-14
	ZSWIM6	0.41	4.20E-17	3.63E-14
	SLC16A5	0.47	5.33E-17	4.19E-14
	HAS3	0.56	1.34E-16	9.50E-14
	TLR4	0.47	1.43E-16	9.50E-14
	UNQ338	0.68	1.76E-16	1.08E-13
	NDEL1	0.21	2.35E-16	1.36E-13
	CKLF	0.42	7.59E-16	4.10E-13
	FAM116B	0.19	9.65E-16	4.91E-13
Down-regulated	*JPH1*	−0.24	2.07E-15	8.94E-13
	ADORA2B	−0.46	1.11E-14	3.55E-12
	PIM3	−0.14	6.76E-14	1.67E-11
	ANKRD57	−0.54	1.86E-13	4.24E-11
	SCNN1G	−0.75	2.38E-13	5.15E-11
	BIRC3	−0.35	3.91E-12	5.73E-10
	ST3GAL4	−0.50	3.42E-11	4.00E-09
	PAG1	−0.18	5.06E-11	5.55E-09
	ATAD4	−0.31	7.17E-11	7.56E-09
	EFNA4	−0.14	1.06E-10	1.06E-08
	TNFRSF21	−0.28	1.57E-10	1.48E-08
	NFKBIZ	−0.19	1.95E-10	1.79E-08
	CBLB	−0.16	2.15E-10	1.96E-08
	SGK1	−0.34	2.49E-10	2.20E-08
	SLC16A9	−0.34	2.90E-10	2.54E-08
	PBEF1	−0.32	3.07E-10	2.66E-08
	WSCD1	−0.22	3.31E-10	2.75E-08

There were 465 genes that were up-regulated and 418 that were down-regulated. Listed here are the top up- and down-regulated genes with log fold change in response to vitamin D treatment, p-values and q-values.

Table 2

Genes significant differences in log fold change (LFC) response to 1α,25$(OH)_2D_3$ treatment between African- and European-Americans

Gene	Description	Location	Effect size of ΔLFC(EA-AA)	p-value	Adjusted p-value
UPP1	*Uridine phosphorylase 1*	7p12.3	0.46	5.73×10^{-7}	1×10^{-4}
UCKL1	*Uridine-cytidine kinase 1 like 1*	20q13.33	0.23	3×10^{-4}	0.02
EPHA2	*EPH receptor A2*	1p36.13	0.21	7×10^{-4}	0.03
MFSD2A	*Major facilitator superfamily domain containing 2A*	1p34.2	0.14	7×10^{-4}	0.03
ERCC1	*ERCC excision repair 1, endonuclease non-catalytic subunit*	19q13.32	0.22	8×10^{-4}	0.03
ZSWIM4	*Zinc finger SWIM-type containing 4*	19p13.13	0.10	3×10^{-3}	0.02
KIAA1324L	*KIAA1324 like*	7q21.12	0.20	2×10^{-3}	0.04
CLRN3	*Clarin 3*	10q26.2	−0.24	2×10^{-3}	0.04

J Steroid Biochem Mol Biol. Author manuscript; available in PMC 2017 October 16.

Table 3

Treatment-specific *cis* regulatory variants associated with transcriptional response to 1α,25(OH)$_2$D$_3$

SNP	Gene	p-value, Ethanol	FDR (Ethanol)	p-value, Vitamin D	FDR (Vitamin D)	*in silico* functional annotation
Vitamin D						
rs10958714	POLB	0.14	0.84	1.84×10^{-8}	1×10^{-3}	in LD with rs3136717 (r^2 = 0.85): active enhancer and promoter histone marks in fetal large intestine, sigmoid colon and colonic and rectal mucosa; rs2272733 (r^2 = 0.8): enhancer histone marks in rectal mucosa
rs12104572	CRIPT	1×10^{-3}	0.31	1.51×10^{-7}	5×10^{-3}	in LD with 2 SNPs: enhancer and promoter histone marks in fetal large intestine, sigmoid colon and colon and rectal mucosa; rs1531133 (r^2 =0.84): open chromatin in fetal large intestine; GTEx eQTL in sigmoid colon
rs17713396	SH3YL1	1×10^{-3}	0.37	3.49×10^{-7}	8×10^{-3}	in LD with rs7595075 (r^2 =0.97): enhancer and promoter histone marks in fetal large intestine, sigmoid colon, colonic and rectal mucosa; open chromatin in fetal large intestine; in LD with rs60484953 (r^2 =0.97) which alters VDR motif; GTEx eQTL in sigmoid and transverse colon
Ethanol						
rs13093517	CCR4	2.10×10^{-8}	1×10^{-3}	0.977	0.96	-
rs6011978	ZNF334	3.94×10^{-8}	2×10^{-3}	1×10^{-3}	0.39	in LD with rs847053 (r^2 =0.95): enhancer and promoter histone marks in fetal large intestine, sigmoid colon, colonic and rectal mucosa; open chromatin in fetal large intestinel GTEx eQTL in sigmoid and transverse colon
rs6808387	SFMBT1	4.64×10^{-8}	2×10^{-3}	1×10^{-3}	0.37	Enhancer and promoter histone marks in fetal large intestine, sigmoid colon, colonic and rectal mucosa; in LD with rs12488768 (r^2 =0.88) which alters VDR motif; GTEx eQTL in transverse colon
rs16989474	YWHAB	7.10×10^{-8}	3×10^{-3}	0.05	0.77	Enhancer histone marks in sigmoid colon, colonic and rectal mucosa; in LD with rs2267850 (r^2 =0.99): enhancer histone and promoter marks in fetal large intestine, sigmoid colon, colonic and rectal mucosa; GTEx eQTL in sigmoid and transverse colon
rs10489821	CD1D	8.08×10^{-8}	3×10^{-3}	3×10^{-3}	0.47	in LD with rs859010 (r^2 = 0.95): enhancer and promoter histone marks in fetal large intestine, sigmoid colon, colonic and rectal mucosa; alters VDR motif; GTEx eQTL in transverse colon
rs13360054	NSA2	3.77×10^{-8}	8×10^{-3}	2×10^{-3}	0.42	GTEx eQTL in sigmoid and transverse colon
rs12411798	CASP7	4.16×10^{-8}	9×10^{-3}	1×10^{-3}	0.31	GTEx eQTL in sigmoid and transverse colon
rs3734737	IYD	4.29×10^{-8}	9×10^{-3}	0.02	0.69	Enhancer and promoter histone marks in rectal and colonic mucosa; open chromatin in fetal intestine, sigmoid colon, colonic and rectal mucosa

J Steroid Biochem Mol Biol. Author manuscript; available in PMC 2017 October 16.

Table 4

Population specific *cis* regulatory variants associated with transcriptional response to 1α,25(OH)$_2$D$_3$ when ancestry and serum 25(OH)D$_3$ levels are removed from the model (FDR ≤ 10%)

Gene	SNP	Location (bp)/distance from gene (Kb)	Effect size	p-value	*In silico* functional annotation*
ZSWIM4	rs73519631	14,141,271 bp/198 Kb	0.97	9.21×10^{-9}	In LD with rs6511905 (r^2= 0.97): Enhancer and promoter histone marks in sigmoid colon, colonic, rectal mucosa and fetal large intestine; open chromatin in fetal large intestine
	rs897785	14,037,094 bp /94 Kb	1.37	6.11×10^{-8}	Enhancer histone marks and open chromatin in rectal mucosa 3 SNPs in LD with rs897785 (r^2=0.5) alter VDR binding
HDAC3	rs9324855	141,073,166 bp/57 Kb	1.24	2.56×10^{-9}	Enhancer histone marks in sigmoid colon, colonic and rectal mucosa; promoter histone marks in colonic and rectal mucosa; open chromatin in colonic mucosa
	rs451491	141,087,721 bp/71 Kb	1.44	1.17×10^{-8}	enhancer histone marks in rectal mucosa and alters VDR motif rs248617 (r^2=0.8 with rs451491): enhancer histone marks in fetal large intestine; rs374769 (r^2=0.99): enhancer histone marks in colonic and rectal mucosa; rs368688 (r^2 = 1): enhancer histone marks in rectal mucosa

*annotation from HaploReg (36) includes data for eQTL and all variants in linkage disequilibrium (r^2 > 0.8)

eQTL, expression quantitative trait loci; SNP, single nucleotide polymorphism; Beta, genotypic effect size; FDR, false discovery rate; LD, linkage disequilibrium

Table 5

Population differences in transcriptional response for *cis* eQTLs within 150 kb of genes with inter-ethnic differences in transcriptional response to 1α, $25(OH)_2D_3$.

			Allele Frequencies					
Gene	**SNP**	**Minor Allele**	**AA**	**EA**	**ASW***	**CEU***	**Observed/Expected Ratio**	**Empirical p-value**
ZSWIM4	rs897785	G	0.54	0	0.42	0	0.96	0.02
HDAC3	rs9324855	G	0.83	0.43	0.67	0.47	0.98	0.01

Shown here are allele frequency of the minor allele from our genotyped data and the ASW and CEU populations from the 1000 Genomes Project for the top associated eQTLs with *ZSWIM4* and *HDAC3* vitamin D response, as well as ratios of observed to expected phenotypic values and an empirical p-value as a measure of significance.

*ASW, African-Americans in Southwest; CEU, Caucasians with European ancestry in Utah