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A genome-wide survey of CD4+ lymphocyte regulatory genetic variants identifies novel asthma genes

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Abstract

Background—Genome-wide association studies have yet to identify the majority of genetic variants involved in asthma. We hypothesized that expression quantitative trait locus (eQTL) mapping can identify novel asthma genes by enabling prioritization of putative functional variants for association testing.

Objective—We evaluated 6,706 cis-acting expression-associated variants (eSNP) identified through a genome-wide eQTL survey of CD4+ lymphocytes for association with asthma.

Methods—eSNP were tested for association with asthma in 359 asthma cases and 846 controls from the Childhood Asthma Management Program, with verification using family-based testing. Significant associations were tested for replication in 579 parent-child trios with asthma from Costa Rica. Further functional validation was performed by Formaldehyde Assisted Isolation of Regulatory Elements (FAIRE)-qPCR and Chromatin-Immunoprecipitation (ChIP)-PCR in lung derived epithelial cell lines (Beas-2B and A549) and Jurkat cells, a leukemia cell line derived from T lymphocytes.

Results—Cis-acting eSNP demonstrated associations with asthma in both cohorts. We confirmed the previously-reported association of *ORMDL3/GSDMB* variants with asthma (combined $p=2.9 \times 10^8$). Reproducible associations were also observed for eSNP in three additional genes: *FADS2* ($p=0.002$), *NAGA* ($p=0.0002$), and *F13A1* ($p=0.0001$). We subsequently demonstrated that *FADS2* mRNA is increased in CD4+ lymphocytes in asthmatics, and that the associated eSNPs reside within DNA segments with histone modifications that denote open chromatin status and confer enhancer activity.

Conclusions—Our results demonstrate the utility of eQTL mapping in the identification of novel asthma genes, and provide evidence for the importance of *FADS2*, *NAGA*, and *F13A1* in the pathogenesis of asthma.

Keywords

Asthma; CD4+; lymphocytes; regulatory variants; Expression Quantitative Trait Locus (eQTL); Haplotype; Integrative Genomics

Introduction

Genetic factors influence an individual's asthma susceptibility, with heritability estimates ranging from 36–79%¹. Among the various genetic mapping techniques used to identify the genetic determinants of complex diseases, genome-wide association studies (GWAS) have

been most fruitful. Individual studies and two meta-analyses^{2, 3} have identified common sequence variants in at least 10 genes that are consistently associated with asthma in children and adults, including the *ZBP2/GSDMB/ORMDL3* locus (17q), *IL1RL1* (2q), *IL33* (9p), and *TSLP* (5q). Though some age- and ethnic-specific trends were noted, these loci are widely reproducible and represent important advances in our understanding of asthma. However, as with virtually all complex diseases, the identified asthma loci explain only a small proportion of the total estimated genetic risk⁴, suggesting that novel approaches are required to identify other genetic variants underlying this “missing heritability.”

Expression quantitative trait loci (eQTL) mapping seeks to identify genetic variants that regulate gene expression by considering the distributions of gene transcript abundance for thousands of genes (measured using microarrays) as individual quantitative traits⁵. From more than 20 such studies, expression-associated variants (eSNPs) have been mapped for thousands of expressed genes, explaining, on average, 5 to 15% of the observed variation in gene expression. Though many eSNP demonstrate wide-spread effects across diverse tissue types, tissue-specific patterns are often observed. Importantly, we and others have demonstrated that, compared to random SNP, identified eSNP are more likely to be associated with complex disease traits^{6, 7}. In a study of the HapMap lymphoblastoid cell lines (LCLs), Nicolae et al. reported ~ 2-fold enrichment for eSNP among disease-associated variants⁶. Similarly, in primary peripheral blood CD4+ T-cells, we found the prevalence of eSNP among disease-associated SNPs was 63% higher than the genome-wide average⁷. These findings suggest that eQTL identification could be leveraged to prioritize SNP for association testing and for localization of functional genetic variants.

Given the central role of the CD4+ lymphocyte in allergic responses and the pathogenesis of asthma^{8, 9}, we hypothesized that eSNP identified from CD4+ lymphocytes would be enriched for asthma-susceptibility variants, thus enabling identification of novel variants and their associated candidate genes from existing GWAS data. We tested this hypothesis in a study of childhood asthma, the results of which we report here. Some of the results of this study have been previously reported in the form of an abstract¹⁰.

Methods

We provide a brief description of the cohorts below, with details in the Online Repository. The Institutional Review Board of Brigham and Women’s Hospital approved these studies. Subject recruitment and procedures for the cohorts have been previously described^{7, 11, 12}

The Childhood Asthma Management Program (CAMP): Discovery Population

The Childhood Asthma Management Program (CAMP) was a multicenter randomized, double-blind, placebo controlled clinical trial established to investigate the effects of inhaled anti-inflammatory asthma medications¹¹. Children enrolled in CAMP had mild-moderate persistent asthma based on the demonstration of airway responsiveness to methacholine PC₂₀ (provocative concentration causing a 20% fall in FEV₁) less than or equal to 12.5mg/ml, and at least two of the following: asthma symptoms at least two times per week, the use of inhaled bronchodilator at least twice weekly, or the use of daily asthma medication for at least six months in the year prior to screening¹¹. DNA was available for

403 parent-child trios and an additional 66 singletons of self-reported non-Hispanic white ancestry with mild-to moderate persistent asthma, therefore, 469 self-reported white CAMP subjects were genotyped with Illumina Infinium II HumanHap 550 or Human610W–Quad BeadChips (San Diego, CA). Non-asthmatic controls were selected from 1,533 subjects in the Illumina’s iControlIDB resource as previously described¹³. We generated a genetically matched case-control cohort using GEM¹⁴, resulting in a final dataset of 359 asthmatic and 846 control subjects, and 547,497 SNPs.

In 200 CAMP participants, we collected peripheral blood CD4+ lymphocyte RNA and performed a genome-wide eQTL analysis for cis-acting eSNP as previously described in detail⁷. Expression profiling was performed using Illumina Human Ref8 v3 array. Similar to observations by others, our prior eQTL analysis demonstrated a precipitous fall in the prevalence of cis-acting eQTL away from the target gene locus, with a substantial increase in the false discovery rates when considering variants more than 50kb from the transcript^{6, 7}. Thus, to limit the potential for type I error inflation, we limited our search space to SNPs of frequency $\geq 10\%$ situated within 50 kb of the transcript. This analysis resulted in the identification of 6,706 cis-acting eSNPs regulating 1,585 genes⁷. Given our prior studies demonstrating enrichment of susceptibility variants for eSNP, in this current study we prioritized these 6,706 variants for asthma-association testing in the CAMP cohort. An overview of our study design is shown in Supplemental Figure E1.

CAMP: Asthma-susceptibility eSNP association testing

Case-control asthma association analysis was performed for the 6,706 eSNP detected in CD4+ lymphocytes using PLINK¹⁵, with EIGENSTRAT adjustment¹⁶, and retested in a subset of 403 available parent-child trios using PBAT. Haplotype association testing was conducted using Haploview (for asthma) and *haplo.score* (for expression traits).

Replication of Genetic Associations in the Genetic Epidemiology of Asthma in Costa Rica Cohort (GACRS)

Replication studies were performed in 579 parent-child trios recruited as part of the Genetic Epidemiology of Asthma in Costa Rica cohort. Details on subject recruitment and study protocols have been published elsewhere¹². In brief, children ages 6 to 14 years were included in the study if they had asthma (a physician’s diagnosis of asthma and ≥ 2 respiratory symptoms or asthma attacks in the previous year) and a high probability of having ≥ 6 great-grandparents born in the Central Valley of Costa Rica, which increased the likelihood that children would be descendants of the founder population of the Central Valley¹².

Only the variants that demonstrated a nominal association with asthma in CAMP ($p < 0.01$) were tested for association with asthma susceptibility in the Costa Rican cohort using family-based methods in PBAT. The transmitted to untransmitted ratio (T:U ratio) for the significant associations were identified in PLINK. Results were considered significant when identical associations (i.e. same allele, phenotype, and direction of genetic effect) were identified in both populations. Fisher’s combined p-value method was applied to assess the cumulative significance of association across populations¹⁷.

Validation: Expression of novel asthma genes between asthmatics and non-asthmatic controls in Asthma BRIDGE

Expression of the *FADS2*, *NAGA*, and *F13A1* genes was compared between asthmatic subjects (n=300) and non-asthmatic controls (n=122) based on gene expression profiling in CD4+ lymphocytes from the Asthma BioRepository for Integrative Genomic Exploration (Asthma BRIDGE). Asthma BRIDGE is a multicenter-collaborative effort to develop well-characterized translational genomic datasets for asthma in North America¹⁸. Samples were collected through October 2011 from among more than 14,000 subjects studied by the EVE Consortium, providing broad representation of the North American asthmatic population. Genome-wide gene-expression data (Illumina Human HT-12 v4 array) was generated from asthma-relevant primary cell types, including peripheral blood CD4+ lymphocytes. Differential expression of *FADS2*, *NAGA*, and *F13A1* was determined using a linear model adjusted for age, gender, and race using the limma package in Bioconductor¹⁹.

Functional Validation: Chromatin enrichment studies

Human Beas-2B (#CRL-9609, ATCC) and A549 (#CCL-185, ATCC) cells were cultured in complete DMEM medium, and Jurkat (TIB-152, ATCC) cells were cultured in RPMI 1640 medium, all supplemented with 10% FBS, penicillin (50 units/ml), streptomycin (50 mg/ml) and gentamicin (10 µg/ml). Additional details can be found in the online repository.

For FAIRE (Formaldehyde-Assisted Isolation of Regulatory Elements) and Chromatin immunoprecipitation (ChIP) followed by real-time PCR, see details in Online Repository. The sequence of primers used in FAIRE-qPCR and ChIP-qPCR was listed in Supplemental Table E1.

Results

Asthma association testing of eSNP

The baseline characteristics of the CAMP and Costa Rican index cases included in this study are presented in Table 1. Baseline characteristics were similar between the CAMP and Costa Rican cohorts including measures of asthma severity and lung function.

Of the 6,706 SNP identified as CD4+ lymphocytes cis-acting expression associated eSNP, 143 were associated with asthma under an additive genetic model in the CAMP cohort in either the case-control or family-based association analyses (Supplemental Table E2). To confirm their associations, we next genotyped these 143 variants in the Costa Rican cohort. Associations were reproduced in this independent, ethnically distinct cohort for multiple variants in four genes (Table 2). Firstly, we were able to replicate the association of the chromosome 17q *ORMDL3/GSDML* locus with asthma²⁰. Specifically, two variants (rs4795405 and rs7216389) were strongly associated with asthma in both cohorts (Fisher's combined p value= 2.9×10^{-8} and 3.4×10^{-7} , respectively). The T allele of rs4795405 allele was associated with decreased *ORMDL3* expression (Figure 1A)⁷, and confers a decreased risk of asthma in two independent cohorts. As previously reported, these variants reside on a haplotype that includes rs1293623, a functional regulatory polymorphism that alters DNA

binding affinity with the insulator CTCF, resulting in differential expression of three surrounding genes: *ORMDL3*, *GSDML* and *ZBP2*²¹.

We also found consistent association in both cohorts for three loci never previously associated with asthma susceptibility: the Fatty Acid Desaturase locus (*FADS1/FADS2*) on 11q, N-acetyl galactosaminidase (*NAGA*) on chromosome 22q, and the factor XIII A subunit (*F13A1*) locus on chromosome 6p. On chromosome 11q12.2, the minor C allele of rs174611 (C) is associated with increased expression of two adjacent members of the fatty-acid desaturase gene cluster (*FADS1* and *FADS2*, FDR $p=0.001$ and 0.0007 , respectively, Figure 1B). The C allele of this variant confers an increased risk of asthma in both CAMP and Costa Rica (Fisher's combined p value= 0.002). On chromosome 22q13.2, the C allele of SNP rs2413669, located ~40kb upstream of the transcription start site of the alpha-N-acetylgalactosaminidase precursor gene (*NAGA*), was associated with decreased *NAGA* expression (FDR $p=0.0003$, Figure 1C), as well as with decreased odds of asthma in both cohorts (Fisher's combined $p=0.0002$). On chromosome 6p25.1, the T allele of variant rs11243081 was associated with increased expression of the coagulation factor XIII A1 gene (*F13A1*, FDR $p=0.0009$, Figure 1D), as well as with increased asthma susceptibility in both cohorts (Fisher's combined $p=0.0001$). Of note, the magnitudes of the effect for each of these genetic variants on asthma susceptibility were similar in both cohorts.

Haplotype testing of asthma-associated regulatory loci

Using genome-wide genotypic data available in CAMP subjects, we next tested haplotype blocks for each of the three novel genes identified in this study for association with gene expression and asthma susceptibility. Figure 2 illustrates the results of the association analyses of the *FADS 2* locus. SNP rs968567, located in a conserved promoter region of the *FADS2* locus, was associated with *FADS2* expression ($p=10^{-15}$), explaining 30% of its variance. In addition, the minor allele of SNP rs174611, which is associated with asthma in CAMP and Costa Rica, explains 11% of the variance in *FADS2* gene expression ($p=0.0007$) and 5.3% of the expression in *FADS1* ($p=0.03$). Figure 3a shows the haplotype structure of the *FADS2* locus. The T allele of SNP rs968567 uniquely tags a common haplotype in block 2 (H2), which is observed in 18.1% of the subjects. SNPs rs174627 and rs174611 are in linkage disequilibrium (LD) with SNP rs968567; all three of these variants and the *FADS* H2 haplotype are associated with asthma susceptibility in the CAMP cohort ($p=0.001$ to 3×10^{-4}). Furthermore, as seen in Figure 3b, haplotype 2 is associated with increased *FADS2* expression ($p=10^{-10}$), suggesting that this is a functional regulatory haplotype in the *FADS* region.

In contrast, the genomic region of eQTL association for *F13A1* is relatively narrow (Supplemental Figure E2) despite of its relative large gene size: 177kb. Although there are three haplotype blocks demonstrating eQTL association (Supplemental Table E4), only one block (Block 9) is strongly associated with both *F13A* expression ($p=0.0007$) and also with asthma ($p=0.003$).

In the *NAGA* association analysis, the minor allele of SNP rs2413669 explains 6% of the variation in expression of *NAGA* (Supplemental Figure E3, $p=0.0003$). However, unlike the *FADS* and *F13A1* loci, haplotype block analysis did not help localize the functional variant:

haplotype blocks in *NAGA* were not significantly associated with either asthma or *NAGA* expression ($p > 0.05$), suggesting the functional regulatory variant likely resides on more than one haplotype, in LD with the asthma-associated eSNP rs2413669.

Validation: Differential *FADS2* expression in asthma in Asthma BRIDGE

Having demonstrated that variants that regulate gene expression in CD4+ lymphocytes were reproducibly associated with asthma, we next assessed whether the expression of target genes were similarly correlated with asthma status. We therefore assessed the expression of *FADS2*, *NAGA* and *F13A1* in 300 asthma cases and 122 non-asthmatic controls participating in Asthma BRIDGE with available expression data in peripheral blood CD4+ lymphocytes (see Supplemental Table E3 for baseline characteristics of the cohort used for this analysis). Of the three genes evaluated, *FADS2* mRNA expression was significantly increased in asthmatics compared to non-asthmatic controls ($p=0.003$, Figure 4). *FADS1* expression was also modestly increased in asthma cases compared to controls ($p=0.04$), and a trend towards increased *NAGA* expression was also observed in asthma cases ($p=0.05$). There was no evidence of asthma-related differential expression of *F13A1* ($p=0.60$).

Functional Validation: Open chromatin enrichment studies

The expression-associated regulatory variants identified by eQTL mapping are often surrogates for functional regulatory variation situated in neighboring regulatory DNA regions^{22, 23}. However, given that the *FADS2* rs968567 regulatory variant uniquely tags the asthma-associated H2 haplotype, and the *NAGA* rs2413669 variant (but not *NAGA* haplotypes) is associated with asthma and *NAGA* expression, it is possible that these two disease associated eSNPs are not merely surrogates or tagging variants, but actually represent the functional variants regulating the target gene expression thus driving the asthma association. To assess this, we first screened for active regulatory activity at the three loci by FAIRE-qPCR, a method used to detect open chromatin regions devoid of nucleosomes²⁴. Given that the eQTL analysis was performed in CD4+ T cells, we chose the Jurkat acute T cell leukemia cell line, in addition to two respiratory epithelial cell lines: Beas-2B (human bronchial epithelial cells) and A549 (human alveolar type II-like pneumocyte). Three SNPs that showed association with asthma in each region were chosen for open chromatin assessments in these cell lines. As shown in Figure 5, the *FADS2* associated SNP rs968567 showed ~15 fold enrichment of FAIRE signals in A549 and Jurkat cells and ~ 5 fold enrichment in Beas-2B cells compared to the negative control region ($p < 0.01$, unpaired t test), consistent with previous finding that rs968567 is a functional SNP with differential binding to transcription factor ELK-1²⁵. Furthermore, in the *NAGA* association region, we observed strong enrichment of FAIRE signals near rs1801311 in Jurkat cells as well as in Beas-2B and A549 cells. In contrast, FAIRE signals in SNPs around the *F13A1* region were relatively weak, suggesting these SNPs are not themselves functional, but rather tag the causal variants within block 9.

Epigenetic modifications on histone tails of nucleosomes usually indicate active or repressive transcriptional regulations²⁶. To further confirm our finding about potential regulatory SNPs indicated by FAIRE, we applied ChIP-PCR with antibodies targeting two enhancer markers H3K4Me1 (indicating enhancer regions) and H3K27Ac (indicating active

enhancer and/or promoter regions) in the *FADS2* and *NAGA* regions in Beas-2B, A549 and Jurkat cells (Figure 6). Consistent with FAIRE enrichment signals, H3K27Ac showed significant enrichment at two SNPs we tested in *FADS2* and *NAGA* regions in almost all three cell lines, indicating active regulatory elements are contained in both the *NAGA* and *FADS2* regions near eSNPs.

Discussion

Although GWAS of complex traits have offered insights into the genetic underpinnings of complex diseases like asthma, the discovered variants explain only a fraction of the genetic contribution to such diseases⁴. In some part, this is likely due to the stringent multiple comparisons burden imposed when testing millions of (mostly functionless) variants. Strategies that guide prioritization of functional genetic markers for testing can address this issue. We and others have demonstrated that eSNP are more likely to be disease-associated variants^{6, 7} suggesting eQTL mapping as a promising strategy. Using asthma as a model, we sought to evaluate this possibility by testing cis-acting regulatory variants previously identified from an eQTL mapping study of peripheral blood CD4+ lymphocytes for association with asthma in two well-characterized populations. Using this integrative genomic approach in CAMP subjects, we have not only replicated the association of the 17q *ORMDL3/GSDMB* locus for association with asthma in two childhood asthma cohorts, but have also defined three novel candidate loci. These later associations did not emerge from prior studies as their nominal association p-values were below the threshold for multiple comparisons correction. Though our application of a functional prioritization strategy using eQTL data led to consideration and successful independent replication of these loci in the Costa Rican cohort, supporting application of such strategies for identification of novel disease candidate genes, they will require follow-up replication testing to assess whether the results are generalizable. We also recognize that the relatively small size of our test and replication cohorts may have precluded identification of additional asthma variants due to limits in statistical power. Application of eQTL approaches to larger populations is warranted. In addition, consideration should be extended beyond cis-acting regulatory variants once adequately powered studies identify validated, reproducible trans-acting eQTLs.

Of three novel genes identified in this study, the greatest biological and epidemiological support is provided for *FADS2* and the fatty acid desaturase gene cluster on chromosome 11. The *FADS1* and *FADS2* genes respectively encode for 5- and 6-desaturase - enzymes that are critical for long chain poly-unsaturated fatty acid (PUFA) biosynthesis, which are the precursors of eicosanoid mediators that are critical to the development and resolution of allergic inflammation^{27, 28}. PUFAs are synthesized from two essential fatty acids: linoleic acid (LA; 18:2n-6) and alpha-linolenic acid (ALA; 18:3n-3). Desaturase enzymes introduce double bonds between defined carbons of the fatty acyl chain. 6-desaturase (*FADS2*) catalyzes the first rate-limiting step in long chain PUFA biosynthesis by causing desaturation of LA and ALA. In our study, we demonstrate association of eSNPs in *FADS2* with asthma in two cohorts, and localize the most robust associations to a haplotype-tagging variant - rs968567, situated in the promoter of *FADS2* that is strongly associated with *FADS2* (and to a lesser degree *FADS1*) expression. We also detected increased expression of

FADS2 in peripheral blood CD4+ lymphocytes of asthmatics in a large population sample, consistent with increased expression of *FADS2* conferred by asthma risk allele in this locus. In addition, rs968567 demonstrates strong enrichment for open chromatin status in both lymphocyte and bronchial epithelial cell lines. In hepatocytes, this same variant exhibits differential binding of the ELK1 transcription factor²⁵. This variant, and others in strong LD with it, have been strongly associated with circulating PUFA²⁹, total cholesterol and high-density lipoprotein, and omega-3 fatty acid levels³⁰. Perhaps most pertinent in the context of asthma pathogenesis, a recent GWAS study in a general population sample²⁹ found this *FADS2* promoter variant to explain 19% of the population variance in serum levels of arachadonic acid (AA) – the precursor for pro- and anti-inflammatory prostaglandins and leukotrienes²⁷.

Due to their roles in the production of pro-inflammatory and anti-inflammatory eicosanoids, and evidence for PUFAs in the alteration of T-cell antigen presentation³¹, others have investigated the role of *FADS1/FADS2* polymorphism in atopic disease, with variable results. In one candidate-gene birth cohort study of 879 children, *FADS* variants that were associated with PUFA and AA serums levels were also associated with increased eczema risk³². In contrast, these same variants were inversely associated with eczema and allergic rhinitis in an adult population³³. In addition, recent work by Standl and colleagues demonstrated that genetic variants in the *FADS* cluster modify the effect of breastfeeding on the development of asthma with the lowest risk noted in children who were exclusively breast fed for the first three months of life³⁴. Although the biologic mechanism underlying this association is unclear, the authors speculate that carriers of the minor allele have lower fatty acid metabolism and, thus, less arachadonic acid levels³⁴. Though replication of these interactions have yet to be reported, if true, such interaction could represent an important modifier of our observed associations between *FADS2* genotype and asthma susceptibility, posing greater challenges to replication in additional populations. Although none of these studies focused exclusively on asthma, making direct comparisons with our initial observations difficult, assuming a direct correlation between asthma and risk for other atopic diseases, our findings are consistent with those in the pediatric cohort, with high *FADS2* expression associated with increased asthma and atopic risk. The associations may be age dependent (explaining the different findings in adults), or modified by dietary PUFA intake. While these possibilities must be tested in other populations, the evidence presented here, together with that from prior studies support *FADS2* as a biologically plausible asthma-susceptibility gene.

In contrast to *FADS2*, the roles of *NAGA* and *F13A1* in asthma pathogenesis are less clear. *NAGA* is a lysosomal glycohydrolase that cleaves the alpha-N-acetylgalactosaminyl moieties from glycol-conjugates. *NAGA* has been implicated as part of the carbohydrate-mediated mechanism of mast cell adhesion to the bronchial epithelium³⁵. Treatment of mast cells of endo- α -N-acytelgalactosaminidase, an enzyme that cleaves disaccharides on the cell surface of the mast cell, results in a significant reduction of mast cell adhesion to bronchial epithelium and changes in airway responsiveness³⁵. Moreover, a guinea pig model demonstrated increased *NAGA* released from bronchoalveolar macrophages in animals with increased airways responsiveness following high ozone exposure³⁶. Though speculative,

given the central role of macrophages and mast cell degranulation in bronchoconstriction, mucus secretion, and airway inflammation in asthmatics³⁷, the asthma-associated *NAGA* eSNP may influence asthma risk through altered glycohydrolase activity.

F13A1 encodes the alpha subunit of factor XIII, the last enzyme generated in the blood coagulation cascade, stabilizing blood clots by cross-linking fibrin. *F13A1* has also been implicated in the inflammatory cascade and in atopy³⁸. When stimulated with dust-mite antigen, peripheral blood mononuclear cells from dust-mite allergic patients demonstrate increased production of *F13A1*³⁹. *F13A1* has also been implicated as a susceptibility locus for obesity⁴⁰. Epidemiologic data demonstrates a consistent link between asthma and obesity⁴¹, with apparent shared genetic determinants⁴². How *F13A1* regulatory variation confers susceptibility to asthma, and whether *F13A1* represents a shared genetic determinant of both asthma and obesity, remains unclear. In addition to replication in additional asthma populations, further characterization of the functional impact of these variants in model systems will be needed to resolve these questions.

Though we have demonstrated the utility of eQTL mapping for the identification of asthma-susceptibility variants, our studies were limited in scope, particularly in regards to the narrow focus on cis-acting regulatory variation in only one cell type – the CD4+ lymphocyte. Although this cell type is relevant to allergic cytokine profiles and asthma, it is only one of many cell types of potential interest. Given that a substantial proportion of cis-acting regulatory variants demonstrate tissue-specific effects, we have likely overlooked some functional variation specific to other relevant cell types, including antigen presenting cells, granulocytes, airway smooth muscle and bronchial epithelium. A recent eQTL study in human lung tissue samples provides complementary evidence at the 17q locus, suggesting a potential role for altered expression of *GSDMA*, in addition to the altered *ORMDL3* and *GSDMB* expression noted by us and others in lymphocytic lines⁴³. Thus, we view these initial efforts as the first step of a more integrative approach to disease gene mapping that considers eQTL variation across multiple tissue types, in diverse patient populations, and under disease-relevant environmental exposures and that also considers other functional annotations, including chromatin modification, CpG methylation differences, and other epigenetic modifications⁴⁴. Such comprehensive, more holistic approaches, combined with powerful systems based analytical tools, should provide a more complete picture of the genetic landscape in asthma.

In summary, we provide new evidence in support of *FADS2*, *NAGA*, and *F13A1* as asthma-susceptibility loci. Similar to variants previously identified through GWAS and other genetic approaches, the relatively modest effect sizes conferred by these loci limit their clinical utility as diagnostic or prognostic markers. Instead, our observations motivate prioritization of these loci and their related pathways for further investigation. Of these, we find strongest support for a common promoter polymorphism in the *FADS2* gene, which regulates *FADS2* expression, is associated with asthma and allergic phenotypes, and has been previously shown to be the major genetic determinant of plasma arachadonic acid levels – the major precursor of asthma-relevant eicosanoids. Though replication of these associations in additional populations is warranted, these findings should prompt revisiting this important pathway as a potential therapeutic asthma target.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CAMP	Childhood Asthma Management Program
ChIP	Chromatin-Immunoprecipitation-PCR
eQTL	Expression quantitative trait loci
eSNP	Expression-associated variant
<i>FI3A1</i>	Factor XIII, A1
<i>FADS1</i>	Fatty Acid Desaturase 1
<i>FADS2</i>	Fatty Acid Desaturase 2
FAIRE	Formaldehyde Assisted Isolation of Regulatory Elements-qPCR
GACRS	Genetics of Asthma in Costa Rica Study
GWAS	Genome-wide association studies
NAGA	N-acetyl-alpha-D-Galactosaminidase
SNP	Single nucleotide polymorphism

Appendix

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Key Messages

- Integrative genomic approaches including expression quantitative trait loci (eQTL) mapping can be used to prioritize genetic variants for disease susceptibility testing.
- Using a genome-wide survey of regulatory genetic variants in peripheral blood CD4+ lymphocytes sampled from subjects with asthma, we identified novel asthma susceptibility loci, including *FADS1/FADS2*, *NAGA*, and *F13A1*.
- Functional variants can be further localized through a combination of eQTL mapping with assessments of chromatin status and modifications nearby eQTL SNPs.

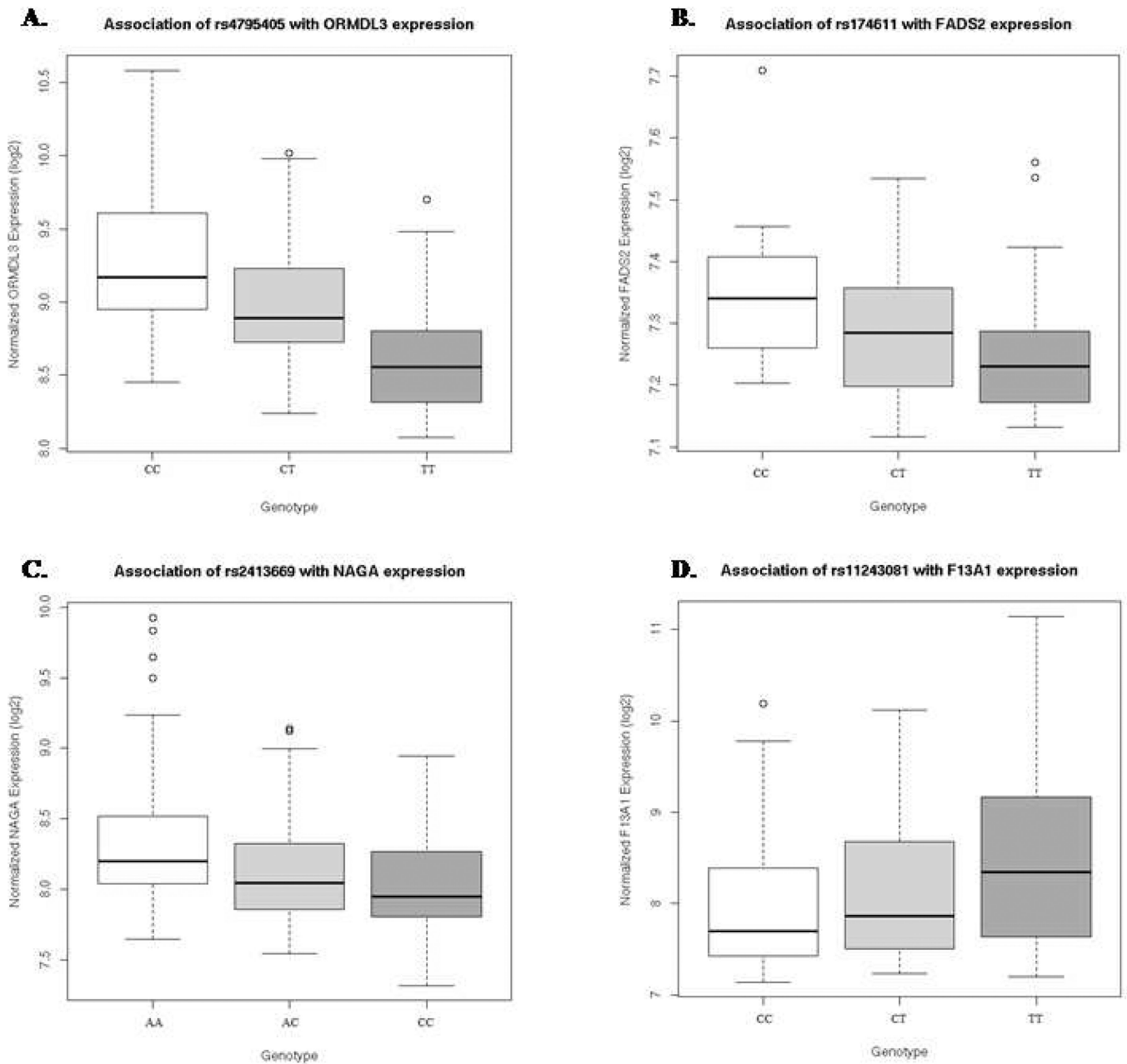


Figure 1.

Genome-wide significant expression quantitative trait loci (eQTLs) previously identified in CD4 positive lymphocytes of asthmatic subjects. A. Association of SNP rs4795405 with the expression of *ORMDL3*. B. Association of SNP rs174611 with *FADS2* expression. C.

Association of SNP rs2413669 with *NAGA* expression. D. Association of rs11243081 with *F13A1* expression.

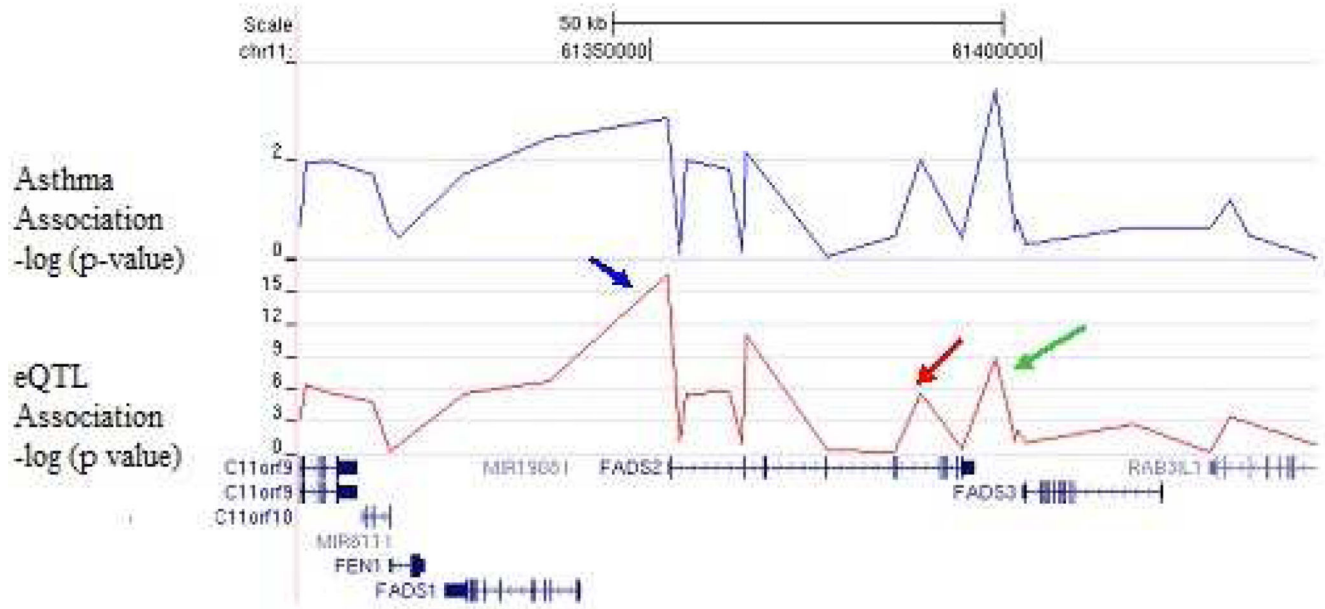


Figure 2. SNP-based eQTL analysis across the *FADS2* locus

SNP-based eQTL analysis across *FADS2*, demonstrating association of rs968567 with *FADS2* expression ($p=10^{-15}$, 30% expression variance explained). SNP rs968567 (blue arrow), located in a conserved region of the promoter, demonstrates association with *FADS2* expression ($p=10^{-15}$). SNPs rs174627 (green arrow) and rs174611 (red arrow) are in LD with rs968567 and are associated with *FADS2* expression and asthma susceptibility.

a)

H1:	AGAGTCCCCAC	.314
H2:	G•GTC•TTT•GA	.181
H3:	GA••••••••	.147
H4:	GA•••T•••••	.137
H5:	G•GTC•TT•TGA	.102
H6:	G•GTC•T••TGA	.041
H7:	G••TC•TT••GA	.028
H8:	G•G•••••••	.019

b)

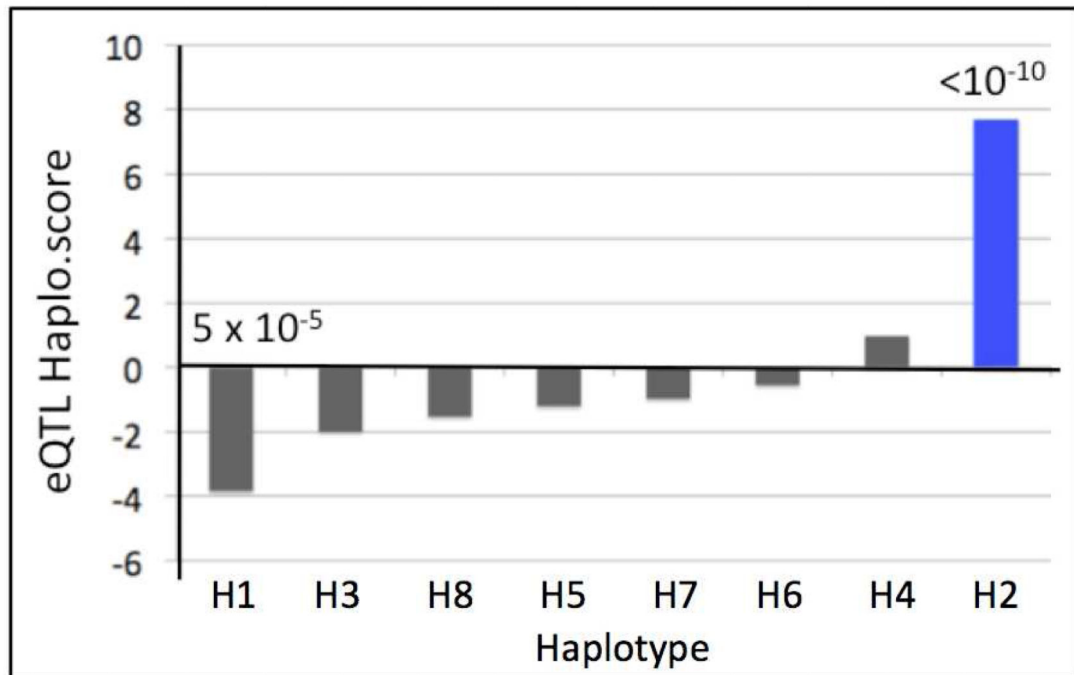


Figure 3. Haplotype analysis of the FADS2 locus

(a) Haplotype structure: Haplotype frequencies observed in at least 1% of CAMP subjects are listed to the right of each haplotype sequence. Dots indicate the allele present in haplotype H1 – the most common haplotype. The asthma-associated rs968567 T allele is unique to, and uniquely tags, haplotype 2 (H2, boxed in blue), observed at 18.1% frequency.

(b) Haplotype-based eQTL analysis: H2 demonstrates strong association with increased *FADS2* expression ($p < 10^{-10}$), while haplotype 1 (H1, frequency 31.4%) is associated with low *FADS2* expression. Others (H3-H8) showed intermediate levels of *FADS2* expression.

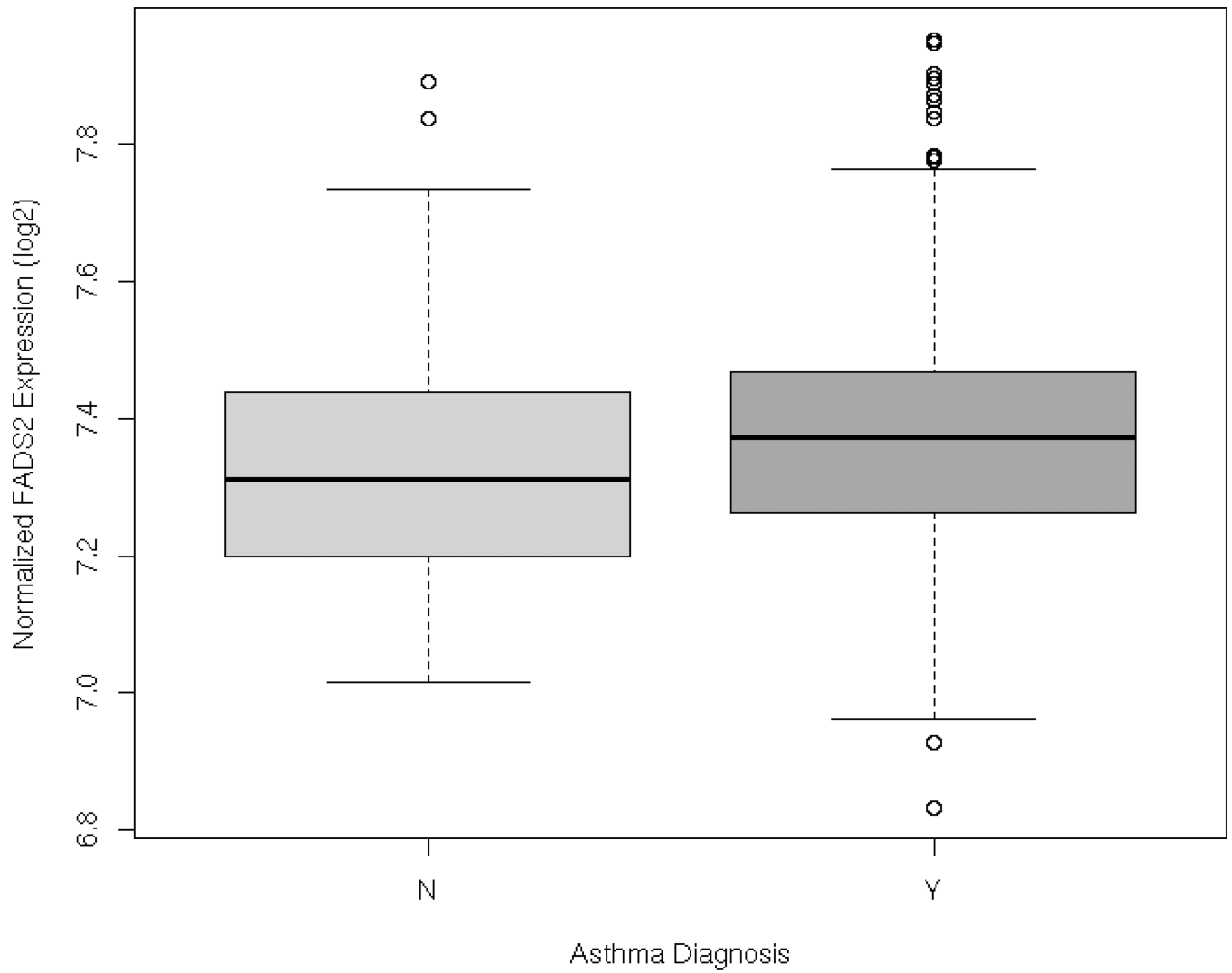


Figure 4. Differential expression of *FADS2* in asthmatic subjects compared to non-asthmatic controls in Asthma BRIDGE

Significantly increased expression of *FADS2* in peripheral blood CD4+ lymphocytes of asthmatic subjects compared to non-asthmatic control subjects ($p=0.003$)

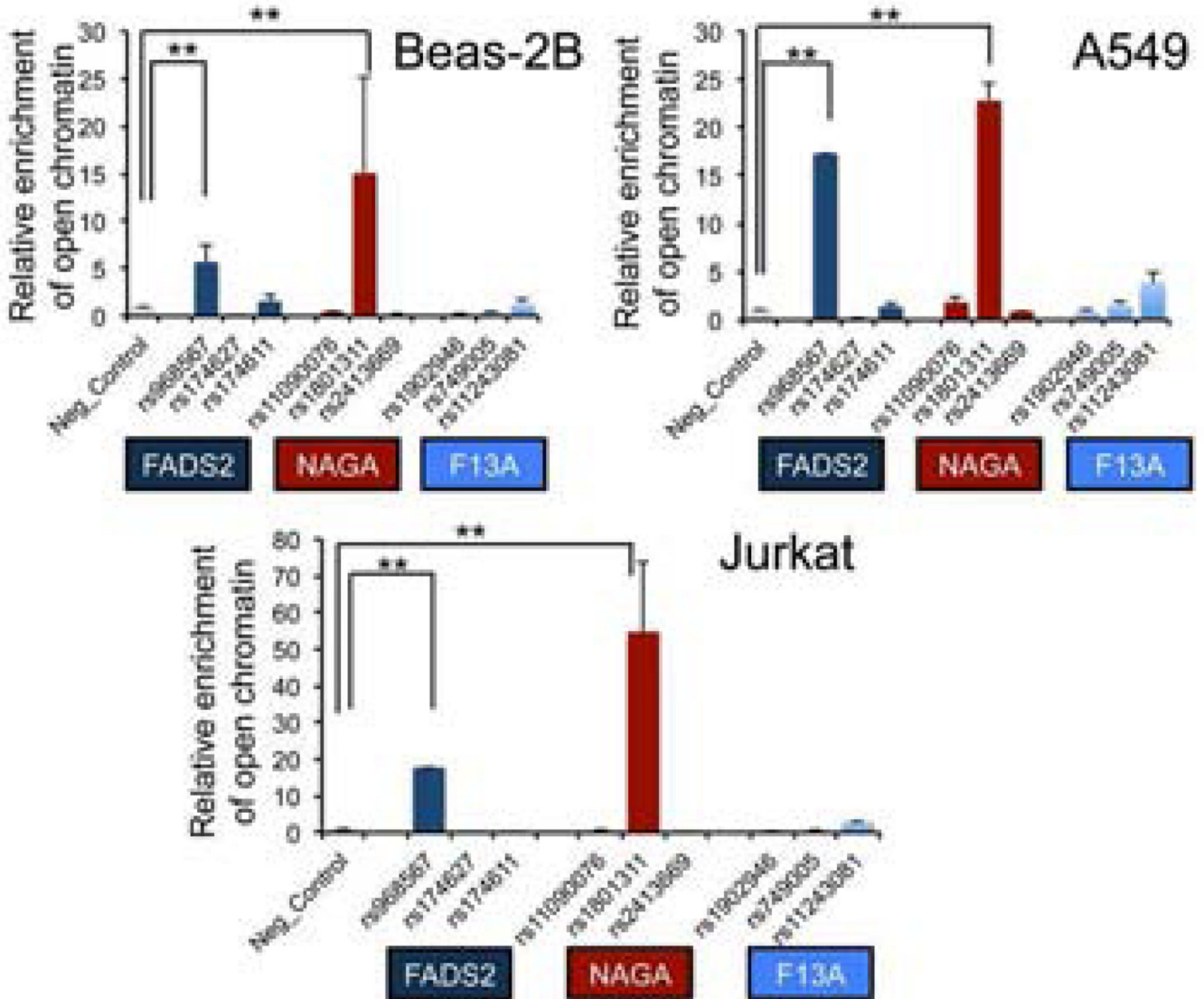


Figure 5. Functional annotation of the three asthma associated regions by FAIRE-PCR analysis Formaldehyde assisted isolation of regulatory enrichment (FAIRE) was performed at three association regions nearby *FADS2*, *NAGA* and *F13A* genes in Beas-2B, A549 and Jurkat cell lines by real-time PCR analysis. The graph depicts the relative FAIRE signal at each locus normalized to input and negative control regions. Each locus contains three SNPs nearby eQTL signals. Mean±SD are from two to four repeats for each SNP. **p<0.01 (unpaired one way *t* test.)

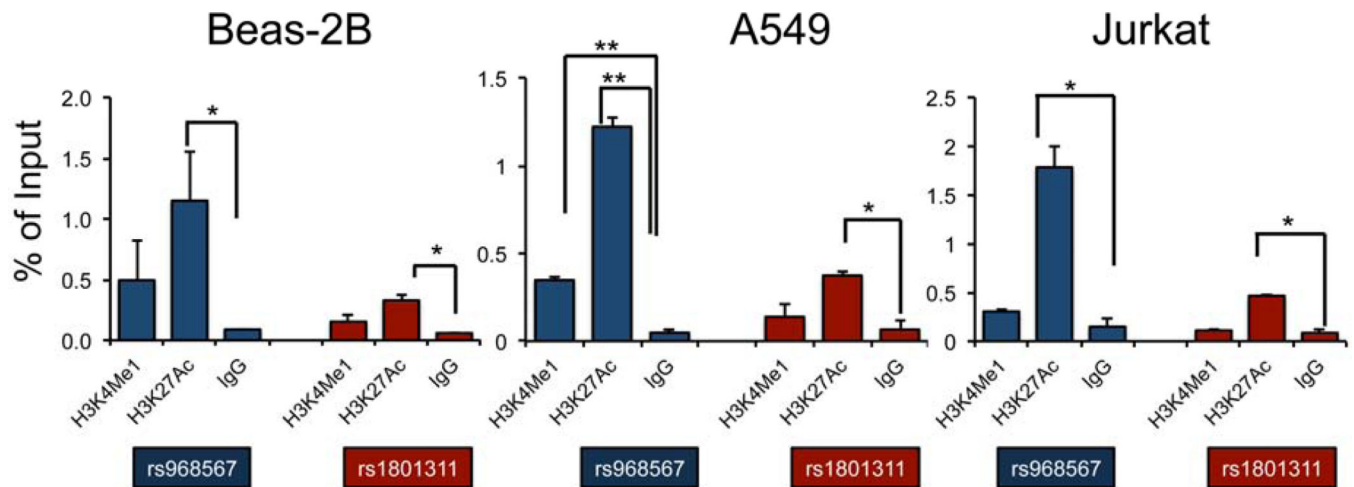


Figure 6. Enrichment of active histone marks in two asthma associated regions detected by ChIP-PCR analysis

Chromatin immunoprecipitation (ChIP) was performed using H3K4Me1 and H3K27Ac antibodies in Beas-2B, A549 and Jurkat cell lines targeting genomic regions nearby two SNPs rs968567 and rs1801311 in *FADS2* and *NAGA* loci respectively. IgG was used as a negative control. The bars represent the average of a two biological replicates with standard deviations. * $p < 0.05$; ** $p < 0.01$ (paired one way t test).

Table 1

Baseline phenotypic characteristics of index children in CAMP and Costa Rica

Variable	CAMP n=359	Costa Rica n=579
Age (years)	8.8 (2.1)	9.1 (1.8)*
Female sex	137 (38%)	222 (41%)
Height (cm)	132.7 (13.3)	131.0 (11.2)
Baseline pre-bronchodilator FEV ₁ (L)	1.6 (0.5)	1.7 (0.5)
Baseline pre-bronchodilator FEV ₁ /FVC (%)	79.1 (8.3)	82.6 (7.2)

* Mean (standard deviation) or count (frequency) reported

Table 2

Replicated associations of eQTLs with asthma susceptibility in the CAMP and Costa Rican cohorts under an additive genetic model

Test of Association	Gene	SNP	Chromosome	Distance (kb from transcript)	eQTL p value (FDR)	Minor Allele	Association p value				
							CAMP	OR	Costa Rica	OR	Fisher's combined p value
Case-Control	<i>ORMDL3</i>	rs4795405	17	-4.6	3.0×10^{-10}	T	0.0007	0.73	2×10^{-5}	0.63	2.9×10^{-8}
	<i>NAGA</i>	rs2413669	22	40.8	0.0003	C	0.0009	0.72	0.02	0.82	0.0002
Family-Based	<i>FADS2</i>	rs174611	11	promoter	0.0007	C	0.009	1.33	0.02	1.2	0.002
	<i>F13A1</i>	rs11243081	6	-16.6	0.0009	T	0.003	1.37	0.003	1.32	0.0001