Functional characterization of the atopy-associated gene *PHF11*

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Background: Polymorphisms in the plant homeodomain finger protein 11 gene *(PHF11)* are associated with increased total serum IgE levels, asthma, and severe atopic dermatitis (AD) in children. Although *PHF11* includes a plant homeodomain, a motif often found in transcriptional regulators, the function of *PHF11* has not been investigated.

Objective: We sought to test (1) whether *PHF11* regulates the transcription of genes involved in allergic disorders and (2) whether polymorphisms in *PHF11* predict changes in the expression or function of this gene.

Methods: Microarray analysis was used to examine the expression of *PHF11* in different immune cell subsets, and the function of *PHF11* was tested by using small interfering RNA–induced knockdown or overexpression of *PHF11* in primary $CD4^+$ T cells or Jurkat T cells. Genotype-dependent effects on *PHF11* expression were tested by using an allele-specific gene expression, and the transcriptional activity of *PHF11* was determined by using luciferase hybrid gene reporter assays and *in vitro* DNA-binding electromobility shift assays.

Results: *PHF11* expression was higher in T_H1 cells relative to that in T_H2 cells, and knockdown of *PHF11* expression reduced expression of the T_H1-type cytokines IFN-γ and IL-2. The G-allele of a 3′ untranslated region polymorphism associated with AD was correlated with reduced abundance of *PHF11* RNA in T_H1 cells, as well as an increase in a *PHF11* isoform lacking exon II. Evidence was also found for a physical and functional interaction between *PHF11* and the p65 subunit of nuclear factor κB.

Conclusion: *PHF11* is a regulator of T_H1-type cytokine gene expression. The reduction in *PHF11* expression seen with an AD-associated genotype could contribute to the strong T_H2 responses that characterize many allergic individuals. (J Allergy Clin Immunol 2008;121:1148-54.)

Key words: *PHF11, atopic dermatitis, asthma, atopy, T_H1, T_H2, small interfering RNA, nuclear factor κB*

Childhood atopic dermatitis (AD) shows considerable heterogeneity in its presentation and duration. Between 27% and 75% of children with AD in Western countries are atopic.[1] Although up to 40% of children show spontaneous remission of dermatitis by the age of 3 years, for children with persistent dermatitis until at least 7 years of age, early atopic sensitization is a significant risk factor. In these cases parental dermatitis also appears to be a contributing factor, indicating an additional genetic component.[2]

Patients with AD show increased numbers of IL-4–secreting and decreased numbers of IFN-γ–secreting $CD4^+$ and $CD8^+$ cells in the peripheral circulation.[3] Decreased IFN-γ expression is reported in cultured T cells from children who are given diagnoses of atopy within the first year of life,[4] and there is evidence for reduced IFN-γ secretion and mRNA expression in young adolescents with severe AD.[5] Therefore in children with severe AD, there is an increased number of circulating T_H2 cells and a suppression of IFN-γ, a cytokine characteristic of T_H1-type T cells.

We have found an association between childhood AD and the plant homeodomain finger protein 11 gene *(PHF11)*, a gene on chromosome 13q14,[6] confirming earlier reports of an association with increased total IgE levels and asthma.[7] However, no functional studies have been done to suggest how *PHF11* might influence the allergic phenotype. *PHF11* is a member of the plant homeodomain (PHD) family, named for the presence of 1 or more PHD-type zinc fingers. Proteins with a PHD finger are commonly found in the nucleus and are implicated in transcriptional regulation.[8] Because *PHF11* is expressed in both T and B cells,[7] an attractive hypothesis is that *PHF11* regulates the transcription of either T- or B-cell genes.

METHODS

Additional methods for T_H1 and T_H2 cultures, cDNA synthesis and cloning, and immune precipitation assays and details of the antibodies used in this study appear in the Methods section of the Online Repository at www.jacionline.org.

Microarray analysis

Microarray analysis of *PHF11* was derived from the data set described by Chtanova et al[9] and Liu et al.[10] T_H1 and T_H2 cells were generated from cord blood with a single round of activation and proliferation, whereas other T-cell subsets were isolated from adult peripheral blood by using MACS beads and flow cytometry.[11]

T_H1 and T_H2 cell culture

All individuals were recruited following guidelines and approval from the Sydney West Area Health Service. Naive $CD4^+$ T cells from healthy adult donors were cultured for 48 hours in the presence of activating α-CD3 and α-CD28 antibodies and T_H1- or T_H2-polarizing cytokines and thereafter for a further 6 days with IL-2 and T_H1- or T_H2-polarizing conditions.

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Abbreviations used
AD: Atopic dermatitis
HEK: Human embryonic kidney
IL-2R: IL-2 receptor
IL-12R: IL-12 receptor
NF-κB: Nuclear factor κB
NFAT: Nuclear factor of activated T cells
PHD: Plant homeodomain
PHF11: Plant homeodomain finger protein 11
PMA: Phorbol 12-myristate 13-acetate
shRNA: Short hairpin RNA
siRNA: Small interfering RNA
SNP: Single nucleotide polymorphism
UTR: Untranslated region

Genotyping and allele-specific gene expression

Allele-specific expression and genotyping was done with SNaPshot methodology, as previously described (ABI Biosystems, Foster City, Calif).[12] The ratio of each allele in cDNA samples was normalized to genomic DNA samples. All reactions were done in triplicate from at least 2 independent cDNA reactions. Our AD cohort, described elsewhere,[6,13] comprised 112 nuclear families recruited from the Dermatology Clinic at the Children's Hospital, Westmead, with institutional ethics approval. All children presented with AD within the first 3 years of life, and the average age at recruitment was less than 4 years of age. The transmission disequilibrium test[14] and haplotype analysis, as well as checking of pedigrees, was done with GENEHUNTER, TRANSMIT, and MERLIN. Primers used for SNaPshot, genomic, and cDNA amplification are available on request.

Nucleofection of primary T cells

Primary human $CD4^+$ T cells were transfected by using nucleofection, essentially as described by Tahvanainen et al.[15] Cells (1×10^7) were transfected with 2 μg of plasmid DNA by using the Human T Cell Nucleofector kit (Amaxa, Cologne, Germany) and program U14. Cells were incubated for 4 to 6 hours in 2 mL of X-Vivo 15 (BioWhittaker, Walkersville, Md)/10% FCS in a 12-well plate before replacing the media and incubating overnight. Dead cells were removed by using a Dead Cell Removal kit (Miltenyi Biotech, Bergisch Gladbach, Germany), and 1×10^5 cells/mL were added to α-CD3–coated wells under either T_H1- or T_H2-polarizing conditions for 48 hours.

Plasmids

MISSION short hairpin RNA (shRNA) plasmids encoding small interfering RNAs (siRNAs) targeting *PHF11* and 1 nonspecific shRNA plasmid were purchased from Sigma (St Louis, Mo). Two plasmids (catalog nos. TRCN0000020109 and 110) that targeted exons II (109) and VI (110) of *PHF11* were effective in knocking down *PHF11* expression. A −436-bp IL-2 promoter fragment was cloned into the pGL3 luciferase reporter plasmid (Promega, Madison, Wis), The PHF11myc expression vector was constructed by amplifying full-length *PHF11* from Jurkat T cells and coligating with the myc tag derived from pcDNAImyc[16] into pcDNA3.1Zeo (Invitrogen, Carlsbad, Calif). The p65 subunit of nuclear factor κB (NF-κB) was constructed by amplifying full-length p65 and cloning into pEGFP-N1 (Clontech, Mountain View, Calif). The NF-κB–Luciferase (pNF-κB-Luc) PathDetect plasmid was purchased from Stratagene (La Jolla, Calif). All primer sequences are available on request.

Jurkat and human embryonic kidney cell transfection

For luciferase assays, cells were transfected and 24 hours later stimulated for a further 16 hours with the indicated stimulus (50 ng/mL phorbol 12-myristate 13-acetate [PMA]/1 mg/mL ionomycin or 20 ng/mL TNF-α). Jurkat cells (1×10^7) were electroporated with 25 μg of plasmid DNA (including 1 μg of a β-galactosidase plasmid driven by the minimal thymidine kinase promoter denoted pTKβgal) by using a Biorad Gene Pulser set at 250 V and 960 μF, whereas 3×10^5 human embryonic kidney (HEK) cells were transfected with 200 ng of pNF-κB-Luc and 25 ng of pTKβgal with up to 500 ng of pPHF11myc, up to 100 ng of pEGFPp65, or both by using Lipofectamine 2000 (Invitrogen). In each case luciferase activity was normalized to cotransfected pTKβgal by using the Dual-Light system (Tropix, Bedford, Mass). Stably transfected shRNA and PHF11myc clones were selected by means of limiting dilution in the presence of puromycin (2.5 μg/mL) or Zeocin (200 μg/mL) (Invitrogen).

Electromobility shift assays

Nuclear extracts were prepared from 1×10^7 cells,[17] and 5 μg of nuclear extract was incubated in a volume of 20 μL in 10 mmol/L Tris-HCl (pH 7.5), 50 mmol/L KCl, 1 mmol/L dithiothreitol, 0.25% Tween-20, and 1 μg poly(dI.dC) with 1 ng of an IRDye 700-labelled NF-κB consensus oligonucleotide (Li-Cor Biosciences, Lincoln, Neb) on ice for 30 minutes. Complexes were resolved on a 4% polyacrylamide gel in 0.5× Tris-borate EDTA (TBE) at 4°C. Gels were analyzed with an Odyssey imaging system (Li-Cor).

RESULTS

Using a well-characterized dataset of gene expression profiles from T cells, B cells, and other immune cells,[9,10] we found robust *PHF11* expression in B- and T-cell subsets, including peripheral blood central ($CCR7^+CD4^+CD45RO^+$) and effector ($CCR7^-CD4^+CD45RO^+$) memory T cells. In the context of allergy, perhaps the most interesting result was the differential expression of *PHF11* between T_H1 and T_H2 cells generated from neonatal $CD4^+$ T cells, with the highest expression seen in T_H1 cells (Fig 1, *A*). There was also a marked increase in *PHF11* expression on maturation of dendritic cells, suggesting a role for *PHF11* in T-cell subset maturation and activation. Basophils also exhibited high *PHF11* expression, although expression in mast cells and eosinophils was low (Fig 1, *A*). Genes associated with allergy tend to show restricted or enriched expression in leukocytes.[10] Using the SymAtlas collection of gene expression profiles,[18] we found high expression of *PHF11* in T and B cells, as well as natural killer and mature dendritic cells, with very low expression in other organs of the body (Fig 1, *B*). The differential expression of *PHF11* in T_H1 and T_H2 cells was confirmed by means of quantitative real-time RT-PCR from T_H1 and T_H2 cultures from healthy adult donors, revealing a significant increase in *PHF11* RNA in T_H1 cells relative to that seen in T_H2 cells after 6 days of differentiation (T_H1: 3.1 ± 0.52; T_H2: 1.1 ± 0.39; n = 8; data not shown).

We next transfected shRNA expression plasmids that generated either *PHF11*-specific or nonspecific control siRNA into primary $CD4^+$ T cells. Transfection of a green fluorescent protein–expressing plasmid showed transfection efficiencies routinely approached 50% (data not shown). Under T_H1-polarizing conditions and relative to control siRNA (Fig 2, *A*, solid bars), transfection of *PHF11*-specific siRNA (Fig 2, *A*, open bars) resulted in a 40% decrease in *PHF11* RNA. This was accompanied by a significant decrease in the expression of the *IL2* and *IFNG* genes. Small nonsignificant decreases were seen in genes encoding the IL-2 receptor α and IL-12 receptor β2. No change was seen in RNA encoding the T_H1-specific transcription factor T-bet (Fig 1, *A*). There was no decrease in RNA encoding IL-4 or IL-13 in parallel cultures grown under T_H2-polarizing conditions (data not shown). Overexpression of myc epitope–tagged PHF11 in primary $CD4^+$ T cells resulted in a 10-fold increase in the basal expression of IFN-γ under T_H1-polarizing conditions (Fig 2, *B*; PHF11myc).

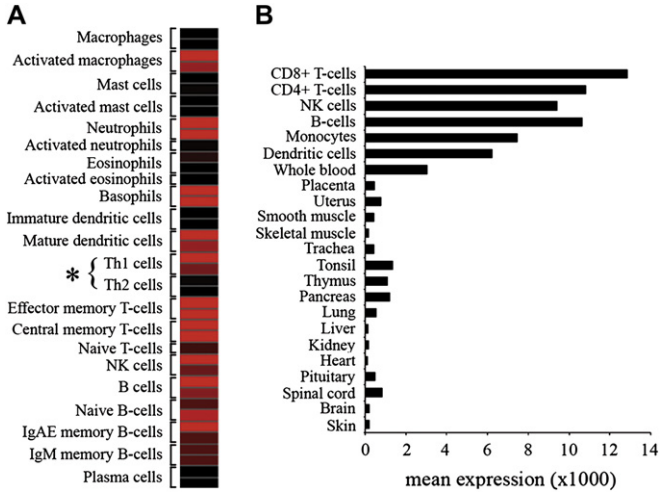

FIG 1. A, Microarray analysis of *PHF11* in different immune cell subsets. *Red* is highest and *black* is lowest expression. T_H1 and T_H2 cells are bracketed and marked by an *asterisk*. Duplicate values for some subsets are also shown. **B**, Expression of *PHF11* in different human tissues. Data are derived from the SymAtlas dataset. *NK*, Natural killer.

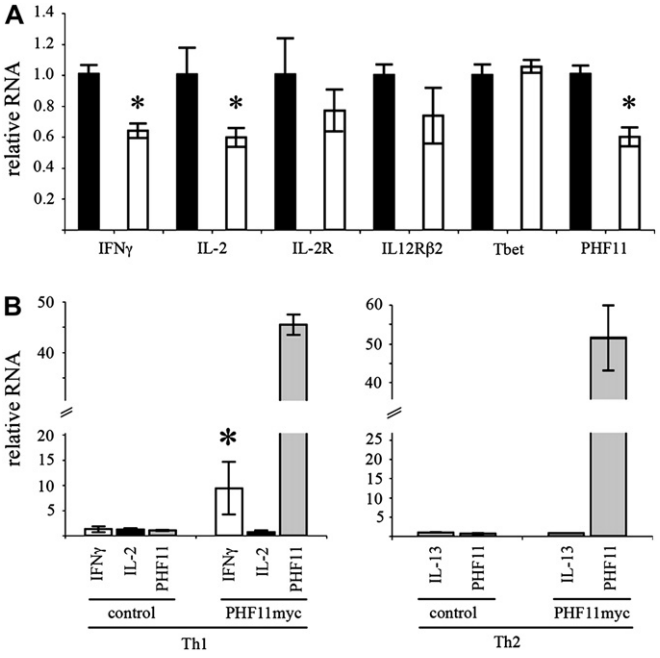

FIG 2. *PHF11* regulates T_H1-type cytokine RNA expression. **A**, T_H1-polarized $CD4^+$ T cells expressing *PHF11*-specific *(open bars)* or a nonspecific *(solid bars)* siRNA (n = 6, results from 2 independent experiments). **B**, T_H1- or T_H2-polarized primary $CD4^+$ T cells (T_H1) overexpressing *PHF11* (PHF11myc) relative to empty vector (control; n = 3, results from 2 independent experiments). Values are presented as means ± SEMs, and *asterisks* indicate significant differences.

No increase in the expression of IL-2 or the T_H2-type cytokine IL-13 was seen (Fig 2, *B*). We confirmed PHF11 is a positive regulator of gene expression by using stably transfected Jurkat T cells expressing either *PHF11* siRNA or PHF11myc (see Fig E1, *A*, in the Online Repository at www.jacionline.org). There was no change in the proliferative response of these cell lines to increasing concentrations of IL-2 (data not shown). In the nucleus of Jurkat T cells (see Fig E1, *B*) and transfected HEK cells, nuclear PHF11myc aggregates were colocalized with PML-containing nuclear bodies (see Fig 1, *C*).

We have now genotyped 3 additional *PHF11* single nucleotide polymorphisms (SNPs; Fig 3, *A*; rs8000149, rs7332573 and rs4942873), adding to those previously genotyped (Fig 3, *A*).[6] The SNPs rs1046295, rs7332573, and rs4942873 are in high linkage disequilibrium (see Fig E2 in the Online Repository at www.jacionline.org), and each SNP approaches (rs7332573) or exceeds significance (rs1046295 and rs4942873) for association with severe childhood AD (Fig 3, *A*). In searching for evidence for genotype-dependent differences in *PHF11* expression, allele-specific expression assays were performed on 2 exonic SNPs in *PHF11*: rs1046295 in the 3′ untranslated region (UTR; exon X) and rs2031532 in exon II (Fig 3, *A*). Although only rs1046295 was associated with childhood AD (Fig 3, *A*), the risk-associated G allele of rs1046295 is inherited with the A allele of rs2031532 in the 3 major haplotypes of *PHF11* (see Fig E2). Allele-specific expression analysis of donors heterozygous for both rs1046295 and rs2031532 revealed a marked underexpression of the G allele transcripts and a significant difference in the average ratio of G/A transcript expression between T_H1 and T_H2 cells for rs1046295 (Fig 3, *B*: rs1046295; T_H1 vs T_H2: 0.84 ± 0.03 vs 1.04 ± 0.05; $P < .05$). In the case of rs2031532, we found evidence for a clear but nonsignificant decrease in the expression of the A allele (Fig 3, *B*; rs2031532).

Because a number of different *PHF11* isoforms have been identified,[7] we next tested whether polymorphisms in *PHF11* could regulate alternate splicing and possibly the function of *PHF11*. First, *PHF11* cDNA was amplified from T_H1 cells. Amplification of exons IV through X produced a single major product of 800 bp (Fig 4, *A*, lane 2), and the absence of any additional major products on amplification of these exons suggested that alternative splicing in the 3′ half of *PHF11* was relatively uncommon. Amplification of exons I through VI produced an expected product of 567 bp, as well as a second smaller product differing in size by approximately 120 bp (Fig 4, *A*, lane 1). Similar results were obtained with T_H2 cells and whole blood (data not shown). Cloning and sequencing of these products identified an isoform of *PHF11* that lacked exon II (denoted I/III) and revealed that although the 3′ end of exon II and the 5′ end of exon III were full triplet codons (Fig 4, *B*; II/III middle), the 3′ end of exon I and the 5′ end of exon II were incomplete triplet codons (Fig 4, *B*; I/II top). This resulted in the I/III isoform having a disrupted reading frame leading to a premature stop codon 27 nucleotides downstream of the splice site in exon III (Fig 4, *B*; I/III bottom) and suggested the I/III isoform was nonfunctional. Quantitative real-time PCR with primers that specifically recognized either the I/III or II/III isoforms showed that transcripts containing exon II were 10- to 20-fold more abundant than the I/III transcripts (data not shown). Grouping individuals based on the rs1046295 genotype, we found a 2-fold increase in the abundance of I/III transcripts in GG versus AA individuals (Fig 4, *C*; AA: 1.4 ± 0.23, n = 8; GG: 2.98 ± 0.64, n = 9; $P < .05$). Similar analysis of transcripts containing exon II showed no genotype-dependent difference in transcript abundance (Fig 4, *C*; II/III). Allele-specific transcription assays in whole blood also revealed a decrease in the abundance of G relative to A alleles of rs1046295 (Fig 4, *C*).

To investigate how PHF11 might regulate gene expression, an IL-2 promoter luciferase expression plasmid was transfected into cell lines stably expressing nonspecific (Fig 5, *A*; NS.1) or

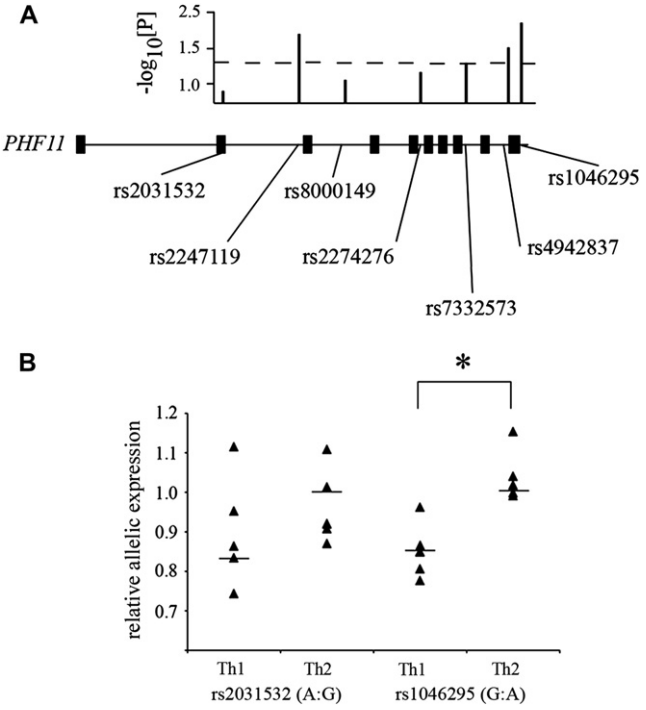

FIG 3. *PHF11* allele–specific expression in T_H1 and T_H2 cells. **A**, Intron/exon structure of *PHF11* and the location and association of *PHF11* SNPs with childhood AD. The *P* value for each SNP and the .05 level of significance *(dashed line)* are indicated. **B**, Allele-specific expression of exon 2 (rs2031532) and 3′UTR (rs1046295) SNPs in T_H1 and T_H2 cells (n = 5). *Asterisk* indicates significant difference.

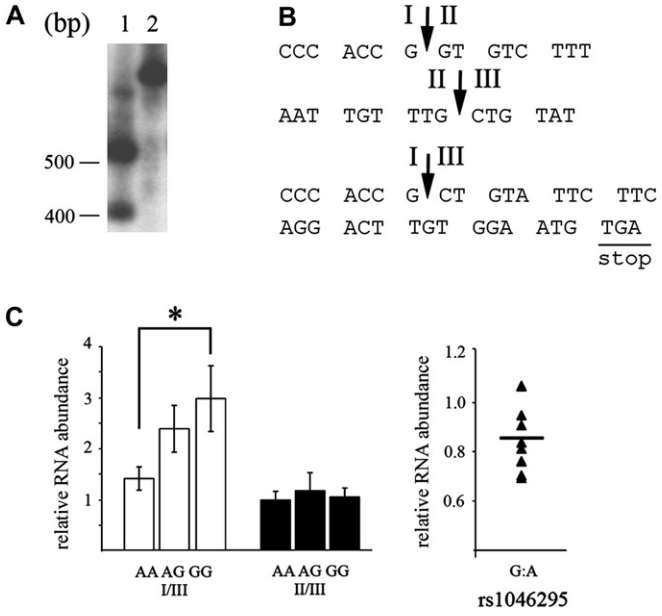

FIG 4. Alternate splicing of *PHF11*. **A**, PCR of exons I through VI *(lane 1)* or IV through X *(lane 2)*. **B**, *Arrows* indicate boundaries of exons I/II and II/III and the I/III isoform with the predicted premature stop codon. Nucleotides are grouped in triplet reading frames. **C**, *Left*: Quantification of the I/III and II/III isoforms grouped by rs1046295 genotype (AA, n = 7; AG, n = 12; GG, n = 9). *Right*: Allele-specific expression of rs1046295 in whole blood. *Asterisk* indicates significant difference.

PHF11-specific shRNA plasmids (Fig 5, *A*; 109.1 and 110.8). There was an approximately 2-fold reduction in the basal activity of the IL-2 promoter/luciferase reporter plasmid in clones 109.1 and 110.8 relative to the control clone NS.1 (Fig 5, *A*). Stimulation with PMA/ionomycin increased luciferase activity 3.5-fold in control NS.1 cells, with only a 1.1- to 1.3-fold increase in 109.1 and 110.8 cells (Fig 5, *A*). The IL-2 promoter contains binding motifs for 2 transcription factors that are critical for inflammatory responses: nuclear factor of activated T cells (NFAT) and NF-κB.[19] Because PHF11 contains a PHD domain that is implicated in protein-protein interactions, we first looked for evidence of a direct interaction between PHF11 and NFATc2 or the p65 subunit of NF-κB. Immunoprecipitation assays on non-stimulated and PMA/ionomycin-stimulated Jurkat/PHF11myc cells failed to detect an interaction with NFATc2 (data not shown), but a stimulation-dependent interaction between PHF11myc and the p65 subunit of NF-κB was detected in nuclear extracts of these cells (Fig 5, *B*; α-p65). Equal loading of samples was verified by probing the samples with an α-PHF11 antibody (Fig 5, *B*).

We next transfected the same Jurkat cell lines, as well as a cell line overexpressing PHF11myc, with a luciferase reporter plasmid driven by 5 copies of an NF-κB response element. After stimulation with TNF-α, a potent inducer of the NF-κB pathway, there was a robust increase in luciferase activity in both the NS.1 and PHF11myc cell lines, although overexpression of PHF11 afforded only a modest 1.3-fold increase in luciferase activity over that seen in the control cell line (Fig 5, *C*, top; NS.1 vs myc). In each cell line expressing *PHF11* siRNA, there was a 3- to 6-fold decrease in luciferase activity relative to NS.1 and PHF11myc cell lines (Fig 5, *C*, top; 109.1 and 110.8). In HEK 293T cells, in which endogenous *PHF11* expression is very low, PHF11myc alone failed to increase basal activity from the NF-κB luciferase plasmid but did result in a 2.6-fold increase in TNF-α–induced luciferase activity relative to that seen in cells not transfected with PHF11myc (Fig 5, *C*, bottom, open vs solid bars). Coexpression of the NF-κB p65 subunit with PHF11myc increased basal luciferase activity, although this was not significantly greater than that seen with p65 alone (Fig 5, *C*, bottom). However, coexpression of p65 and PHF11myc, together with stimulation with TNF-α, did result in a 1.5-fold increase in luciferase activity over that seen with p65 alone (Fig 5, *C*, bottom).

An electrophoretic mobility shift assay with an NF-κB consensus oligonucleotide showed maximal NF-κB binding in control and PHF11myc Jurkat cell lines after 30 minutes of stimulation with 5 ng/mL TNF-α, with no change or a small decrease in DNA binding at 25 ng/mL TNF-α (Fig 5, *D*; NS.1, lanes 1-3; myc, lanes 4-6). In nuclear extracts prepared from PHF11 siRNA–transfected cells, there was a marked reduction in the intensity of the NF-κB/DNA complexes (Fig 5, *D*; compare lanes 1-3 and 4-6 with 7-9). This was accompanied by a small decrease in nuclear p65 expression (Fig 5, *D*). These results were seen in 3 independent experiments and in the second siRNA-transfected cell line, 110.8 (data not shown). Although the NF-κB protein–DNA complex was abolished by preincubation with an α-p65 antibody, preincubation of nuclear extracts from TNF-α–stimulated PHF11myc cells with an α-myc antibody had no effect on the formation of the NF-κB complex (Fig 5, *D*, lanes 10 and 11, respectively).

DISCUSSION

In this article we show that (1) *PHF11* expression is higher in T_H1 than in T_H2 cells; (2) siRNA knockdown of endogenous

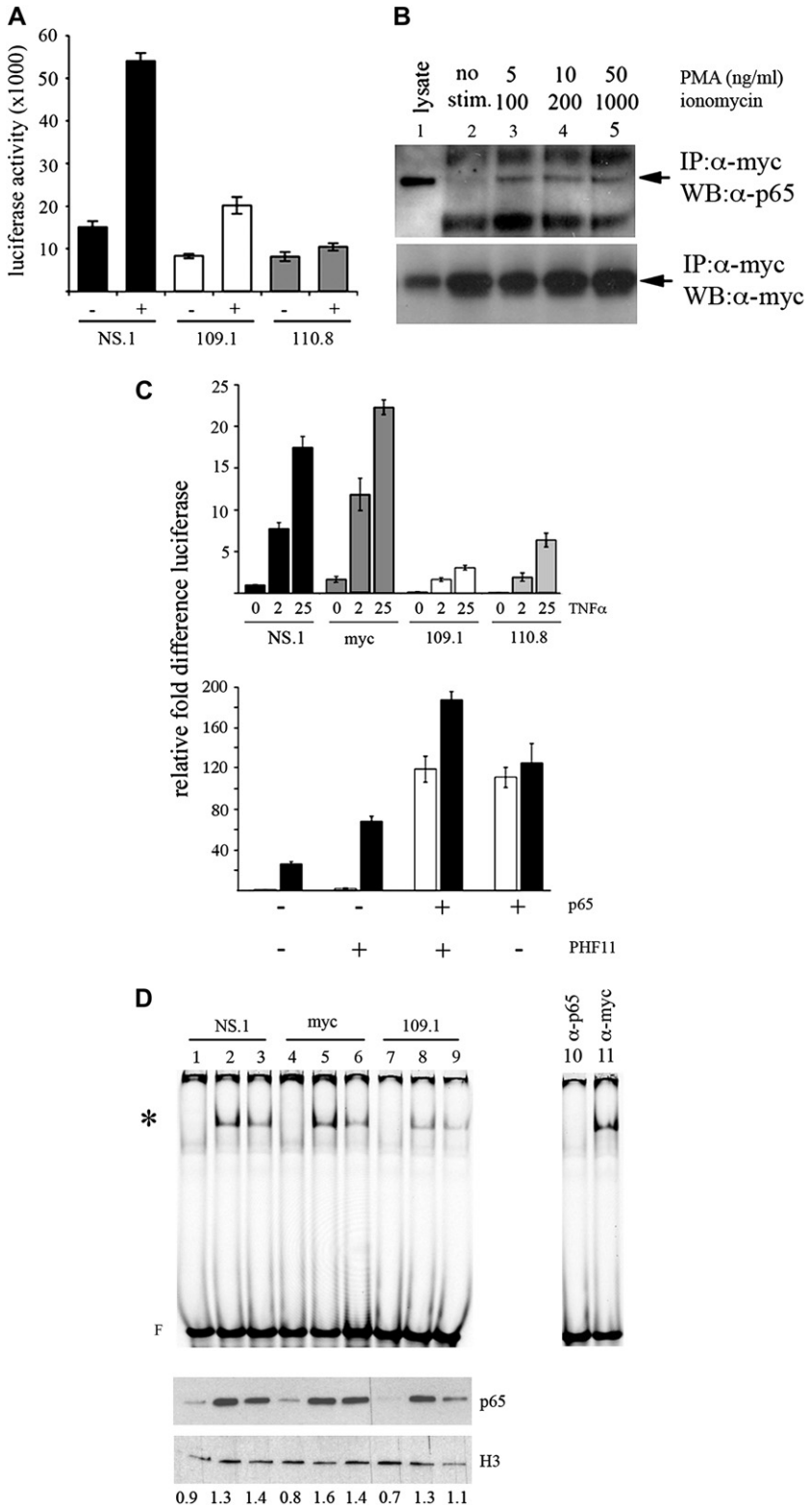

FIG 5. *PHF11* acts through the NF-κB pathway. **A**, IL-2 promoter/luciferase activity in Jurkat cell lines in the absence *(−)* or presence *(+)* of PMA/ionomycin. **B**, Immunoprecipitation *(IP)* of PHF11myc and Western blotting *(WB)* of p65 or PHF11myc *(myc)* in the presence or absence *(no stim.)* of PMA/ionomycin. *Arrows* indicate 65- and 42.5-kd p65 and PHF11myc bands, respectively. *Lane 1*, Lysate loading control. **C**, Luciferase activity of pNF-κB-Luc in Jurkat *(top)* or HEK *(bottom)* cells with coexpression of PHF11myc, p65, or both in the presence *(solid bars)* or absence *(open bars)* of TNF-α. **D**, *Top*: NF-κB DNA binding *(asterisk)* in nuclear extracts from cells expressing nonspecific *(NS.1)* or *PHF11*-specific *(109.1)* siRNA or PHF11myc *(myc)* treated with no TNF-α *(lanes 1, 4,* and *7)*, 5 ng/mL TNF-α *(lanes 2, 5, and 8)*, or 25 ng/mL TNF-α *(lanes 3, 6, 9, 10,* and *11). F*, Free DNA. *Bottom*: Western blot of nuclear p65 normalized to Histone H3. *Numbers* indicate p65 abundance.

PHF11 or overexpression of *PHF11* either decreased or increased the expression of T_H1-type genes, respectively; (3) The G allele of the 3′UTR SNP rs1046295 that is preferentially transmitted to children with AD was associated with decreased *PHF11* RNA in T_H1 cells, and a predicted nonfunctional *PHF11* isoform lacking exon II was more common in individuals carrying this G allele; and (4) *PHF11* is a transcriptional activator that acted in concert with the NF-κB pathway. Based on these results, we propose that *PHF11* is a positive regulator of T_H1-type gene expression and that the inheritance of certain genetic variants decreased *PHF11* expression, possibly contributing to the T_H2 bias that characterizes children with severe AD.

A recent study identified a strong genetic component in T-bet–dependent regulation of T_H1-type cytokines but failed to find evidence for a genetic influence on the regulation of T_H2-type cytokines.[20] In addition, circulating T_H1 cells from atopic individuals are more prone to apoptosis, leading to a skewed T_H2 profile.[21] Both these studies support the hypothesis proposed here that defects in T_H1 cells could underlie the observed T_H1/T_H2 imbalance in allergic individuals.

Allele-specific gene expression studies of rs1046295 showed that the AD-associated G allele was expressed at a lower level than the alternate A allele in T_H1 cells but not in T_H2 cells. One reason for this difference might be the higher expression of *PHF11* in T_H1 cells, thereby amplifying any differences in gene expression associated with different genotypes. The 3′UTR of *PHF11* lacks any readily identifiable regulatory motifs, such as AU-rich elements, that control RNA stability, and no regulatory motifs were identified with the alternate alleles of rs1046295, including a search of new 3′UTR motifs.[22] Experiments in which the *PHF11* 3′UTR was fused to the luciferase reporter gene under control of the IL-2 promoter also failed to detect any differences in expression associated with alternate alleles of rs1046295 (data not shown). However, a splicing variant of *PHF11* that lacks exon II, resulting in a frame-shift mutation and the introduction of a premature termination codon in exon III, was more common in individuals carrying the G allele of rs1046295. Alternate splicing events that generate such nonfunctional transcripts are relatively common in the human genome and are often the targets of nonsense-mediated RNA decay. This has led to the suggestion that such alternate splicing is a form of gene regulation.[23] A more recent article, although confirming the existence of nonfunctional transcripts, suggests the majority of these transcripts are not targeted by nonsense-mediated decay but instead represent a class of low-abundance transcripts. The formation of nonfunctional transcripts is more common in species-specific splicing events,[24] and inspection of human and mouse *PHF11* genes shows that human exon I is not present in mice and that all mouse *PHF11* transcripts begin at an exon homologous with human exon II (University of California, Santa Cruz Genome Web Browser).

Although the T allele of rs2247119 is also associated with childhood AD[6] and is located close to the 5′ end of exon III, this SNP is not part of the splice recognition site, and resequencing of our AD cohort failed to detect novel SNPs flanking exons I, II, or III that might regulate RNA splicing (data not shown). Introns 1 and 2 are 10.5 and 6.3 kb long, respectively, and it is possible that polymorphisms in regulatory elements within these large introns regulate the alternate splicing of exons I through III. Because the G allele of rs1046295 is coinherited with the T allele of rs2247119 in 1 of the major and 2 of the minor haplotypes of *PHF11* (see Fig E2), this might explain why we detected the correlation between inheritance of the G allele of rs1046295 and increased levels of isoform I/III.

In human subjects nuclear import and DNA binding of the NF-κB subunits p65 and c-Rel is impaired in a group of patients with AD.[25] Further evidence linking perturbation in NF-κB signaling with impaired T_H1-type responses comes from transgenic mice. Mice that express a form of IκBα that is resistant to proteosomal degradation have decreased T_H1-type cytokines and increased T_H2-dependent inflammation,[26] and mice functionally deficient for CARMA-1, a scaffolding protein that regulates NF-κB activation after T- and B-cell receptor activation, have atopy and dermatitis.[27]

The NF-κB p65 subunit interacts with an impressive number of proteins that act as transcriptional coactivators in directing NF-κB–dependent gene expression.[28] Although PHF11 increased luciferase activity from an NF-κB plasmid, we failed to detect any evidence for PHF11 forming a DNA-binding complex with p65 *in vitro*, suggesting that PHF11 could regulate NF-κB by an indirect mechanism. The protein MYBBP1 interacts with the p65 subunit of NF-κB but affects neither the DNA binding nor nuclear import of p65. Instead, MYBBP1 competes for the binding of the transcriptional coactivator p300 to p65.[29] To elucidate the precise mechanism by which *PHF11* regulates gene transcription, microarray analysis of $CD4^+$ T cells transfected with PHF11 siRNA and the identification of other PHF11-associated proteins will be required, especially because PHF11 itself appears to lack recognizable domains that catalyze posttranslational modifications, such as acetylation or ubiquitinylation, both of which are known to regulate p65 activity.[30,31]

In summary, we have presented evidence showing the atopy-associated gene *PHF11* is a regulator of T_H1-type cytokine gene expression through NF-κB, a pathway already implicated in atopy and atopic eczema from mouse and human studies. Future studies aimed at identifying additional proteins that interact with *PHF11* and genes regulated by *PHF11* will further clarify the role of *PHF11* in T_H1 T-cell biology and in allergic disorders.

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Clinical implications: Our data might lead to better understanding of the genetic and molecular basis of the T_H1/T_H2 T-cell imbalance that characterizes many allergic individuals.

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METHODS

T_H1 and T_H2 cell culture

Naive $CD4^+$ T cells were negatively selected by using the $CD4^+$ T-Cell Isolation Kit II and CD45RO microbeads (Miltenyi Biotech) and plated onto α-CD3 (5 μg/mL; R&D Systems, Minneapolis, Minn)–coated, round-bottomed, 96-well plates at a concentration of 1×10^6 cells/mL in X-Vivo 15 serum-free media (BioWhittaker) with 1 μg/mL soluble α-CD28 (R&D Systems) under either T_H1 (30 ng/mL rIL-12, 0.5 μg/mL neutralizing α-IL-4) or T_H2 (20 ng/mL rIL-4, 2.5 μg/mL neutralizing α-IFN-γ) conditions. Nonpolarized cells were cultured with α-CD3/α-CD28 costimulation only. After 48 hours, cells were washed and replated in round-bottom wells in the absence of α-CD3/α-CD28 but containing 200 U/mL rIL-2 under T_H1-polarizing, T_H2-polarizing, or nonpolarizing conditions for a further 6 days.

cDNA synthesis and real-time PCR

Total RNA was isolated by using the PureLink (Invitrogen) or PAXgene (whole blood; PreAnalytiX, Franklin Lakes, NJ) systems. First-strand cDNA was synthesized from 100 ng of RNA by using SuperScriptIII (Invitrogen) with a mixture of Oligo(dT) and random hexamers in 25 μL, according to the manufacturer's instructions. PCR products were resolved on an agarose gel and analyzed by means of Southern blotting with a *PHF11* digoxygenin-labeled PCR product to analyze *PHF11* isoforms. PCR fragments were cloned directly into pGEM-T Easy (Promega, Madison, Wis) and sequenced. Quantitative real-time PCR was performed on a Corbett Rotagene (Corbett Lifescience) by using Platinum SYBR Green Supermix (Invitrogen). Amplification efficiencies of different primer pairs were determined, and melt-curve analysis was performed at the end of each PCR cycle to check for nonspecific amplification products. All reactions were normalized against either actin or 18S RNA and then to nonstimulated/control cells by using the formula $2^{-\Delta\Delta Ct}$. Primer sequences are available on request.

Immune precipitation assays

Nuclear lysate from 8×10^6 stably transfected PHF11myc Jurkat T-cell clones was adjusted to 150 mmol/L NaCl and 10 mmol/L KCl and incubated with monoclonal α-myc antibody 9E10 for 2 hours at 4°C with rotation. Fifty microliters of a 50% vol/vol solution of protein G–sepharose beads was added, and incubation was continued for a further 3 hours. The beads were then washed 4 times in 20 mmol/L HEPES (pH 7.9), 2 mmol/L $MgCl_2$, 150 mmol/L NaCl, 10 mmol/L KCl, and 0.5% NP40.

Antibodies and cytokines

Antibodies and suppliers were as follows: rabbit α-p65 (C-20; Santa Cruz Biotechnology, Santa Cruz, Calif); rabbit α-*PHF11* (BCO17212; ProteinTech Group, Inc, Chicago, Ill); α-IL-4–phycoerythrin, IFN-γ–fluorescein isothiocyanate, CD28 (MAB342), CD3 (MAB100), neutralizing α-IL-4 (MAB304), and IFN-γ (MAB2852; R&D Systems); rabbit α-myc Tag (Upstate Biotechnology, Lake Placid, NY); α-Histone-H3 (Cell Signaling Technologies, Danvers, Mass); recombinant human IL-12 (R&D Systems); and recombinant human IL-4 (Apollo Cytokine Research, Beaconsfield, Australia).

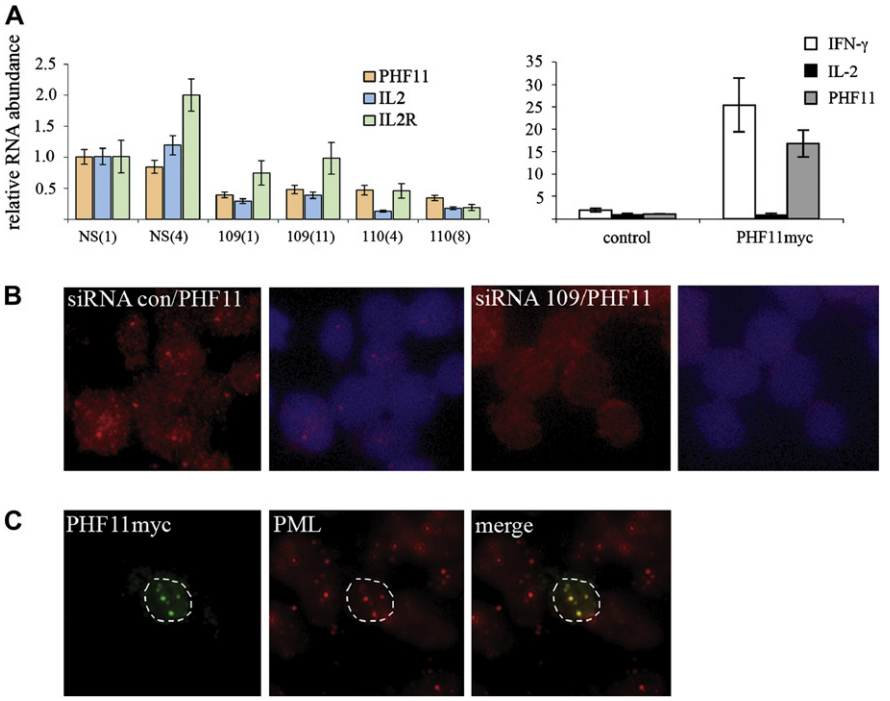

FIG E1. **A**, *Left*: *PHF11* siRNA decreases *IL2* gene expression in Jurkat T-cell clones. Nonspecific siRNA controls (*NS[1]* and *NS[4]*) and clones expressing each of 2 different *PHF11* siRNAs (*109[1]* and *109[11]* and *110[4]* and *110[8]*) are shown. Expression of *PHF11*, IL-2, and IL-2 receptor α *(IL2R)* are shown. *Right*: Increased IFN-γ RNA, but not IL-2, in Jurkat T cells overexpressing PHF11myc. **B**, *PHF11* is present as aggregates in the nuclei of Jurkat T cells expressing a nonspecific siRNA (*siRNA con, red*) but is absent from the nuclei of cells expressing *PHF11*-specific siRNA *(siRNA 109)*. Merged images of *PHF11* and nuclei are also shown. **C**, PHF11myc forms aggregates in the nucleus of transfected 293T HEK cells and colocalizes with PML. *Dotted lines* indicate the location of the nucleus.

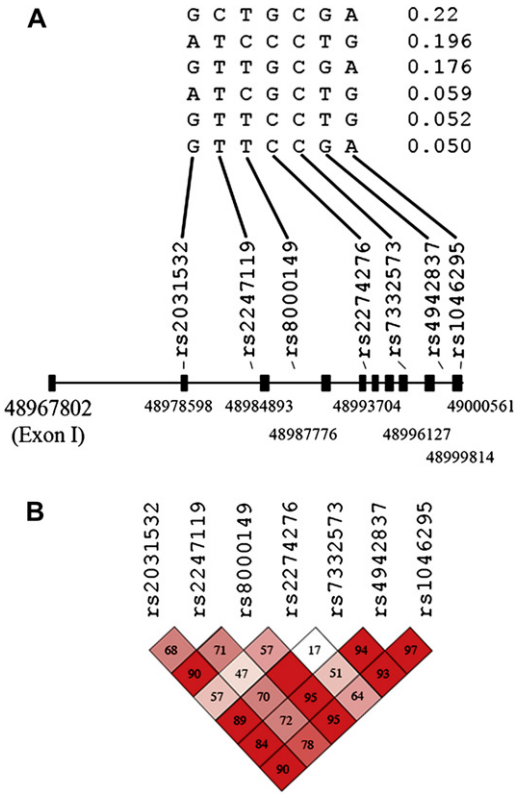

FIG E2. **A**, Major haplotypes and haplotype frequencies of *PHF11*. The *horizontal line* and *boxes* represent the arrangement of *PHF11* introns and exons. Major haplotypes and haplotype frequencies are indicated, as are the rs numbers and locations of each SNP genotyped. The genomic position of each SNP (University of California, Santa Cruz Genome Browser March 2006) is indicated below the diagram of *PHF11*. **B**, Linkage disequilibrium plot of genotyped *PHF11* SNPs. *Numbers* are values of D′, and *red* is the highest and *white* is the lowest linkage disequilibrium. All results were derived by using the cohort of 112 children with AD and their parents, as described in the online-only Methods section and in the main text.