Association of TRPV4 gene polymorphisms with chronic obstructive pulmonary disease

Guohua Zhu1, ICGN Investigators, Amund Gulsvik2, Per Bakke2, Srinivas Ghatta3, Wayne Anderson1, David A. Lomas4, Edwin K. Silverman5 and Sreekumar G. Pillai1,*

1Genetics, GlaxoSmithKline R&D, 5 Moore Drive, Research Triangle Park, NC 27709, USA, 2University of Bergen, Bergen, Norway, 3Center for Excellence in Drug Discovery (Respiratory), GlaxoSmithKline, Upper Merion, PA, USA, 4Cambridge Institute for Medical Research, Cambridge, UK and 5Brigham and Women’s Hospital, Boston, MA, USA

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Chronic obstructive pulmonary disease (COPD) is characterized by airway epithelial damage, bronchoconstriction, parenchymal destruction and mucus hypersecretion. Upon activation by a broad range of stimuli, transient receptor potential vanilloid 4 (TRPV4) functions to control airway epithelial cell volume and epithelial and endothelial permeability; it also triggers bronchial smooth muscle contraction and participates in autoregulation of mucociliary transport. These functions of TRPV4 may be important for the regulation of COPD pathogenesis, so TRPV4 is a candidate gene for COPD. We genotyped 20 single nucleotide polymorphisms (SNPs) in TRPV4, and tested qualitative COPD and quantitative FEV1 and FEV1/(F)VC phenotypes in two independent large populations. The family population had 606 pedigrees including 1891 individuals, and the case–control sample included 953 COPD cases and 956 controls. Family-based association tests were performed in the family data. Logistic regression and linear models were used in the case–control data to replicate the association results. In the family data, seven out of 20 SNPs tested were associated with COPD (2.5 \times 10^{-4} \leq P \leq 0.04) and six SNPs were associated with FEV1/VC (0.02 \leq P \leq 0.03) from family-based association tests (PBAT) analysis. Four out of the seven SNPs associated with COPD demonstrated replicated associations with the same effect directions in the case–control population (0.02 \leq P \leq 0.03). Significant haplotype associations supported the results of single SNP analyses. Thus, polymorphisms in the TRPV4 gene are associated with COPD.

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is one of the major causes of death worldwide, showing an increasing prevalence and morbidity. Although it is well known that cigarette smoking is a major risk factor for COPD, only a minority of smokers develops the disease (1), which suggests that additional risk factors including genetic factors can contribute to COPD susceptibility. Evidence for genetic factors in COPD susceptibility is derived from twin studies (2,3) and familial aggregation studies (4–7).

COPD is characterized by epithelial cell damage, bronchoconstriction, lung parenchymal destruction and mucus hypersecretion (8,9). Ca^{2+} channels might impact COPD pathogenesis; for upon activation, they produce Ca^{2+} influx to trigger a variety of important physiological activities in the airways. Transient receptor potential vanilloid 4 (TRPV4), a member of the TRP channel family in vertebrates, is a non-selective cation channel with a high permeability to Ca^{2+} (10,11). In humans, the TRPV4 gene is located on chromosome 12q24.1 (12), and it is widely expressed in various respiratory tract tissues including epithelia, endothelia and smooth muscle (13–16).

TRPV4 channel can be activated by hypotonicity, mechanical stress, heat, acidic pH, the synthetic phorbol ester 4\alpha-phorbol 12,13-didecanoate (4\alpha-PDD) and epoxyeicosatrienoic acids (EETs)—metabolites of arachidonic acids (AAs) (17). TRPV4 activation leads to diverse physiological effects in airways, depending on the nature of the stimuli, the intensity and duration of stimulation and the specificity of tissues. In human tracheal and bronchial...
epithelial cells, TRPV4 activation by hypotonicity functioned to control cell volume by inducing an effective regulatory volume decrease (RVD) response, a process that the cells shrank toward their original volumes after swelling (18,19); TRPV4 activation by low-level shear stress enhanced epithelial barrier function of human bronchial cells by decreasing paracellular permeability (20). TRPV4 activation by 4α-PDD and 5,6- or 14,15-EET disrupted alveolar septal walls by increasing lung epithelial and endothelial permeability in rats (16); TRPV4 activation by ventilation at 35 or 40°C also induced lung injury by augmenting lung endothelial permeability in mice (21). In addition, TRPV4 might account for hypotonicity-induced contraction of human bronchial smooth muscle cells, for among limited types of Ca$^{2+}$ channels expressed in these cells, TRPV4 appeared to be the only Ca$^{2+}$ channel sensitive to hypotonic stimulation (15). Furthermore, TRPV4 activation by 4α-PDD increased the ciliary beat frequency (CBF), and viscosity-induced TRPV4 activation participated in the CBF autoregulation in hamster oviductal ciliated epithelial cells (22). Since mammalian airway and oviductal ciliated epithelial cells used a common mechanism to regulate mucociliary transport through CBF (23), TRPV4 activation might contribute to maintaining the capability of mucociliary transport under the viscous stress from airway mucus secretion. Overall, TRPV4 plays a crucial role in regulating airway epithelial barrier function, epithelial and endothelial permeability, smooth muscle contraction and mucociliary transport, which are likely fundamental to COPD pathophysiology. Therefore, we hypothesized that TRPV4 might be a COPD susceptibility gene.

Here, we present our comprehensive evaluation of TRPV4 variants in a family-based association study by investigating a large population from the International COPD Genetics Network (ICGN) consisting of 606 families and 1891 subjects and a large replication population including 953 cases and 956 controls from Norway. Our results suggest that genetic variants in TRPV4 affect COPD-related phenotypes.

RESULTS

Single nucleotide polymorphism association analyses

We investigated the genetic association of 20 single nucleotide polymorphisms (SNPs) in the TRPV4 region with COPD, which was part of a larger panel of 384 SNPs typed in a few other COPD candidates. The nucleotide positions and genotype information of these SNPs are listed in Table 1, and the results of single SNP association analyses with COPD in two populations are summarized in Table 2. With family-based association tests (FBAT) analysis, seven SNPs—SNP 8 (rs12578401), 10 (rs3825396), 11 (rs12579553), 12 (rs16940583), 18 (rs3742030) and 20 (rs6606743)—showed significant association with COPD (2.5 × 10$^{-4}$ ≤ $P$ ≤ 0.04); we obtained similar results from Disease Family-based Association (DFAM) analysis (1.3 × 10$^{-4}$ ≤ $P$ ≤ 0.04 for the above seven SNPs). Four of these SNPs—SNP 8 (rs12578401), 10 (rs3825396), 11 (rs12579553) and 12 (rs16940583)—were significantly associated with COPD even after a Bonferroni correction for the number markers analyzed (Table 2). We performed a genetic association analysis in a COPD case–control population from Norway in order to replicate the association results in the family-based analyses for TRPV4. In the case–control data, four SNPs in TRPV4 were significantly associated with COPD (Table 2). SNP 8 (rs12578401), 11 (rs12579553), 12 (rs16940583) and 18 (rs3742030) revealed significant association with COPD (SNP 8 $P$ = 0.03 and OR = 1.26, SNP 11 $P$ = 0.03 and OR = 1.25, SNP 12 $P$ = 0.03 and OR = 1.26, SNP 18 $P$ = 0.02 and OR = 1.62). The significant associations between the four SNPs and COPD in the case–control population replicated the results with the same effect directions (same risk allele in both populations) in the family data. Moreover, SNP 8 (rs12578401), 11 (rs12579553), 12 (rs16940583) and 18 (rs3742030) showed trends to significant association with COPD (SNP 8 $P$ = 0.13, SNP 11 $P$ = 0.13, SNP 12 $P$ = 0.13, SNP 18 $P$ = 0.11) from the corrected $P$-values using permutation in the case–control data. Table 2 shows combined $P$-values for significant SNPs from the two populations (9.6 × 10$^{-5}$ ≤ $P$ ≤ 1.9 × 10$^{-2}$), which are lower than the $P$-values from individual populations.

The results of single SNP association analyses with pulmonary function traits including FEV$_1$ and FEV$_1$/FVC in two populations are summarized in Table 3. From the analysis of FBAT, six SNPs—SNP 8 (rs12578401), 10 (rs3825396), 11 (rs12579553), 12 (rs16940583), 18 (rs3742030) and 20 (rs6606743)—demonstrated significant association with FEV$_1$/VC (0.02 ≤ $P$ ≤ 0.03), which was confirmed in the analysis using Merlin (0.01 ≤ $P$ ≤ 0.02 for the above six SNPs). In the case–control data, three SNPs in TRPV4 were significantly associated with FEV$_1$ within the COPD cases (Table 3). SNP 2 (rs4766631) ($P$ = 0.01), 3 (rs10774894) ($P$ = 6.0 × 10$^{-7}$) and 6 (rs10735104) ($P$ = 4.0 × 10$^{-7}$) showed significant association with FEV$_1$ in COPD cases. Moreover, we detected significant SNPs 3 (rs10774894) ($P$ = 0.03) and 6 (rs10735104) ($P$ = 0.02) from the corrected $P$-values using permutation.

Linkage disequilibrium analysis

Figure 1 displays pair-wise linkage disequilibrium (LD) ($r^2$) values of the 20 SNPs in the 64 kb sequence encompassing TRPV4 and its flanking regions. We detected three LD blocks in ICGN family data; Block 3 covered five significant SNPs—SNP 10 (rs3825396), 11 (rs12579553), 12 (rs16940583), 18 (rs3742030) and 19 (rs7971845). Blocks 1 and 2 did not include any significant SNPs, and significant SNPs 8 (rs12578401) and 20 (rs6606743) were not part of these 3 blocks. We identified four LD blocks in the COPD case–control population. All four significant SNPs with COPD were located in Blocks 3 and 4: SNP 8 (rs12578401), 11 (rs12579553) and 12 (rs16940583) in Block 3; and SNP 18 (rs3742030) in Block 4. All three significant SNPs with FEV$_1$ were found in Blocks 1 and 2: SNPs 2 (rs4766631) and 3 (rs10774894) in Block 1; and SNP 6 (rs10735104) in Block 2.

Haplotype-based association analyses

We performed adjacent three-SNP sliding window haplotype analysis for the COPD binary phenotype. Table 4 shows the significant results of haplotype-based association analysis in
the two populations. In the ICGN family population, we detected eleven adjacent SNP combinations with a significant score test $(1.4 \times 10^{-4} \leq P\text{-global} \leq 0.04$ and $4.0 \times 10^{-3} \leq P\text{-specific} \leq 0.03$) with COPD; four of them (haplotypes 7-8-9, 10-11-12, 11-12-13 and 12-13-14) were still significant after Bonferroni correction for multiple testing. In the case–control population, four haplotypes (7-8-9, 10-11-12, 11-12-13 and 18-19-20) demonstrated significant association $(0.02 \leq P\text{-global} \leq 0.04$ and $0.01 \leq P\text{-specific} \leq 0.03$) with COPD, several of these sliding window haplotype associations replicated the haplotype association results of the family-based population.
Table 3. Results of genetic association of single TRPV4 SNPs with pulmonary function traits (PFT) under the additive model in two datasets

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<th>FEV1 (FVC) ICGN family data P-value</th>
<th>Case–control data Risk allele P (β (SE)a)</th>
<th>FEV1 (FVC) ICGN family data P-value</th>
<th>Case–control data Risk allele P (β (SE)a)</th>
<th>Combined P-valueb</th>
<th>FEV1 (FVC) ICGN family data P-value from Merlin</th>
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aEffect estimates are β (regression coefficient) for quantitative and quantitative phenotypes, respectively.

bFisher’s combined method was applied to combine the P-values from PBAT in ICGN family data and P-values in the case–control Bergen cohort.
Figure 1. Linkage disequilibrium map across the TRPV4 region. Pattern of linkage disequilibrium between 20 SNPs in the TRPV4 region in the ICGN family-based population (A) and COPD case–control population (B). Values of $r^2$ ($\times 100$) are shown. Black squares, $r^2 = 1$; white squares, $r^2 = 0$; squares in shades of gray, $0 < r^2 < 1$ (the intensity of the gray is proportional to $r^2$). Haplotype block structure was estimated with the Haplovew program.
The phenotypic expression of COPD is likely influenced by multiple genes, but severe alpha 1-antitrypsin deficiency is the only proven genetic risk factor identified so far. Though TRPV4 is highly expressed in the respiratory system, and several lines of evidence show its possible role in obstructive lung diseases, the study of genetic association of COPD has not been reported previously. To assess the impact of COPD-related airway physiological activities. Hypotonic activation of TRPV4 was necessary for maintaining airway epithelial barrier function by inducing a full RVD response (18,19). However, in airway epithelia affected with cystic fibrosis (CF) bearing the most typical $\Delta F 508$ (p.Phe508del) mutation of the CF transmembrane conductance regulator (CFTR) gene, hypotonic activation of TRPV4 was lost, which resulted in the absence of the RVD response under hypotonic stress (18). The CFTR mutation might lead to the disruption of hypotonic activation of TRPV4 through modulating phospholipid $A_2$ (PLA$_2$). Cell-swelling activated PLA$_2$ which transformed membrane phospholipids to AA, and AA was subsequently metabolized by cytochrome P450 epoxygenase to 5,6-EET, a direct activator of TRPV4 (25). Both PLA$_2$ and AA levels were altered in CF cells (18), which would affect the formation of 5,6-EET, causing loss of TRPV4 activation by hypotonicity. The deficiency of TRPV4 activation by hypotonicity in the CF airway epithelium with the $\Delta F 508$ mutation might account for the association between this CF variant and bronchial hypersecretion, a COPD characteristic (26). cTRPV4 activation by hypotonicity participated in the regulation of the abundance of aquaporin-5, a water channel that played a critical role in airway space liquid (ASL) secretion (27).

Unrestricted TRPV4 activation by hypotonicity might induce bronchoconstriction in COPD patients. Human ASL was normally ~80% of isotonic body fluids and lower under pathologic conditions (15). The normal bronchial epithelial cells with tight junctions serve as a barrier to separate smooth muscle from hypotonic ASL. However, due to epithelial cell damage in COPD patients, the underlying bronchial smooth muscle cells could be exposed to hypotonic ASL, which would activate TRPV4 in these cells to trigger their contraction (15). On the other hand, normal airway epithelial cells release nitric oxide (NO) to relax smooth muscle, but damaged epithelial cells had a deficiency of releasing NO. Consequently, the bronchial smooth muscles of COPD patients kept contracting under hypotonic stimulation, which led to bronchoconstriction (8).

Genetic association studies provide a powerful tool to identify genetic variants that affect susceptibility to common complex diseases, but results are often inconsistent (28). The inconsistency may result from small sample sizes, population stratification, genetic heterogeneity among different populations and the lack of correction for multiple testing (29,30). To limit the impact of these potential factors, several experimental and analytical strategies were used in this study. We analyzed two large populations (one family-based and the other case–control) that had enough power to detect associations with modest genetic effects. The FBAT are immune to false-positive results caused by population stratification, and genomic control methods were used to assess for population stratification in our case–control association analysis (31).

In the present study, three of the five SNPs showing replicated association with COPD (SNPs 8, 11 and 12) were in strong LD and located in introns 5–7. Thus, the associated variants might affect TRPV4 splicing, resulting in dysfunctional TRPV4 proteins with deletions in ANK domains, which could influence COPD susceptibility.

TRPV4 may have multiple functions in the regulation of COPD-related airway physiological activities. Hypotonic activation of TRPV4 was necessary for maintaining airway epithelial barrier function by inducing a full RVD response (18,19). However, in airway epithelia affected with cystic fibrosis (CF) bearing the most typical $\Delta F 508$ (p.Phe508del) mutation of the CF transmembrane conductance regulator (CFTR) gene, hypotonic activation of TRPV4 was lost, which resulted in the absence of the RVD response under hypotonic stress (18). The CFTR mutation might lead to the disruption of hypotonic activation of TRPV4 through modulating phospholipid $A_2$ (PLA$_2$). Cell-swelling activated PLA$_2$ which transformed membrane phospholipids to AA, and AA was subsequently metabolized by cytochrome P450 epoxygenase to 5,6-EET, a direct activator of TRPV4 (25). Both PLA$_2$ and AA levels were altered in CF cells (18), which would affect the formation of 5,6-EET, causing loss of TRPV4 activation by hypotonicity. The deficiency of TRPV4 activation by hypotonicity in the CF airway epithelium with the $\Delta F 508$ mutation might account for the association between this CF variant and bronchial hypersecretion, a COPD characteristic (26). cTRPV4 activation by hypotonicity participated in the regulation of the abundance of aquaporin-5, a water channel that played a critical role in airway space liquid (ASL) secretion (27).

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with the same SNPs for the same phenotypes in two independent datasets will likely make our results reliable. Demonstrating significant associations with multiple SNPs also increases the likelihood that our results are valid. Several single and haplotype associations remained significant after Bonferroni multiple correction in the family-based data (32). Since we used a larger panel of SNPs in the genotyping run, most of which were not used in testing this particular hypothesis, it can be argued that the conservative correction should be based on the total number of SNPs genotyped and not the ones used in testing the hypothesis. Replication in a second cohort was our primary approach to address the limitations of multiple statistical testing and we chose to adjust our P-values for 20 SNPs used in this study.

On the other hand, some limitations are present in this study. First, we could not completely exclude the possibility of population stratification in the case-control population. However, since the subjects were recruited from a single clinical center, this population was more homogeneous, so the possibility of population stratification was decreased. Moreover, we did not find evidence of population stratification using the genomic control method (31), which, however, is not well-powered to detect population stratification in European populations. Secondly, the non-synonymous coding polymorphism SNP 18 (rs3742030) showed nominal significance but lost the significance after a conservative Bonferroni correction for multiple testing in the ICGN family data. It has to be noted that the number of informative families is a function of the allele frequency of the SNP analyzed and there were only 21 informative families available for the analysis of rs3742030 (Table 2). So if we test this SNP in a larger population, it is possible to detect a much stronger association. A larger panel of SNPs was genotyped in the experiment, but we conducted multiple testing correction only for the SNPs used to test this specific hypothesis. We only detected four correction for multiple testing in the ICGN family data. It has to be noted that the number of informative families is a function of the allele frequency of the SNP analyzed and there were only 21 informative families available for the analysis of rs3742030 (Table 2). So if we test this SNP in a larger population, it is possible to detect a much stronger association. A larger panel of SNPs was genotyped in the experiment, but we conducted multiple testing correction only for the SNPs used to test this specific hypothesis. We only detected four

In summary, the present study using single SNP and combined analyses discovered significant association of polymorphisms in the TRPV4 gene with COPD phenotypes. The results suggest that the modulation of TRPV4 protein structure and activity may impact COPD pathogenesis, which reveals the hope of treating COPD through targeting TRPV4. Further functional studies are needed to clarify the molecular mechanism of TRPV4 variants in the pathophysiology of COPD.

MATERIALS AND METHODS

Study populations

The details about subject recruitment, assessment and characteristics were reported previously (31). Briefly, in the multicenter ICGN COPD family study, ascertainment criteria for probands were airflow limitation (post-bronchodilator FEV1 < 60% predicted and FEV1/VC (vital capacity) <90% predicted) at a relatively early age range (45–65 years old), smoking history ≥5 pack-years and at least one eligible sibling (with ≥5 pack-years smoking history). All siblings and available parents of probands were invited to participate. COPD in siblings was defined as a post-bronchodilator FEV1 < 80% predicted and FEV1/VC <90% predicted. These criteria are similar to GOLD Stage 2, with two differences: (i) VC was assessed as the higher of SVC and FVC since FVC often underestimates VC in COPD subjects and (ii) we used 90% predicted to define reduced FEV1/VC (based on prediction equations for FEV1/VC), because the predicted values for FEV1/VC change with age and a fixed value of 0.7 can overestimate the presence of airflow obstruction in older individuals. In this analysis, 606 pedigrees including 1891 Caucasian individuals were genotyped.

The case-control replication population was recruited from Bergen, Norway. The entry criteria for COPD case group were post-bronchodilator FEV1 < 80% predicted and FEV1/VC < 0.7. The control group included only the subjects without evidence of airflow obstruction (FEV1 > 80% predicted and FEV1/VC > 0.7). This corresponds to GOLD Stage 2. The population used for the case-control association analysis consisted of 953 COPD cases and 956 controls. Both cases and controls were required to have a minimum of 2.5 pack-years of smoking. Clinical characteristics of the subjects in two populations are shown in Table 5.

SNP selection and genotyping

For the selection of SNPs, LD bins were established using an r² threshold of 0.8. The tagging SNP selection was based on HapMap data for European-Americans (CEU) with a minor allele frequency (MAF) > 5% from the public database (http://www.hapmap.org), and non-synonymous SNPs with any MAFs were included. Eighteen SNPs within the TRPV4 gene and two more SNPs flanking TRPV4 were selected. These 20 SNPs were genotyped in both the ICGN family population and the Norwegian case-control population using the Illumina array-based custom SNP genotyping platform. Hardy-Weinberg equilibrium (HWE) was performed for all SNPs in the control data by using the χ² goodness-of-fit test with SAS software 8.2; HWE for all SNPs was also tested in the family data using PBAT version 3.6 (36). All TRPV4 SNPs (P > 0.05) were in HWE in both the family and case-control data. COPD family data were evaluated for inconsistent Mendelian inheritance using the PedCheck program (37).

Statistical analysis

FBAT. Association analyses of single SNP with the COPD binary phenotype, as defined above, were performed using the FBAT version 1.7.3 (38) in the ICGN family study. The same phenotype was also analyzed using DFAM function from PLINK version 1.05 (39). The analyses of quantitative traits (FEV1 and FEV1/VC) were performed with the adjustment of covariates including center, age, sex, height and pack-years of cigarette smoking using PBAT version 3.6 (36); these two phenotypes were also analyzed using association function
from Merlin version 1.1.2 (40). Biallelic tests were conducted for SNPs under an additive genetic model. In the analyses, \( P \)-values < 0.05 were considered as nominally significant. Because 20 SNPs were tested, \( P \)-values < 2.5 \times 10^{-3} \) (0.05/20) were reported as adjusted-significance after a very conservative Bonferroni correction for multiple statistical testing. In consideration of high correlation between phenotypes, we chose not to correct for testing three phenotypes in order to avoid being overly conservative. Haplotype-based analyses were conducted using the HBAT function of the FBAT program with Monte Carlo sampling (up to 100,000 permutations) for COPD, FEV1 and FEV1/VC (41) in the family data. Using a SNP sliding window approach (adjacent three SNPs), the results of global and haplotype-specific statistics were reported. In the analyses, \( P \)-values < 0.05 were considered as nominally significant. Because 18 haplotypes were tested, the global \( P \)-values of haplotypes < 2.77 \times 10^{-3} \) (0.05/18) were considered significant after Bonferroni correction of multiple testing.

**Case–control replication analysis.** The case–control data were analyzed for association using two statistical models. A logistic regression model for the COPD binary phenotype and a linear regression model for the quantitative phenotypes (FEV1 and FEV1/VC) were performed with the adjustment of potential confounders including age, sex, height and pack-years of smoking. Only COPD cases were included for the quantitative trait analyses, which were conducted using SAS software 8.2 under an additive genetic model. \( P \)-values < 0.05 in the analyses were considered as nominally significant. We also corrected these \( P \)-values with 100,000 permutations for multiple testing using PLINK 1.05 (39). Haplotype-based association analysis was assessed using the expectation-maximization algorithm and score tests with 10,000 permutations, implemented in Haplo.stats program (42). Using a SNP sliding window approach (adjacent three SNPs), the results of global and haplotype-specific statistics were reported. \( P \)-values < 0.05 in the analyses were considered as nominally significant. Because we used the case–control population to replicate the significant results of the family-based association analyses, we provided both uncorrected and corrected \( P \)-values from permutation for multiple testing. Fisher’s combined method was applied to the calculation of combined \( P \)-values from FBAT (PBAT) in ICGN family data and uncorrected \( P \)-values in the case–control data. In the case–control data, we had previously genotyped a panel of 221 unlinked SNPs to detect population stratification by estimating an inflation factor \( \lambda \) for genomic control (43,44), which did not show significant evidence of population stratification (inflation factor was 1.027 for the mean test statistic of the genomic control SNPs) (31).

The LD structure was assessed with the software package, Haploview version 4.0 (45). Strength of LD between pairs of SNPs was calculated, and LD blocks were defined according to the criteria of Gabriel et al. (46).

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