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# Identification of *ZNF313/RNF114* as a novel psoriasis susceptibility gene

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**Psoriasis is an immune-mediated skin disorder that is inherited as a multifactorial trait. Linkage studies have clearly identified a primary disease susceptibility locus lying within the major histocompatibility complex (MHC), but have generated conflicting results for other genomic regions. To overcome this difficulty, we have carried out a genome-wide association scan, where we analyzed more than 408 000 SNPs in an initial sample of 318 cases and 288 controls. Outside of the MHC, we observed a single cluster of disease-associated markers, spanning 47 kb on chromosome 20q13. The analysis of two replication data sets confirmed this association, with SNP rs495337 yielding a combined *P*-value of $1.4 \times 10^{-8}$ in an overall sample of 2679 cases and 2215 controls. Rs495337 maps to the *SPATA2* transcript and is in absolute linkage disequilibrium with five SNPs lying in the adjacent *ZNF313* gene (also known as *RNF114*). Real-time PCR experiments showed that, unlike *SPATA2*, *ZNF313* is abundantly expressed in skin, T-lymphocytes and dendritic cells. Furthermore, an analysis of the expression data available from the Genevar database indicated that rs495337 is associated with increased *ZNF313* transcripts levels ($P = 0.003$), suggesting that the disease susceptibility allele may be a *ZNF313* regulatory variant tagged by rs495337. Homology searches indicated that *ZNF313* is a paralogue of *TRAC-1*, an ubiquitin ligase regulating T-cell activation. We performed cell-free assays and confirmed that like TRAC-1, ZNF313 binds ubiquitin via an ubiquitin-interaction motif (UIM). These findings collectively identify a novel psoriasis susceptibility gene, with a putative role in the regulation of immune responses.**

## INTRODUCTION

Psoriasis (MIM #177900) is a chronic inflammatory skin disorder, affecting up to 4% of the general population. Familial recurrence of the disease is well established and psoriasis is widely regarded as a multifactorial disease (1). Linkage studies have provided overwhelming support for a primary disease locus (*PSORS1*), lying within the major histocompatibility complex (MHC) on chromosome 6p21 (2). The assignment of non-MHC susceptibility loci has proved more problematic, in all likelihood owing to their smaller phenotypic effect. Genome-wide linkage scans have mapped at least nine non-MHC disease regions (*PSORS2-10*), but efforts to validate these findings have generated conflicting results (3). High-resolution genetic analyses have also identified a putative susceptibility allele within the *PSORS2* locus (4), but follow-up

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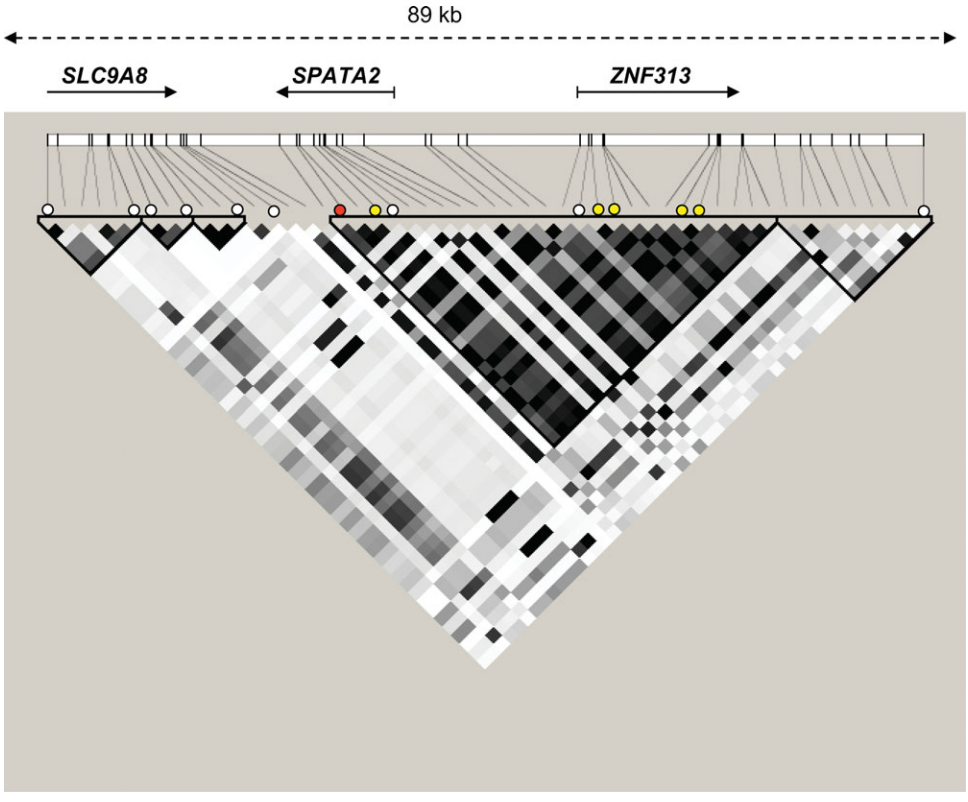


**Figure 1.** Linkage disequilibrium conservation across the disease-associated region. The $r^2$ plot generated by Haploview has been annotated to show the position of the *SLC9A8*, *SPATA2* and *ZNF313* genes. Circles indicate the localization of analyzed SNPs, with markers showing association in the GWA scan and tag SNPs, respectively, represented by yellow and white symbols. Marker rs495337 is represented by a red circle.

studies have failed to replicate this observation (5–7). Lack of reproducibility is thought to reflect the fact that linkage studies have inadequate power to detect disease loci of small effect (8).

Genome-wide association (GWA) scans hold the promise of reliably identifying genetic determinants of modest effect and are rapidly uncovering large numbers of complex disease susceptibility alleles (9). For example, a small-scale psoriasis GWA, involving the analysis of 25 000 gene-centric SNPs has been carried out by Cargill *et al*. (10) and has convincingly identified *IL23R* and *IL12B* as disease susceptibility genes.

Here, we describe a larger GWA scan, involving the analysis of more than 408 000 SNP loci. We also report the identification and preliminary characterization of a novel disease susceptibility gene, coding for a C3HC4 RING domain protein that binds ubiquitin via a UIM.

## RESULTS

### Outcome of the GWA scan

The scan, which was conducted on DNA pools, generated genotypes for 99% of the analyzed markers. The distribution of the resulting *z*-scores was close to normality, matching the pattern expected for two samples drawn from the same population ($k = 0.81$). Importantly, this demonstrates the absence of stratification within our case–control sample.

As expected, the most significant association scores generated by the scan corresponded to MHC markers. The highest-ranking SNP was rs3134792 ($z = 6.08$; $P = 1.0 \times 10^{-9}$), mapping centromeric to HLA-C. As reported elsewhere, the initial results of this scan also confirmed the presence of association at the *IL23R* and *IL12B* psoriasis susceptibility loci (11).

Outside of the MHC, we found 244 *z*-scores$>$3.5 ($P < 0.0005$) and 55 *z*-scores$>$3.89 ($P < 0.0001$) (Supplementary Material, Table S1). As the use of pooled DNA samples precludes the implementation of quality control checks (e.g. analysis of call rates and Hardy–Weinberg equilibrium), we opted to follow-up genomic regions with clusters of disease-associated SNPs, rather than focusing on single markers generating very significant *P*-values.The largest non-MHC cluster of associated markers was found within a 47 kb interval on chromosome 20q13. This region contains two genes (*ZNF313/RNF114* and *SPATA2*) and spans a total of six correlated SNPs (rs495337, rs2235617, rs1056198, rs6125829, rs2235616, rs636987), all generating *z*-scores$>$3.5 ($P < 0.0005$) (Fig. 1).

### Genetic analysis of the disease-associated interval

To confirm and extend the results obtained on pooled-DNAs, we arbitrarily selected one of the six associated markers (rs495337) for follow-up. We first carried out individual genotyping of rs495337, in an expanded data set, including the case and control samples used to generate the pools and an

additional 1429 patients and 987 controls. The analysis of this combined UK sample (1747 cases versus 1275 controls) confirmed that genotypes were distributed according to Hardy–Weinberg equilibrium and that SNP rs495337 is significantly associated with psoriasis ($P = 4.05 \times 10^{-5}$; OR: 1.25; 95% CI: 1.12–1.39) (Table 1). To exclude the possibility that these results may reflect population stratification, we queried the 1958 Birth Cohort regional variation database (http://www.b58cgene.sgul.ac.uk/). We obtained data for rs2235617, an SNP highly correlated to rs495337 ($r^2 = 1.0$) and found that the allele frequencies of this marker are homogenous across all UK regions ($P = 0.82$). Thus our association results are unlikely to reflect hidden population structure.

To refine the localization of the susceptibility allele underlying the association signal, we genotyped our expanded data set for nine tag SNPs, capturing a total of 49 variants ($r^2 > 0.95$) across 90 kb of genomic DNA (Fig. 1). The most significant single-marker association remained the one originally observed for rs495337 (Table 2). A sliding-window analysis also confirmed that the haplotypes showing the highest association with psoriasis were those including rs495337 (Supplementary Material, Fig. S1).

Having identified rs495537 as a critical marker for the associated region, we typed this SNP in a second replication cohort, including 932 patients and 940 controls of German origin. We observed a *P*-value of $8.3 \times 10^{-4}$ (OR of 1.25; 95% CI: 1.10–1.43), providing further validation for our association results. As we failed to detect any evidence for heterogeneity between the UK and German data sets ($I^2 = 0\%$), we combined the two samples, obtaining a *P*-value of $1.4 \times 10^{-8}$ for the overall resource (2679 cases versus 2215 controls).

To ensure that we had annotated all the variation captured by SNP rs495337, we re-sequenced the exons and putative regulatory regions (i.e. conserved non-coding sequences identified on the UCSC genome browser) of *SPATA2* and *ZNF313*, in 15 heterozygous individuals. This effort identified four sequence variants that had not been typed by the HapMap Consortium. An analysis of 96 control individuals confirmed that all four markers were in high LD ($r^2 > 0.9$) with previously genotyped tag SNPs (Supplementary Material, Table S1). Taken together, the data that we generated and that available from the HapMap Consortium indicate that rs495337 captures eight SNPs, (seven in the *ZNF313* gene region, one in *SPATA2*), with $r^2 > 0.9$. None of these variants results in an amino acid change (Table 3).

**Table 1.** Association results for marker rs495337

| | MAF (allele counts) Cases | Controls | OR | *P*-value |
|---|---|---|---|---|
| UK sample | 0.37 (1242/3320) | 0.43 (1055/2468) | 1.25 | 0.000045 |
| German sample | 0.42 (735/1742) | 0.48 (861/1802) | 1.25 | 0.00083 |
| Combined sample | 0.39 (1977/5062) | 0.45 (1916/4270) | 1.25 | $1.4 \times 10^{-8}$ |

**Table 2.** Association analysis of tag SNP spanning the disease-associated region

| Marker | Location | MAF (allele counts) Cases | Controls | *P*-value |
|---|---|---|---|---|
| rs6020100 | SLC9A8 | 0.43 (1421/3304) | 0.39 (967/2454) | 0.006 |
| rs645544 | SLC9A8 | 0.44 (1483/3334) | 0.48 (1173/2462) | 0.02 |
| rs7270636 | SLC9A8 | 0.08 (266/3294) | 0.08 (174/2304) | 0.48 |
| rs1062840 | SLC9A8 | 0.10 (262/2686) | 0.09 (221/2452) | 0.36 |
| rs13433386 | SLC9A8 | 0.05 (175/3350) | 0.05 (123/2472) | 0.67 |
| rs683954 | Intergenic | 0.13 (441/3286) | 0.12 (296/2466) | 0.11 |
| **rs495337** | **SPATA2** | **0.37 (1242/3320)** | **0.43 (1055/2468)** | **0.000045** |
| rs2769982 | SPATA2 | 0.43 (1437/3330) | 0.48 (1172/2454) | 0.0005 |
| rs2038127 | ZNF313 | 0.31 (1061/3418) | 0.29 (712/2486) | 0.05 |
| rs6020157 | Intergenic | 0.19 (604/3090) | 0.18 (454/2470) | 0.27 |

**Table 3.** Summary of variants captured by rs495337

| Marker | Location | $r^2$ |
|---|---|---|
| rs632376[a] | *SPATA2* 3′-UTR | 0.92 |
| rs612586 | *ZNF313* intron 1 | 1.0 |
| rs2235617 | *ZNF313* intron 1 | 1.0 |
| rs1056198 | *ZNF313* intron 1 | 1.0 |
| rs2235616 | *ZNF313* 3′-UTR | 1.0 |
| rs6125829 | *ZNF313* 3′-UTR | 1.0 |
| rs6067284[a] | *ZNF313* downstream region | 0.97 |
| rs4810997 | *ZNF313* downstream region | 1.0 |

[a]SNPs identified by re-sequencing.

## Expression analyses

Given the high correlation between *ZNF313* and *SPATA2* SNPs, we sought to refine the localization of the disease susceptibility allele by assessing the effect of SNP rs495337 on the expression levels of the two genes. We queried the Sanger Institute Genevar database (12) (http://www.sanger.ac.uk/humgen/genevar/) and obtained normalized expression data relating to the lymphoblastoid cell lines of 210 unrelated HapMap individuals (Fig. 2). We found that the minor allele of rs495337 was associated with increased levels of *ZNF313* (unpaired *t*-test $P = 0.003$) but had no effect on *SPATA2* expression ($P > 0.05$).

We also assessed the expression of the two transcripts in tissues that are relevant to the pathogenesis of psoriasis. Using real-time PCR, we demonstrated that *ZNF313* is clearly expressed in skin, CD4+ T-lymphocytes and dendritic cells, whereas *SPATA2* transcripts are barely detectable in these cell types (Fig. 3A). Taken together, these findings suggest that the disease susceptibility allele may be a *ZNF313* regulatory variant tagged by rs495337.

To further characterize the distribution of *ZNF313* transcripts, we carried out real-time PCR analyses of multiple tissues cDNA panels. We detected *ZNF313* mRNA in a wide range of cell types, with the highest transcript levels found in testis (Fig. 3B).

## Analysis of recombinant GST-ZNF313

The *ZNF313* gene (also known as *RNF114*) consists of six exons, encoding a 2.4 kb transcript and a 25.7 kDa protein. Homology searches have indicated that ZNF313 belongs to a

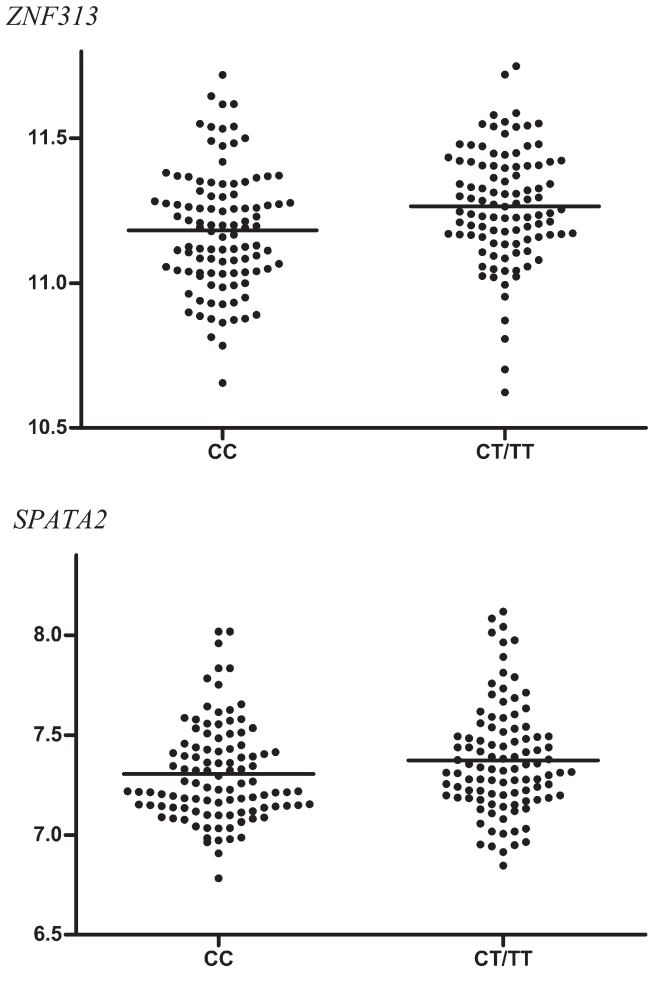


**Figure 2.** Plots of *ZNF313* and *SPATA2* expression levels measured in HapMap Individuals bearing different rs495337 genotypes.

recently defined family of RING domain E3 ubiquitin ligases, characterized by the presence of three zinc-fingers and an UIM (13). In order to establish whether ZNF313 binds ubiquitin, we carried out a pull-down assay by incubating free poly-ubiquitin chains with a GST-ZNF313_UIM protein, bound to glutathione beads. We found that the recombinant protein could efficiently pull down K48-linked poly-ubiquitin chains (i.e. molecules where ubiquitin residues are linked to each other via lysine 48), as well as K63-linked ones (Fig. 4).

## DISCUSSION

We report here the characterization of a novel psoriasis susceptibility gene, identified by whole genome association analysis. Our scan was carried out on a relatively small initial sample, including only 318 cases and 288 controls. We appreciate the limitations of this approach and recognize that modestly powered data sets tend to generate upwardly biased estimates for susceptibility alleles odds ratios (14). In genome-wide scans, however, this bias is most pronounced for variants showing extreme statistical significance (and consequently extreme odd ratios) (14). As SNP rs495337 ranked only 210th among disease-associated markers, the estimate of its genetic effect is less likely to be inflated. Moreover, the odds ratio observed for rs495337 in the initial UK sample (OR: 1.25, CI: 1.12–1.39) was remarkably similar to that obtained in the German replication data set (OR: 1.25, CI: 1.10–1.43), which would indicate that the genetic effect of the associated variant is unlikely to have been over-estimated.

To maximize the cost efficiency of our experiment, we have carried out our initial association scan on pooled DNA samples, having previously demonstrated that this approach can reliably detect genuine susceptibility alleles (15). Indeed, our scan has readily identified *HLA-C* as the locus conferring the highest disease risk and has detected significant associations at the *IL23R* and *IL12B* psoriasis susceptibility loci. Conversely, the use of pooled DNA samples prevents the implementation of fundamental quality controls, such as the analysis of Hardy–Weinberg equilibrium. Thus, our scan can be regarded as a first pass study, the results of which need to be validated by individual genotyping of the most significantly associated loci. With this study, we chose to follow-up a chromosome 20q13 genomic region harboring a cluster of six disease-associated markers. We validated our initial observations by genotyping two independently ascertained replication samples of British and German origin. The analysis of our entire data set, including a total of 2679 cases and 2215 controls, identified SNP rs495337 as the marker showing the most significant association with the disease. Rs495337 maps to the *SPATA2* transcript and is in high LD with several SNPs lying within the adjacent *ZNF313* gene. Having reached a limit in the resolution of genetic analyses, we used publicly available expression data, to differentiate the effect of these highly correlated variants. We were able to show that the minor allele of SNP rs495337 is associated with an increased expression of *ZNF313*, suggesting that the disease susceptibility allele is likely to be a *ZNF313* regulatory variant tagged by rs495337.

As GWA scans have identified significant overlaps between genetic determinants for clinically distinct inflammatory diseases (16,17), it is tempting to speculate that *ZNF313* might also be involved in the pathogenesis of other immune-mediated conditions. In this context, the analysis of SNP rs495337 in Crohn's disease (CD) data sets would be of particular interest, as we and others have documented the existence of several risk alleles conferring susceptibility to both psoriasis and CD (10,11,18).

Since the function of the ZNF313 protein is unknown, we used expression studies and database searches to gain an insight into its physiological role. Our real-time PCR analysis demonstrated that *ZNF313* is clearly expressed in disease relevant cell types, including CD4+ T-lymphocytes, dendritic cells and skin. At the same time, we detected abundant transcript levels in testis, pancreas, kidney and spleen, indicating that the activity of the ZNF313 protein is unlikely to be restricted to the immune system.

Homology searches have shown that ZNF313 belongs to the same family of RING domain E3 ubiquitin ligases as TRAC-1, a positive regulator of T-cell activation (19). We were able to show that ZNF313, like TRAC-1 (13), can bind ubiquitin *in vitro*. Our experiments clearly demonstrated that recombinant GST-ZNF313_UIM can pull down both K48- and K63-linked

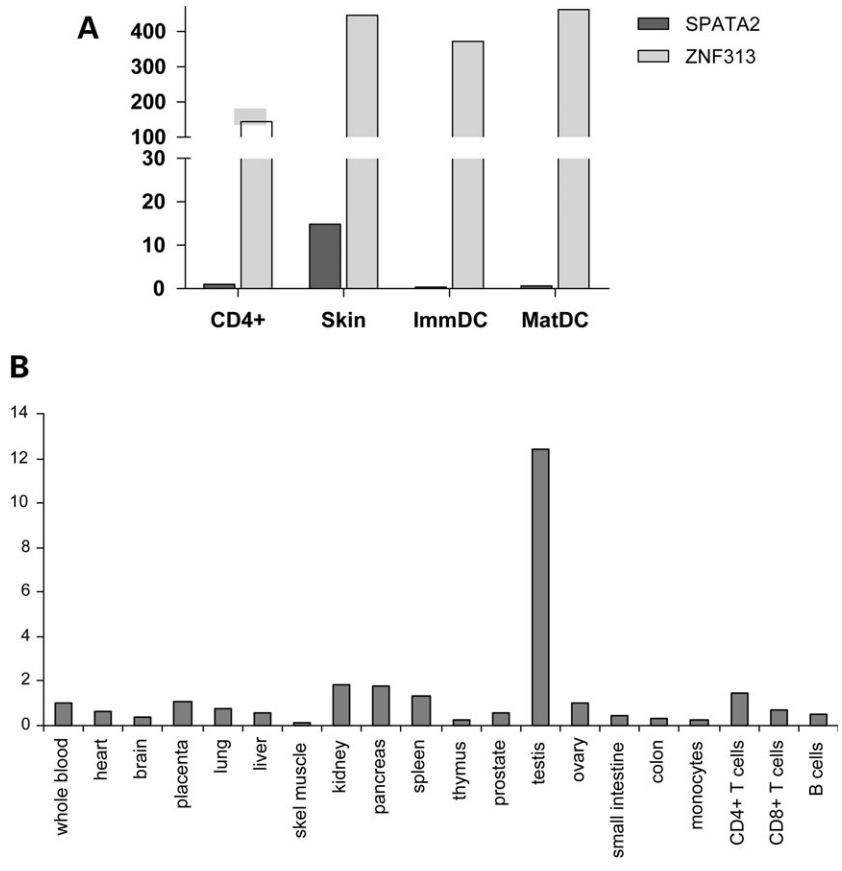


**Figure 3.** Real-time PCR analysis of *SPATA2* and *ZNF313* expression levels. (**A**) Analysis of *SPATA2* and *ZNF313* expression in disease-relevant cell types. The *SPATA2*/*GAPDH* ratio for CD4+ T-lymphocytes was set as a baseline value to which all other transcripts were normalized. ImmDC, immature dendritic cells; MatDC, mature dendritic cells. (**B**) Analysis of *ZNF313* expression in multiple tissue cDNA panels. The *ZNF313*/*GAPDH* ratio for whole blood was set as a baseline value to which all other transcripts were normalized.

polyubiquitin chains. These two molecules have distinct physiological roles, as K48 chains are a signal for proteasomal degradation, whereas K63-linked poly-ubiquitins participate in a variety of cellular pathways, including endocytosis, autophagy and DNA repair (20–22).

Protein ubiquitinylation plays an important role in the regulation of immune responses by modifying the activity or promoting the degradation of many signaling molecules (23). E3 ligases are the enzymes that confer substrate specificity to this process (24) and have been repeatedly involved in the pathogenesis of auto-immune conditions (25–27). In this context, the identification of ZNF313 physiological substrates is of great interest, as it will shed new light on the regulation of the pathways that underlie epithelial inflammation.

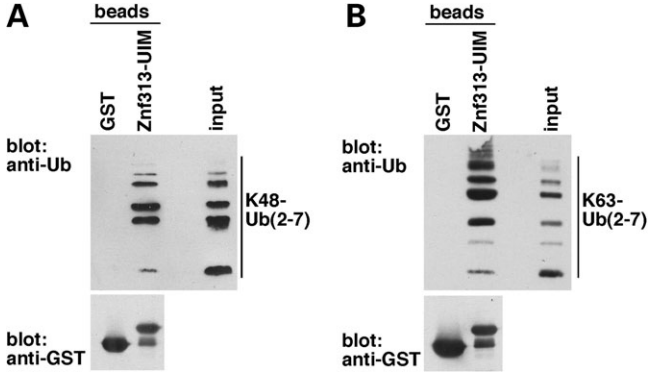


**Figure 4.** ZNF313 binds both K48- (**A**) and K63-linked (**B**) ubiquitin chains. Poly-ubiquitin molecules were incubated overnight with GST-ZNF313_UIM or GST beads. Beads washed in PBS were analyzed by western blotting for ubiquitin and GST, respectively.

## MATERIALS AND METHODS

### Subjects

The GWA scan was carried out on 318 British patients of North-European descent and 288 ethnically matched controls. All patients had early-onset psoriasis vulgaris (occurring before 40 years of age) and were recruited at St John's Institute of Dermatology (London, UK). The control individuals were part of the European Collection of Cell Cultures (ECACC) Human Random Control Panel, a cohort of healthy Caucasian blood donors whose parents and grandparents were born in the UK. The UK replication sample included 1429 British patients with early onset psoriasis and 987 unrelated controls. The patients were recruited through St John's Institute of Dermatology ($n = 365$), Glasgow Western Infirmary ($n = 310$) and the Dermatology Centre of

Manchester University ($n = 754$). Four hundred and fifty-nine British Caucasian controls were recruited from St John's Institute of Dermatology and the remaining 528 were randomly sampled from the 1958 British Birth Cohort, a nationally representative data set, including individuals prospectively ascertained in 1958, in England, Wales and Scotland (28). The German replication sample included 932 patients recruited through two psoriasis rehabilitation hospitals and 940 geographically matched, healthy blood donors. All patients and controls gave their informed consent for use of their DNA in genetic epidemiological analyses. This study was approved by the Guy's and St Thomas' Hospitals Ethics Committee of King's College London, the Salford and Trafford Local Research Ethics Committee, North Glasgow University Hospitals NHS Trust Local Research Ethics Committee and the Ethical Committee of the University of Munster.

### Genotyping

The GWA scan was carried out on case and control DNA pools, generated as previously described (15). Briefly, DNA was diluted to 50 ng/μl based on Quanti-iT Picogreen (Invitrogen) quantitation. The Picogreen analysis was repeated on the diluted DNA and concentrations were adjusted based on these results. This process was repeated until all samples consistently measured 50 ng/μl. Four replicates of each pool were prepared and hybridized to HumanHap300 and Human-1 BeadChips (Illumina, San Diego). Although only three pool replicates need to be produced to address the possibility that duplicated chips may yield discordant allele frequencies estimates, we included a fourth replicate to make our estimation more robust. We did not analyze more than four chips as the effect on allele frequencies estimates would have been marginal and for most SNPs, the sampling error (determined by the size of our resource) would have exceeded any technical inaccuracies in allele frequency measurement.

Estimates of allele frequencies for each pool were obtained based on the hybridization intensities from the two probes corresponding to each SNP allele. The Illumina genotype calling algorithm was modified as previously described (15), in order to maximize its accuracy in the context of pooled DNA genotyping.

In the follow-up to the GWA scan, chromosome 20q13 SNPs were typed using Applied Biosystems TaqMan assay, according to the manufacturer protocol.

### Direct sequencing

Primers were designed to amplify all *SPATA2* and *ZNF313* exons, as well as exon–intron junctions, conserved non-coding regions and putative gene promoters (primer sequences and cycling conditions available on request). Sequence reactions were loaded on an ABI 3730*xl* automated sequencer (Applied Biosystems) and nucleotide changes were detected by visual inspection of chromatograms.

### Real-time PCR

Ficoll-Hypaque density-gradient centrifugation was used to isolate peripheral blood mononuclear cells (PBMCs) from 50 ml buffy coats. CD4+ T-cells were isolated from PBMCs by using the RosetteSep human CD4+ T-cell enrichment kit (StemCell Technologies). To obtain dendritic cells, monocytes were isolated from PBMCs using CD14 microbeads (Miltenyi Biotec.) and cultured for 7 days in complete medium plus 1% (v/v) single donor plasma, 20 ng/ml GM-CSF (Peprotech) and 20 ng/ml IL-4 (R&D system). Maturation of DCs was induced on day 6, using a cocktail of cytokine containing TNFα, IL-1β, and IL-6 (all from R&D system) plus prostaglandin $E_2$ (Sigma-Aldrich). Punch skin biopsies (6 mm) were obtained from healthy volunteers undergoing plastic surgery. Total RNA was extracted from all tissues using the Trizol reagent (Invitrogen) and reverse transcription was carried with the Reverse-it cDNA first-strand synthesis kit (ABgene). Multiple tissue cDNA panels (Human MTC I and II, Human MTC blood fractions) were purchased from Clontech. Real-time PCR reactions were set up using TaqMan Gene Expression assays (Applied Biosystems) that had been designed to detect cDNA but not genomic DNA. *SPATA2* and *ZNF313* transcript levels were quantified by the ΔΔCt method (29), using *GAPDH* as an endogenous control. All reactions were carried out in duplicate.

### Ubiquitin pull-down assay

Recombinant GST-ZNF313_UIM (amino acids 183–228) proteins were purified from 10 ml *E.Coli* BL21 cultures, using glutathione–sepharose beads (Amersham Pharmacia Biotech). For the ubiquitin pulldown, 20 μl GST-ZNF313_UIM beads were incubated overnight at 4°C, with 1 μg K48- or K63-linked poly-ubiquitin$_{2-7}$ (Biomol), in PBS/0.1% BSA. Beads were washed three times in PBS, heated to 65°C in SDS-sample buffer and analyzed by western blotting, using an anti-ubiquitin (Covance) or anti-GST (Amersham Pharmacia Biotech) antibody.

### Statistical analyses

The differences in allele frequencies between case and control pools were expressed in terms of *z*-scores, a statistic incorporating errors due to imprecise measurements within a DNA pool. Under the null hypothesis of no association, the *z*-scores are normally distributed, so that they can be readily converted into *P*-values (15). To assess the presence of population stratification within the GWA data set, we used software written in-house to analyze the observed *z*-score distribution. The program computes the value of *k*, a multiplier that is used to adjust the *z*-score distribution, in the presence of population stratification. If cases and controls are drawn from the same population, the *z*-score distribution is very close to normality and the value of *k* approaches 1.0. Conversely, when the two samples are drawn from ethnically different populations, the *z*-score distribution is much wider than expected and adjusting to normality requires a *k*-value that is somewhat different from 1. We have applied this program to the analysis of samples of known ethnicity and observed a *k*-value of 0.8 for a comparison between two US populations of North-European descent. As expected, the analysis of samples of divergent ethnicity (North-Europeans versus Ashkenazi Jews) generated a markedly different *k*-value ($k = 0.15$).

Tag SNPs were identified using the Haploview software (30). The difference in allele frequencies between individually genotyped cases and controls were assessed using Fisher's exact test. Haplotype-based association analysis was carried out on three-marker sliding windows, using the PLINK software (31). The presence of heterogeneity between replication samples was assessed using the $I^2$ statistic (32). Differences in gene expression levels between individuals carrying different rs495337 genotypes were assessed using an unpaired *t*-test.

## SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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*Conflict of Interest statement*. As employees of Myriad Genetics Inc., Drs Timms, Abkevich, Gutin and Lanchbury receive compensation and stock options from the Company.

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