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Genetic and Physical Interaction of the B-Cell SLE-Associated Genes *BANK1* and *BLK*

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Abstract

Objectives—Altered signaling in B-cells is a predominant feature of systemic lupus erythematosus (SLE). The genes *BANK1* and *BLK* were recently described as associated with SLE. *BANK1* codes for a B-cell-specific cytoplasmic protein involved in B-cell receptor signaling

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Competing Interest
Please list Competing Interests if they exist if not please include the following statement; Competing Interest: Dr Jerome Wojcik is an employee of Merck Serono International, SA.

CONFLICT OF INTEREST STATEMENT
JW is an employee of MerckSerono Inc, and as such, the data or software he has developed belongs to MerckSerono Inc.

and *BLK* codes for an Src tyrosine kinase with important roles in B-cell development. To characterize the role of *BANK1* and *BLK* in SLE, we performed a genetic interaction analysis hypothesizing that genetic interactions could reveal functional pathways relevant to disease pathogenesis.

Methods—We Used the method GPAT16 to analyze the gene-gene interactions of *BANK1* and *BLK*. Confocal microscopy was used to investigate co-localization, and immunoprecipitation was used to verify the physical interaction of BANK1 and BLK.

Results—Epistatic interactions between *BANK1* and *BLK* polymorphisms associated with SLE were observed in a discovery set of 279 patients and 515 controls from Northern Europe. A meta-analysis with 4399 European individuals confirmed the genetic interactions between *BANK1* and *BLK*.

As BANK1 was identified as a binding partner of the Src tyrosine kinase LYN, we tested the possibility that BANK1 and BLK could also show a protein-protein interaction. We demonstrated co-immunoprecipitation and co-localization of *BLK* and *BANK1*. In a Daudi cell line and primary naïve B-cells the endogenous binding was enhanced upon B-cell receptor stimulation using anti-IgM antibodies.

Conclusions—Here, we show a genetic interaction between *BANK1* and *BLK*, and demonstrate that these molecules interact physically. Our results have important consequences for the understanding of SLE and other autoimmune diseases and identify a potential new signaling pathway.

Keywords

systemic lupus erythematosus; genetics; polymorphism; B-cells; autoantibodies

INTRODUCTION

Systemic lupus erythematosus (SLE) is a complex autoimmune disease where B-cell activity plays a major role in its development and clinical expression through the production of auto-antibodies and antigen presentation. Therefore, susceptibility genes co-expressed in B-cells are interesting candidates to be tested for genetic and functional interactions.

In humans, polymorphisms of the *BANK1 gene* have been associated with susceptibility for SLE in European and Asian populations (1–3). *BANK1* is located on chromosome 4q24 and codes for an adaptor/scaffold protein of 785aa (full length isoform) primarily expressed in B cells. BANK1 protein has 13 tyrosines susceptible of phosphorylation, two ankyrin repeats, a conserved Dof, BCAP, and BANK (DBB) domain, and a coiled-coil motif. It was identified as a binding partner of LYN, and it is also phosphorylated by SYK (4). BANK1 protein binds the IP3 receptors type 1 (IP3R-1) and 2 (IP3R-2) and promotes their LYN-mediated phosphorylation to induce Ca2+ mobilization from endoplasmic reticulum stores (4). However, Ca2+ mobilization was not impaired in a *Bank1* knock-out mouse (5). Further, the *Bank1* deficient mouse showed slight increase in germinal center formation and increased T-dependent responses with activation of Akt dependent on CD40 signaling. These features were subtle and no autoimmune phenotype was investigated. *BLK* was also recently identified as a susceptibility gene for SLE (6–8). The genetic polymorphisms of *BLK* associated with SLE, rs1327713 and its proxy rs2736340, are located in the promoter of *BLK* and the risk genotypes are correlated with reduced gene transcript levels. *BLK* is a Src tyrosine kinase specifically expressed in the B cell lineage (9). A knockout mouse for *Blk* did not show any phenotype and BLK was deemed to be redundant in B cell development and immune responses (10).

In this study we tested whether *BANK1* and *BLK* polymorphisms associated with SLE showed a genetic epistatic interaction, but we also extended our study to analyze whether BANK1 and BLK, like LYN and BANK1 (4), could show a protein-protein interaction. While we identified an interaction between polymorphisms in both genes, we also found that both proteins immunoprecipitated and their co-expression influenced the sub-cellular location of the kinase. As the genetic interaction involves risk variants correlated with gene expression, the genetic interaction might reflect an imbalance in gene expression. The relative amounts of the gene products could be important to maintain the homeostasis of a common pathway.

MATERIALS AND METHODS

Patients and controls

We extracted data from an Affymetrix® 100k SNPs genome-wide association scan conducted in 279 cases with SLE and 515 controls from Northern Europe (1). Individuals used for the 100k GWAS have been described (1). Two independent sets of cases and controls were used for replication. Set 1 (“USA”) is a European-American multicenter cohort of 621 cases and 774 controls. The second set (“Europe”) comprised 1697 SLE cases and 1550 sex- and ethnically-matched controls from a European multicenter collection (BIOLUPUS) including Germans, Italians, Argentineans and Spanish individuals.

Genetic outliers with <90% European ancestry were removed, as estimated using principal component analysis and the clustering algorithms implemented in EIGENSTRAT and STRUCTURE software, respectively, based on genotype data from 350 Ancestry Informative Markers or genome-wide data (available for the Argentineans and North Europeans). All SLE cases met at least 4 of the 11 classification criteria of the American College of Rheumatology (11). All individuals provided informed consent as approved by the recruiting site Institutional Review Boards at each of the affiliate Institutions. All clinical investigation has been conducted according to the Declaration of Helsinki.

Genotyping

The Swedish individuals were genotyped using the 100k Affymetrix® SNP array as described (1). The previously associated SNPs for *BLK* (rs2736340), which is not included in the 100k Affymetrix® SNP array, was genotyped by TaqMan® (ABI, Foster City, CA) pre-designed genotyping assays. SNPs showing genetic interaction with *BANK1* in the 100k were selected for replication. The replication set 1 (“USA”) SNPs were genotyped on the BeadExpress Illumina system. SNP rs10516483 (*BANK1*) was not available for this data set. Genotyping of set 2 (“Europe”) was performed for SNPs rs10516487 and rs10516483 (*BANK1*), rs1478895 and rs2736340 (*BLK*) also using TaqMan®. Only individuals with a genotyping rate >90% were used for analysis.

Statistical Analysis

From the 100k Affymetrix® SNP array data, nine tag SNPs in *BANK1* (rs7675129, rs10516487, rs10516483, rs2850390, rs1872701, rs10516490, rs1395306, rs871153 and rs238486) were individually tested for 16 types of interaction against 7 tag SNPs in BLK (rs1478895, rs1478890, rs2252534, rs1382566, rs9329246, rs7014565 and rs2061830). SNPs were filtered as following Hardy-Weinberg equilibrium (HWE) in controls ($p>0.01$) and having missing data rate per SNP <5%. Only markers with minor allele frequencies >30% in controls and >10% in cases, and minor genotype frequencies >10% in controls and >5% in cases were used. The rationale was that we wanted to screen only common variants of the general population (controls) in order to have enough 2-SNPs combinations and we did not want to miss some SNP that would be less common in the SLE population. Linkage

disequilibrium (LD) blocks were determined using the method of Gabriel *et al* (12) and tag SNPs were selected not to be in strong LD (r^2 <.80). BLK SNPs covered 22% while BANK1 SNPs covered 44% of the alleles in each genomic region at a r^2 >95%.

For the replication stage, SNPs following Hardy-Weinberg Equilibrium (HWE) in controls (p>0.001) and with missing data rates per SNP <10% were included in the analysis. None of the SNPs had significant differences in missing data between cases and controls (p>0.05).

Genetic Interaction Analysis—We used the GPAT16 method of Wirapati *et al*. (13). In brief, this method tests the genetic interaction between every pair of non-correlated SNPs (r^2<0.8) by recording the 16 possible contingency tables formed by the combinations or co-occurrences of alleles or genotypes of both SNPs under dominant and recessive models. For each contingency table, a Pearson score S is computed with its corresponding *P* value. A $P<1\times10^{-5}$ was considered significant. A significant interaction reflects the sum of additive (or main effects) and epistatic effects for a specific genotype combination (dominant or recessive). In this particular experiment our total number of tests performed was 504 (9 *BANK1* bait SNPs x 7 *BLK* SNPs x 16 tests / 2). GPAT16 makes 16 tests, but the total number is divided by 2 because each interaction is tested only in one direction. To determine the epistatic effect, that is, the increase in risk and an association odds ratio higher than expected under the null hypothesis of independence, each interaction is computed as the difference between the observed Pearson score S of each contingency table and the expected Pearson score S_0 under the null hypothesis of no epistasis (14). By doing so, it derives an epistasis-like score ($S_e=S-S_0$). An epistasis *P* value (P_e) is obtained through permutation. A $P_e<1\times10^{-3}$ was considered significant. This score is the difference of two dependent scores, each one following asymptotically a 1-df c^2. Therefore it does not follow any known statistical law and *p*-values p^e_t have to be empirically determined by permutation. If two genotypes when combined have a significant association (S score significant, P<1×10-5) but there is no significant epistatic effect (*Pe*>1×10-3) we conclude that such association is mainly due to the sum of the individual or marginal effects of the associated genotypes. If the epistatic effect is significant (*Pe*<1×10-3) we then refer to it as a genetic epistatic interaction.

Protein Interaction Experiments

Antibodies—The synthesized peptide ETKHSPLEVGSESSC was used to immunize rabbits to generate polyclonal anti-human *BANK1* anti-sera (ET-BANK antibody) and affinity purified using the SulfoLink Kit (Pierce). Additional antibodies include anti-mouse and anti-rabbit Alexa Fluor488, anti-mouse and anti-rabbit Alexa Fluor647, anti-V5 (Invitrogen, Carlsbad, CA); anti-Flag M2 monoclonal and rabbit anti-Flag (Sigma); anti-rabbit and anti-mouse IgG HRP (Zymed, San Francisco, CA). Mouse anti-human BLK antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA) and anti-β-tubulin from Sigma-Aldrich (St. Louis, MO).

DNA Cloning—*BANK1* and *BLK* sequences were amplified by PCR using cDNAs from human blood and the BJAB cell line, respectively and ORFs were cloned in pcDNA3.1D/V5-His (Invitrogen) and confirmed by sequencing. Proteins tagged by V5 and His epitopes at the C-terminal were produced by stop codon deletion. The N-terminal FLAG-tagged *BANK* plasmids were constructed by sequential PCR using overlapping primers. The amplified product coding FLAG fused to *BANK1* variants was cloned into pCR4-TOPO (Invitrogen) excised by EcoRI and BamHI and directionally sub-cloned into pIRESS2-EGFP (Clontech, Mountain View, CA). Sequences of the constructs are available upon request.

Co-immunoprecipitation and immunoblot analysis—Embryonic kidney HEK293T cells were seeded on 6-well plates and transfected with 4 μg of expression plasmids containing FLAG-tagged *BANK1* and V5-tagged *BLK* using Lipofectamine 2000. At 40 hrs cells were solubilized in Triton X-100 buffer (1% Triton X-100, 50mM HEPES pH 7.1, 150 mM Nacl, 1 mM EDTA, 2 mM Na_3VO_4, 10 % Glycerol, 0.1% SDS) containing protease inhibitors (Roche, Indianapolis, IN) and 1mM PMSF. Aliquots of pre-cleared lysates were saved for input analysis and the remaining lysate was incubated with rabbit anti-FLAG or mouse anti-V5 and immobilized with A or G-Sepharose beads (GE Heathcare, Uppsala, Sweden), respectively. Beads were washed with 1:1 Triton X100 buffer:PBS and immunoprecipitates eluted with SDS sample buffer boiling 5min. SDS-PAGE and immmunoblotting were carried out using standard protocols.

Primary B-cell separation and purification

Peripheral blood mononuclear cells (PBMCs) from buffy coats were isolated by Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden) density gradient centrifugation. For preparation of purified, unmanipulated naïve B cells, PBMCs were subjected to negative selection using naïve B cells isolation kit II (Miltenyi Biotec, Auburn, CA). For depletion of CD10+ transitional B cells from negatively selected CD19+CD27- naïve B cells, selected cells were incubated with anti-human CD10 microbeads (Miltenyi, Biotec, Auburn, CA). Cells were magnetically separated with MACS Columns and MACS Separator (Miltenyi, Biotec, Auburn, CA). The negatively selected naïve B cells consisted of >95% CD19+CD27- cells.

Endogenous Co-immunoprecipitation

Primary naïve B-cells (3×10^6 per condition) were treated without (−) or with (+) aIgM (10μm/ml) for 10 minutes in serum-free RPMI medium. Cell extracts were made from the treated cells and subjected to immunoprecipitation and Western analysis. Antibodies against human *BANK1* and *BLK* were purchased from Santa Cruz Biotech, Inc., and Abnova Corporation (Heidelberg, Germany), respectively. Recombinant Protein–G sepharose 4B beads were obtained from Invitrogen. Cell extracts were prepared using the lysis buffer containing 1% TritonX100, 50mM Tris pH7.4, 50 mM NaCl, 1 mM EDTA 2 mM Na3V04 and protease inhibitor cocktail from Roche. Immunoprecipitation was carried out using anti-human *BANK1* antibody overnight. The immunocomplexes were precipitated using Protein-G beads and washed three times with lysis buffer.

The precipitated complexes were mixed with SD-PAGE sample buffer from Invitrogen and the proteins were resolved in 4–12% gradient NuPAGE gel (Invitrogen). Western blot was carried out using standard protocols.

Confocal Microscopy

Transfected cells were fixed for 20 min at room temperature (RT) with 3,7% paraformaldehyde in PBS/0.18% Triton-X and permeabilized in ice-cold 50:50 methanol-acetone at −20°C for 10 min. After blocking in 3% BSA, 3% goat serum in PBT antibodies were diluted in blocking buffer and incubated overnight at 4°C. Fluorochrome-conjugated secondary antibodies were incubated for 2 hrs at RT and counterstained with SlowFade antifade with DAPI (Invitrogen). Fluorescence fusion proteins were visualized directly after fixation, FX enhancer treated (Invitrogen) and mounted in Vectashield (Vector Laboratories, Burlingame, CA).

Confocal microscopy was performed using a Zeiss 510 Meta confocal scanning microscope with Zeiss plan-Apochromat 63x oil-immersion objective. Dual- or triple- color images were

acquired by consecutive scanning with only 1 laser line active per scan to avoid cross-excitation. Image analysis was prepared using ImageJ and Adobe Photoshop.

RESULTS

Genetic interactions with BANK1

In the initial gene interaction analysis performed in the North European set, we observed a genetic interaction between *BLK* and *BANK1*, with the strongest epistatic effect between the *BANK1* SNP rs10516483 and the *BLK* SNP rs1478895 (*Pe* = 0.0001) (Table 1). Two SNPs in *BANK1* (rs10516483 and rs10516487, D'=0.86, r^2=0.36) and two in *BLK* (rs1478895 and rs2736340, D'=0.93, r^2=0.06) were involved in significant interactions although they did not reach the $Pe<10^{-3}$ threshold. Given the moderate sample size of the North European data set, we chose these four SNPs for replication in two larger and independent sets of cases and controls of European ancestry. We observed significant interactions between *BANK1* and *BLK* across all data sets (Table 1). The strongest association was displayed by the combination of recessive genotypes of *BANK1* rs10516487 (GG) and dominant genotypes of *BLK* rs2736340 (TT+TC) ($P_{meta\text{-}analysis}$ =1.75 x 10^{-15}) by using a total of 4399 samples. A significant *Pe* was demonstrated for this association in the replication set 2 from Europe (*Pe*=0.0013) (Table 1). In this set, a significant epistatic interaction was also observed between *BANK1* rs10516483 (CC) and *BLK* rs2736340 (TT+TC) genotypes (P_e = *0.0024*).

Biochemical Interaction between *BANK1* and *BLK* Proteins

The fact that BANK1 was identified as a partner of LYN (4), a Src tyrosine kinase, led us to test whether BANK1 would show a similar interaction with Blk, also a Src tyrosine kinase. We found that BANK1 and BLK co-immunoprecipitated each other in co-transfected HEK293T cells (Figure 1a,b). As the products of co-transfection could result in an enhanced artifactual binding, we then tested whether the endogenous proteins co-immunoprecipitate in the B-cell line Daudi and in isolated naïve B cells. We demonstrated co-immunoprecipitation between the endogenous BANK1 and BLK in the B-cell line (Figure 1c) and in primary, naïve B-cells (Figure 1d). We further showed that the binding was enhanced by stimulation through the B-cell receptor using anti-IgM antibodies (Figure 1c,d) suggesting that activation of BANK1 or BLK may be required to enhance protein-protein interaction.

BANK1 is classified as an adaptor/scaffold protein and as such, could function to direct other molecules towards specific sub-cellular compartments. Confocal microscopy showed that both BANK1 and BLK co-localized in the cytoplasm when co-expressed (Figure 2a–d). Interestingly, BLK localized preferentially to the plasma membrane in the absence of BANK1 (Figure 2e–g) while it was mostly retained in the cytoplasm when BANK1 was co-expressed in the same cell (Figure 2g). In fact, BLK was located at the plasma membrane in 95% of cells when the protein was expressed alone contrary to 27% of cells co-expressing both BLK and BANK1 (Figure 2h). Our results suggest that BANK1 could modulate the subcellular localization of BLK, which would be in agreement with the function of BANK1 as an adaptor/scaffold protein.

DISCUSSION

Here, we demonstrate that two SLE susceptibility genes showing a genetic interaction, namely *BANK1* and *BLK*, also interact physically.

We used the GPAT16 method to test for associated genotypic interactions, a method in principle similar to the Multifactor Dimensionality Reduction (MDR) (16) and slightly more powerful than standard algorithms (17). According to simulations, GPAT16 is at least as

powerful as the method of Marchini, *et al.* (18). The GPAT16 method enumerates exhaustively genetically relevant genotype combinations under dominant and recessive inheritance models, resembling the Batesonian definition of epistasis. This method is different from methods that consider the Fisherian definition of epistasis such as that implemented in PLINK (19), which test the interaction term in a logistic regression model.

The genetic interactions between *BANK1* and *BLK* observed in the North European and European data sets follow a recessive model for the *BANK1* genotypes (rs10516483 CC or rs10516487 GG) and a dominant model for *BLK* (rs2736340 TT+TC) genotypes. The interactions described here were not observable using logistic regression as implemented in PLINK (19) (supplementary Table 2), except for a weak significant interaction using the discovery set.

True epistatic interactions have been very difficult to detect and replicate (20, 21). We observed in the North European set a strong epistatic effect. As there is no established P-value for genetic interaction analysis, we used replication with independent sets of cases and controls. We replicated some of the epistatic effects (represented by the *Pe* value) that however did not reach our stringent *Pe* limit of $<10^{-3}$ (22). Due to the computational characteristics of the method, a meta-analysis cannot be done.

We chose to study the interaction between *BANK1* and *BLK* because of their functional interest in relation with SLE and their role in B-cell signaling. We believe that this way of analyzing genetic interactions fits our purpose of prioritizing candidate interacting genes for biological validation (23–25). In fact, a recent paper by Sun, *et al.* (25) analyzed human genome protein-protein interactions and found that physical connections were preferentially involved in gene-gene interactions. Thus, we believe that statistical genetics may guide the identification of true functional pathways in complex diseases.

Our findings point to a B-cell specific pathway that might be relevant in lupus pathogenesis. We showed that B-cell receptor stimulation enhances BANK1 and BLK binding. Because the engagement of the B-cell receptor with anti-IgM leads to tyrosine phosphorylation of numerous proteins including BANK1, it is likely that the interaction between BANK1 and BLK is regulated by cellular kinases. In chicken cell lines, SYK is a major player in phosphorylation of BANK1 upon BcR stimulation (4). BANK1 is a proline- and tyrosine-rich protein containing several predicted motifs for binding the SH2 and SH3 domains of Src-kinases. The binding of BANK1 to the Src-kinase LYN has been demonstrated but the precise protein domains involved in the interaction have not been defined (4). Detailed mutational analyses of BANK1 and BLK would be required to understand how BANK1 interacts with this family of kinases.

The change in sub-cellular distribution when BLK and BANK1 are expressed simultaneously suggests two possible functional scenarios. First, BANK1 as an adaptor protein could curb the positioning of BLK at the BcR by arresting it in intracellular compartments or, alternatively BANK1 could remove BLK from the BcR to restrict a sustained signaling. In both cases BANK1 could play an inhibitory role in B-cell activation. Supporting this idea, the *bank1* deficient mouse shows an increase in B-cell activation illustrated by an increased IgM production in response to T-dependent antigens (5).

It is important though to remember that the interacting SNPs in *BLK* are located in non-coding regions. The risk genotypes of rs2736340 in *BLK* correlate with gene expression (6). The interacting SNP of *BANK1* rs10516487 is located in exon 2 and leads to a R61H substitution but it is also a proxy of an intron 1 variant (rs17266594, $r^2 = 0.90$) associated with higher level of expression of BANK1 (1). In summary, the risk allele of *BLK* is associated with lower level of gene expression while the risk alleles of *BANK1* are coupled

with higher level of their own gene expression (Supplementary Figure 3). At this point we are unable to draw the precise mechanistic pathway to explain how the risk allele interactions lead to B-cell abnormalities. A hypothesis is that alleles affecting gene expression could impair the homeostasis of the B-cell by a combinatorial inhibition model as proposed by Ferrell (26). This model claims that the signaling is impaired due to alteration of the relative concentration of the interacting proteins.

The interacting variants of *BANK1* and *BLK* presented in this study might not be the functional variants responsible for the biological interaction effect as more extensive fine mapping and re-sequencing are required. Also, the SNP coverage would need to be increased although in detriment of multiple testing issues, particularly for whole-genome interaction analyses, which will be possible with new high-density arrays, so replication of the interactions will become even more important.

In summary, we describe here the use of a genetic interaction approach to reveal biologically relevant interactions and demonstrate that such approach can serve to define new pathways of disease, in this particular case a B cell-specific signaling pathway, which might be impaired in lupus patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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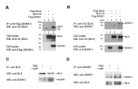

Figure 1. BANK1 and BLK Display a Protein-Protein Interaction

a) Immunoprecipitation and western blot showing protein-protein binding of *BANK1* and *BLK*. FLAG-BANK1 and BLK-V5 were co-transfected into HEK293T cells and immunoprecipitation was done using anti-FLAG antibodies. Western blot was performed using anti-V5 antibodies and confirmed with anti-FLAG antibodies. Lanes show: 1. Untransfected cells; 2. Co-transfection of FLAG-mock vector and BLK-V5; 3. Co-transfection of FLAG-BANK1 and BLK-V5.

b) Immunoprecipitation of cell extracts from co-tranfections showing recovery of BANK1 with the anti-V5 antibody directed to BLK-V5. Lanes show: 1. Untransfected cells; 2. Co-transfection of FLAG-Mock vector and FLAG-BANK1; 3. Co-transfection with FLAG-Mock and BLK-V5; and 4. Co-transfection of BLK-V5 and FLAG-BANK1.

c) Immunoprecipitation of endogenous BANK1 and BLK in the human cell line Daudi. Cell extracts were immunoprecipitated using anti-human BLK and the immunoprecipitates analyzed by Western blot.

d) Immunoprecipitation of endogenous BANK1 and BLK in naïve primary B cells. Cells were treated with anti-human IgM (SouthernBiotech) in a final concentration of 10 ug/ml for 10 minutes in serum-free RPMI medium or left unstimulated. Cell extracts were immunoprecipitated with anti-human BANK1 antibody (sc-133357, Santa Cruz Biotech) and analyzed by Western blot.

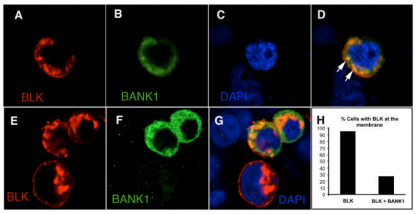

Figure 2. BANK1 co-localization with BLK and modulation of the subcellular localization of BLK by BANK1

Confocal images of HEK293 cells co-transfected with plasmids expressing BLK-V5 and BANK1 detected with immuno-fluorescence staining using antibodies against V5 and BANK1. Figures **2a–2d** and **2e–2g** represent two separate selected fields in two separate experiments. **a)** BLK (in red), **b)** BANK1 (in green); **c)** The nucleus stained with DAPI; **d)** merging showing co-localization of BANK1 and BLK in cytoplasmic compartments (arrows) and some BLK in the plasma membrane; **e)** Three cells expressing BLK (in red); **f)** Of the three cells, two co-express BANK1 (in green); **g)** Cell not expressing BANK1 shows BLK at the plasma membrane, while co-expression leads to its retention in cytoplasmic compartments, reduced at the plasma membrane. **h)** Diagram showing the proportion of cells harboring BLK at the plasma membrane when co-expressing or not BANK1. Approximately 200 cells were counted blindly in two independent experiments. BLK was detected with mouse anti-V5 followed by anti-mouse Alexa-647; BANK1 was detected using the rabbit anti-human BANK1 polyclonal antibody ET-BANK and anti-rabbit Alexa-488.

Table 1

Summary of the two-gene interactions between *BANK1 and BLK* in three independent sets of cases and controls

Gene SNP1	Genotype SNP1	Gene SNP2	Genotype SNP2	Population set	OR	OR L95	OR U95	*P*	Se	*Pe*	Freq. Cases	Freq. Controls	a	b	c	d	N
BANK1		***BLK***															
rs10516483	CC	rs1478895	CC	North Europe 100k	2.38	1.69	3.36	4.83E-07	8.9	**0.0001**	35%	18%	88	167	92	416	763
				Set 1 (USA)	-	-	-	-	-	-	-	-	-		-		-
				Set 2 (Europe)	1.42	1.17	1.72	0.0003	−0.3	0.8256	25%	19%	261	773	285	1200	2519
				Meta-analysis [b]	1.60	1.36	1.89	7.61E-12		nc			349	940	377	1616	3282
rs10516487	GG	rs1478895	CC	North Europe 100k	1.82	1.35	2.45	8.27E-05	3.7	**0.0063**	48%	33%	131	144	171	342	788
				Set 1 (USA)	1.33	1.06	1.66	0.0124	2.7	**0.0170**	38%	31%	233	388	241	533	1395
				Set 2 (Europe)	1.33	1.13	1.57	0.0005	−1.0	0.9578	42%	35%	434	605	527	980	2546
				Meta-analysis [a]	1.40	1.24	1.58	1.97E-10		nc			798	1137	939	1855	4729
rs10516483	CC	rs2736340	TT+TC	North Europe 100k	2.32	1.44	3.76	4.68E-04	4.6	**0.0111**	24%	12%	56	178	31	229	494
				Set 1 (USA)	-	-	-	-	-	-			-		-		-
				Set 2 (Europe)	1.70	1.36	2.13	3.65E-06	6.3	**0.0024**	18%	12%	184	824	169	1287	2464
				Meta-analysis [b]	1.80	1.47	2.21	1.18E-11		nc	19%	12%	240	1002	200	1516	2958
rs10516487	GG	rs2736340	TT+TC	North Europe 100k	1.82	1.22	2.72	0.0031	2.6	**0.0373**	32%	21%	80	170	54	209	513
				Set 1 (USA)	1.57	1.22	2.02	4.36E-04	−1.6	0.9850	27%	19%	168	453	148	626	1395
				Set 2 (Europe)	1.62	1.34	1.95	3.55E-07	6.7	**0.0013**	29%	20%	293	720	297	1181	2491
				Meta-analysis [a]	1.63	1.41	1.87	1.75E-15		nc	29%	20%	541	1343	499	2016	4399
BANK1	GG	rs10516487		Meta-analysis [a]	1.37	1.22	1.54	4.33E-08			56%	48%					4791
	CC	rs10516483		Meta-analysis [b]	1.52	1.31	1.76	1.66E-12			36%	27%					3334
BLK	TT+TC	rs2736340		Meta-analysis [a]	1.33	1.21	1.46	4.31E-14			30%	24%					8862

The epistasis P value (*Pe)* measures only the statistical significance of the epistatic effect of the interaction. For every 2 SNP-genotype combination tested, a contingency table under the null hypothesis of independence between both SNPs (no epistasis) is derived and an expected Pearson S score is calculated (S_0). The epistatic score is then defined as $Se = S - S_0$. This score is the difference of two dependent scores, each one following asymptotically a one-degree of freedom chi-square distribution. *Pe* are empirically determined by permutations (100,000 case/control label shuffling). nc: Due to the nature of the P_e statistic, no permutation p-value can be calculated for meta-analysis.

North Europe 100k: discovery set, genotype data extracted from a 100.000 SNPs genome-wide scan in Swedish. The SNP rs2736340 was genotyped *a posteriori*. Replication Set 1 (USA): European-American set. Replication Set 2 (Europe): A combined set of German, Italian, European-Argentine and Spanish cases and controls.

a = cases with genotype SNP1 AND genotype SNP2, b = cases with other genotype, c = controls with genotype SNP1 AND genotype SNP2, d = controls with other genotype. N: number of cases and controls with non-missing genotypes for both SNP 1 and SNP 2. Frequencies of the 2 SNP-genotype combinations in cases and controls are shown.