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### ► **To cite this version:**

Angelika Erhardt, Ludwig Czibere, Darina Roeske, Susanne Lucae, Paul G Unschuld, et al.. TMEM132D: a new candidate for anxiety phenotypes - evidence from human and mouse studies. *Molecular Psychiatry*, 2010, <10.1038/mp.2010.41>. <hal-00523674>

**HAL Id: hal-00523674**

**<https://hal.science/hal-00523674v1>**

Submitted on 6 Oct 2010

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## **TMEM132D: a new candidate for anxiety phenotypes - evidence from human and mouse studies**

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***Running title: TMEM132D - a new candidate gene for panic disorder***

***To be submitted to: Molecular Psychiatry***

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**ABSTRACT**

The life-time prevalence of panic disorder (PD) is up to 4% worldwide and there is substantial evidence that genetic factors contribute to the development of panic disorder. SNPs in TMEM132D, identified in a whole-genome association study, were found to be associated with panic disorder in three independent samples, with a two SNP haplotype associated in each of three samples in the same direction, with a p-value of  $1.2 \times 10^{-7}$  in the combined sample (909 cases and 915 controls). Independent SNPs in this gene were also associated with the severity of anxiety symptoms, in patients affected by panic disorder or panic attacks as well as patients suffering from unipolar depression. Risk genotypes for panic disorder were associated with higher TMEM132D mRNA expression levels in the frontal cortex. In parallel, using a mouse model of extremes in trait anxiety, we could further demonstrate that anxiety-related behavior was positively correlated with *Tmem132d* mRNA expression in the anterior cingulate cortex, central to the processing of anxiety/fear-related stimuli, and that in this animal model a *Tmem132d* SNP is associated with anxiety-related behavior in an F2 panel. TMEM132D may thus be an important new candidate gene for panic disorder as well as more generally for anxiety-related behavior.

## INTRODUCTION

Panic disorder (PD) is characterized by sudden episodes of acute anxiety or intense fear that may occur without any apparent reason or stimulus. PD is often accompanied by agoraphobia, an avoidance of situations where a person may feel trapped and unable to escape. The life-time prevalence of PD is up to 4% worldwide, and there is substantial evidence that genetic factors contribute to the development of PD, with heritability estimates ranging from 30-40% for PD and over 50% for agoraphobia.<sup>1,2</sup> Twin, family, and segregation analysis studies indicate a complex genetic model for PD with the involvement of multiple genes, each of modest effect.<sup>3</sup> In the past, genetic studies were mainly designed as candidate gene association or linkage studies. Linkage studies have revealed several potentially interesting loci located on chromosomes 1, 12 and 14.<sup>4-6</sup> Of these, only the locus on chromosome 1q has been identified by different groups independently.<sup>4,5,7</sup> Candidate gene association studies so far have mostly tested monoamine- and neuropeptide-related genes, giving inconsistent results, which often failed to replicate in independent studies, most likely due to phenotypic diversity, genetic heterogeneity and small sample sizes.<sup>8</sup> In summary, previous genetic studies in PD using linkage or hypothesis-driven candidate gene approaches have produced a number of findings, but have so far failed to bring up definitive results.

Following the development of high-throughput chip-based genotyping technologies, cost-effective simultaneous genotyping of a large number of single nucleotide polymorphisms (SNPs) across the entire genome has now become possible. These genomewide association (GWA) scans represent a new opportunity to identify common genetic variants predisposing to human mental disorders.

In this study, we report the results of a genomewide case-control association analysis in patients with PD in a multiple stage design<sup>9</sup> using a 317,000 SNP genotyping array in the initial sample and two independent German PD and anxiety disorder case-control samples for replication. Furthermore, to test for complementary inter-species effects, the clinical data were supported and extended by expression and association analyses in a mouse model of extremes in trait anxiety.<sup>10,11,12</sup>

## **METHODS**

### **Recruitment and sample characterization**

#### **Stage 1: PD discovery sample from the Max Planck Institute (MPI)**

216 patients consecutively admitted to the Anxiety Disorders Outpatient Clinic of the Max Planck Institute of Psychiatry in Munich (MPI) for diagnosis and treatment of PD, mostly presenting with a PD with agoraphobia (83.3%) and PD without agoraphobia (16.7%) as their primary psychiatric diagnoses, were recruited for the study (**Table 1**). The diagnosis was ascertained by trained psychiatrists according to the Diagnostic and Statistical Manual of Mental Disorders (DSM)-IV criteria. All patients underwent the Structured Clinical Interviews for DSM-IV (SKID I and II). PD due to a medical or neurological condition or a comorbid Axis II disorder was an exclusion criterion. All patients underwent a thorough medical examination including EEG, ECG and detailed hormone laboratory assessment. The mean age at onset of the disorder was 27.4 (SD: 11.6). The Bandelow Panic and Agoraphobia Scale<sup>13</sup> indicated a moderate to high severity of panic and agoraphobia (mean score (SD) rating assessed by clinicians: 29.8 (9.8)). The mean Hamilton Depression Scale score was 13.9 (6.7) and mean Hamilton Anxiety Scale score was 23.9 (9.9), indicating low depression and moderate anxiety in the patients.<sup>14,15</sup> At the time of assessment 83.0% of the cases were not medicated. 12% of the panic cases were medicated with SSRI's with or without benzodiazepine and the remaining patients (5%) were on tricyclic antidepressants, mirtazapin or benzodiazepine.

Ethnicity was recorded using a self-report sheet for perceived nationality, mother language and ethnicity of the subject itself and all 4 grandparents. All included patients were Caucasian: 83.0 % of German origin, 17.0% of European origin from countries other than Germany (Western European 5.1%: Switzerland, Austria, UK; Eastern European 7.6 %: Poland, Czechoslovakia, Rumania, Russia or former Yugoslavia and Mediterranean 4.0 %: Italy or Greece), excluding Turkish origin. The study was approved by the local ethics committee. Written informed consent was obtained from all subjects.

222 controls matched for ethnicity (using the same questionnaire as for patients), gender and age were recruited (**Table 1**). The included controls were Caucasian, 90.1 % of German origin, 2.2 % Western European origin, 7.3 of Eastern European origin and 0.4 % Mediterranean. Controls were selected randomly from a Munich-based community sample and screened for the presence of mood disorders,

psychotic symptoms, anxiety disorders, alcohol dependence, drug abuse, obsessive/compulsive disorders, PTSD, dissociative disorders, somatoform disorders, and eating disorders using the Munich version of the Composite International Diagnostic Interview (M-CIDI).<sup>16</sup> The M-CIDI is an updated version of the World Health Organization's CIDI version 1.2 (WHO-CIDI; World Health Organization, 1992), which incorporates questions to cover DSM-IV (American Psychiatric Association, 1994) and ICD-10 (World Health Organization, 1990) diagnostic criteria. Only individuals with a negative life-time history for the above-named disorders were included in the sample. These subjects thus represent a group of individuals from the general population who has never been mentally ill.

### **Stage 2: PD replication sample**

225 patients with PD with or without agoraphobia as their primary diagnoses as well as 240 anonymous blood donor controls matched for ethnicity, gender and age were used as a replication sample (**Table 1**). Patients were recruited at the Universities of Münster, Bonn, Göttingen and Würzburg. The diagnosis was ascertained by trained psychiatrists according to the Diagnostic and Statistical Manual of Mental Disorders (DSM)-IIIR criteria in 37.5 % of patients and according to DSM-IV in 62.5% of patients on the basis of structured interviews (SADS-LA and CIDI) and clinical records as previously described.<sup>17,18</sup>

### **Stage 3: PD and anxiety patients with psychiatric comorbidity**

#### **Patients from Bonn, Germany**

468 cases (33.3 % male, 66.7 % female; mean age male/SD: 42.9/14.3; mean age female/SD: 43.7/12.4) were recruited from consecutive admissions to psychiatric inpatient units in Bonn, Göttingen, Dresden, Mainz and Munich and as part of a population-based sample established with the help of the Census Bureaus of the cities of Bonn (North Rhine-Westphalia, Germany) and Mannheim (Baden-Württemberg, Mannheim) (**Table 1**). DSM-IV lifetime diagnoses were made by a consensus best-estimate procedure, based on all available information, including a structured interview (SCID-I), medical records, and the family history method. OPCRIT system was used to obtain detailed

polydiagnostic documentation of symptoms. The details of the recruitment and phenotype characterization procedures are outlined elsewhere.<sup>19</sup> The major part of the cases has PD with agoraphobia (39.0%) or PD without agoraphobia (20.4%), following by specific phobia (16.1%), social phobia (12.8%) and other anxiety diagnosis (11.7%). 84.0% of the cases were comorbid with other psychiatric disorder: depression disorder (51.6%), bipolar disorder (21.8%) and schizophrenic disorder (10.6%).

### **Controls: epidemiological sample (Popgen), Germany**

For research groups within the German National Genome Research Network (NGFN) genotypes from 550k Genotyping BeadChips (Illumina Inc., San Diego, USA) were available of 700 participants from PopGen (Population Genetic Cohort), an on-going cross-sectional epidemiological survey of a population from Northern Germany.<sup>20</sup> For the present study, only controls negative in screening questions regarding depressive mood or general anxiety were selected. This resulted in total of 452 controls (50.9% male, 49.1% female; mean age male/SD: 41.2/11.3; mean age female/SD: 42.5/13.9).

### **Unipolar depression sample, Max Planck Institute**

Data from 313 patients recruited within the Munich Antidepressant Response Signature (MARS; [www.MARS-depression.de](http://www.MARS-depression.de)) study (44.4% males, 55.6% females) were used for replication of association with anxiety severity (see Binder et al., 2004 for further details). Patients were diagnosed according to DSM-IV. Only Caucasian patients over 18 years old with depressive episodes were included. Exclusion criteria were the presence of manic or hypomanic episodes, the presence of a lifetime diagnosis of intravenous drug abuse and depressive symptoms only secondary to alcohol or substance abuse or dependence, or to a medical illness or medication. 77.6% of MD patients were currently treated with antidepressants (42.5% monotherapy, 57.5% two or more antidepressant substances). Ethnicity was recorded using a self-report sheet for perceived nationality, first language and ethnicity of the subject himself, parents and all four grandparents. All included patients were Caucasian and 91.2% were of German origin. The mean age was  $48.5 \pm 14.1$  years.

**EDSP, Max Planck Institute**

Data from 140 probands (34.6 % male, 63.6% female) come from the prospective longitudinal Early Developmental Stages of Psychopathology (EDSP) study comprising a baseline and three follow-up waves that cover an overall time period of about 10 years. Onset, development, and correlates of DSM-IV mental disorders were assessed in a community sample of adolescents and young adults in Munich, Germany. The baseline sample (n=3021) was drawn randomly from the 1994 government registries of all residents aged 14-24 years in metropolitan Munich / Germany and the surrounding counties. All participants were Caucasians of German descent. Detailed descriptions of the EDSP sample and design have been presented elsewhere.<sup>21</sup>

Diagnostic assessment was based on the computer-assisted version of the Munich-Composite-International-Diagnostic-Interview (DIA-X/M-CIDI).<sup>16</sup> The M-CIDI allows for the standardized assessment of symptoms, syndromes, and diagnoses of DSM-IV disorders along with information about onset, duration, and severity. Inclusion criteria were a history of panic attacks and a positive score in the M-CIDI anxiety section, which measure the severity of anticipatory anxiety in response to previous panic attacks (M-CIDI anxiety section D5, 4 items). The individuals did not necessarily fulfill the diagnosis of PD according to DSM-IV criteria (n=40: PD; n=45: subthreshold PD; n=55 no PD).

All studies have been approved by respective local ethic committees, and informed consent was obtained from all participants prior to inclusion.

**Quantitative analysis of severity of panic and anxiety symptoms:**

The quantitative analysis of anxiety severity parameters was done in the extended MPI anxiety sample containing the initial PD sample (n=216) and additional 18 patients having social/simple phobia as primary diagnosis and concomitant syndromal panic attacks. We used the Panic and Agoraphobia Scale (PAS)<sup>22</sup> which assesses the severity of the worst experienced period of panic symptoms (using the clinician rated scale). Association with SNP genotypes were first performed with the total score on

this scale and if  $p < 0.01$ , the 5 different subscales (severity of panic attacks, agoraphobia, anticipatory anxiety, disability and health concerns) were then tested for genotypic association too.

In the unipolar depression sample from the MPI, we used the subscore “Emotional Anxiety” (HAMAA; 7 items, number 1-6 and 14 on the scale) of the Hamilton Anxiety scale (HAMA)<sup>14</sup> to test psychopathological symptoms that might be similar to the symptom complex defined as anticipatory anxiety by the PA Scale. To evaluate whether the effects are specific for anxiety, we also performed the analysis with Hamilton Depression severity scores ascertained in both samples.<sup>15</sup> For the depression samples, scores obtained at the initial study assessment within 5 days of in-patient admission were taken for this evaluation.

### **DNA preparation**

On enrolment in the study, up to 40 ml of EDTA blood were drawn from each individual and DNA was extracted from fresh blood using standard DNA extraction procedures (Puregene whole blood DNA-extraction kit, Gentra Systems Inc; MN), in the MPI.<sup>23</sup>

### **SNP Genotyping**

Genomewide SNP genotyping for all patients from the MPI panic sample and MPI depression sample was performed on Sentrix BeadChip Array HumanHap300 Genotyping BeadChips (Illumina Inc., San Diego, USA), using the InfiniumII assay, according to the manufacturer’s standard protocols. On the Illumina HumanHap300 microarrays, more than 317,000 SNPs can be interrogated, which had been selected from the entire genome, mainly based on tagging SNPs as identified in Phase I of the International HapMap Project, and slightly enriched in genes as compared to intergenic regions. Genotype calling was performed with the BeadStudio software (Illumina Inc., San Diego, USA). The average call rate achieved was better than 99%, with samples below 98% being either retyped or excluded from the study. The reproducibility for samples (n=3) genotyped twice was better than 99.999%. Fluorescence intensity clusters of associated SNPs were inspected individually and the correct genotype assignment was controlled.

The genotyping in the second stage (replication samples, EDSP individuals) was performed on a MALDI-TOF MassArray® system (Sequenom Inc., San Diego, USA) employing the AssayDesigner software for primer selection, multiplexing and assay design, and the homogeneous mass-extension (hMe) process for producing primer extension products. Genotype calling was done with the MassArray Typer Software, after visual inspection of clusters. All MALDI-TOF SNP genotyping was performed on instruments of the Gene Analysis Center, GAC, at the Helmholtz Zentrum Munich, Neuherberg, Germany. All primer sequences used are available upon request.

### **Human TMEM132D expression:**

To explore possible effects of the SNPs associated with PD (rs7309727 and rs11060369) and anxiety severity (rs879560 and rs900256), we examined their association with TMEM132D mRNA expression in two publicly available datasets containing gene expression data from lymphoblastoid cell lines in the HapMap population<sup>24</sup> and human postmortem cortex.<sup>25</sup> For both samples, gene expression was assayed using the humanRefSeq8 Illumina expression arrays that contain two probes for TMEM132D - GI\_27484731-S and GI\_42734372-S, both targeting the 3' UTR of the gene. In lymphoblastoid cell lines, both probes gave equivalent signal, whereas for frontal cortex, normalized signal intensities were about twice as high for GI\_27484731-S than the other probe. For cortical expression data, p-values are thus reported for this probe as it is likely that GI\_42734372-S displayed sub-optimal hybridization. Nonetheless, for rs879560 and the SNPs tagging rs900256, nominally significant and similar associations were found for both probes. While the Stranger et al., data set contained genotypes for all SNPs in question, SNP genotyping in the Myers et al., data set was performed using the Affymetrix V5 SNP array. On this array, rs11060369 and rs879560 are directly represented and rs900256 is tagged by rs4759607 and rs4760030 with an  $r^2 = 1$ , rs7309727, however, is not tagged. Genetic associations of these SNPs with gene expression were performed using linear regression and normalized expression data and SNP genotypes as available for download from the respective websites (<ftp://ftp.sanger.ac.uk/pub/genevar/> and <http://labs.med.miami.edu/myers/LFuN/data.html>). For expression in lymphoblastoid cell lines, only HapMap parents and unrelated individuals were used (n=210). Data were first analyzed for CEU parents (n=60) only and then also for

all populations together, co-varying for population origin as expression levels were different among the four populations. For expression in cortical regions, we focused on the data available from frontal cortex (n=41) for maximal comparability to the mouse experiments as well as for the relevance of this brain region in fear and anxiety. All samples were neuropathologically healthy controls of European origin. Of the available covariates (gender, age at death, post-mortem interval and origin of samples), only age at death was significantly associated with TMEM132D expression levels and was thus used as covariate in all analyses.

### **Measuring *Tmem132d* expression in an animal model of pathological anxiety:**

#### ***Animals***

Animals for the following studies were inbred high (HAB) and low (LAB) anxiety related-behavior mice<sup>10,1112</sup> or unselected outbred CD1 controls (Charles River Laboratories, Sulzfeld, Germany). All mice were tested for parameters indicative of anxiety-related and depression-like behaviors as well as locomotor activity on the EPM, in the tail-suspension test and in the open field.<sup>10</sup> The animal studies were both approved by local authorities and conducted according to current regulations for animal experimentation in Germany and the European Union (European Communities Council Directive 86/609/EEC).

#### ***Microarray analysis***

The microarray experiment was performed in 6 male HAB and LAB mice aged 10 weeks under baseline conditions (EPM test > 72 hours before the experiment) and analyzed as previously described by Krömer et al.<sup>10</sup> RNA was extracted from the cingulate cortex, basolateral amygdala, central amygdala, dentate gyrus and hypothalamic paraventricular nucleus. RNA was amplified and labeled using the Illumina TotalPrep RNA Amplification kit (Ambion, Houston, TX) and hybridized onto MouseWG-6 v1.1 Expression BeadChips (Illumina, San Diego, CA) following the manufacturer's instructions. Fluorescence signals were scanned on a BeadStation (Illumina) and analyzed by in-house software routines. The manufacturer's built-in controls have been analyzed including hybridization

controls and sample dependent parameters. Only microarrays fulfilling Illumina's recommendations for quality control have been used for further evaluation.

### ***Real-time (RT)-qPCR***

Tissue samples from the cingulate cortex were obtained by the micropunching technique<sup>26</sup> using micropunchers of 1mm diameter (Fine Science Tools GmbH, Heidelberg, Germany). Punches were collected from 200µm sections between 1.18mm to 0.74mm from bregma, medially above the genu of the *corpus callosum*. All coordinates were based on the Mouse Brain Atlas.<sup>27</sup> Tissue punches were immediately frozen and kept on -80°C until further processing. For whole brain analyses, five coronal sections of 200µm were taken from different parts of the brain. Tissue punches or brain sections were homogenized with a pipette in 300µl Trizol (Tri Reagent, Sigma-Aldrich), then 30µl bidistilled water, 1µl linear acrylamide (5mg/ml, Ambion, Houston, TX), and 60µl chloroform were added before continuing with a standard protocol, using n-propanol for RNA precipitation overnight. The yield of total RNA was between 0.3 and 1.5µg. A maximum of 1µg of total RNA was reverse transcribed with Superscript II (Invitrogen, Karlsruhe, Germany) after DNase treatment according to the manufacturer's protocol. For quality control, a small aliquot of each cDNA was analyzed on an agarose gel.

cDNA of male or female HAB (n=10), unselected CD1 mice (n=5), or LAB mice (n=4) was analyzed by qPCR, using the QuantiFast SYBR Green PCR Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions, the respective oligonucleotide primers for *Tmem132d* (fwd: 5' CATCCCTTCTTCAGCCAGAG 3', rev: 5' AGTGAGAACCGCTGAATGCT 3'), and the house keeping gene *Gapdh* (fwd: 5' CCATCACCATCTTCCAGGAGCGAG 3', rev: 5' GATGGCATGGACTGTGGTCATGAG 3'). Experiments were performed in duplicates on the Lightcycler®2.0 instrument (Roche Diagnostics, Mannheim, Germany) under the following PCR conditions: initial denaturation at 95°C for 10min, followed by 40 cycles of denaturation (95°C for 10s) and a combined annealing and extension phase (60°C for 30s). At the end of every run, a melting curve (50-95°C with 0.1°C/s) was generated to ensure the quality of the PCR product.

Crossing points (Cp) were calculated by the LightCycler®Software 4.0 (Roche Diagnostics GmbH) using the absolute quantification fit points method. Threshold and noise band were set to the same level in all compared runs.

Relative gene expression was determined by the  $2^{-\Delta\Delta CT}$  method<sup>28</sup> using the real PCR efficiency calculated from an external standard curve. Cp was normalized to the housekeeping gene *Gapdh*, fold regulation values were calculated relative to the expression mean of LAB mice.

### ***Association study in an F2 panel***

A total of 510 F2 offspring of a HABxLAB intercross (F1) progeny were phenotyped (anxiety-related behavior: EPM; locomotor activity: EPM and open field; depression-like behavior: tail-suspension test; see **Figure 5C-F**) and genotyped based on DNA isolated from tail tips as described recently.<sup>12</sup>

Genotypes for SNP rs13478518 were determined on an Illumina Sentrix Array Matrix platform (Illumina, San Diego, CA) with the GoldenGate assay. The SNP was part of a custom designed panel with 384 SNPs. Genotypes were called from fluorescence intensity clusters using the Illumina BeadStudio software Ver. 3.1.0.0, with the genotyping module Ver. 3.1.12. All intensity clusters were inspected individually and adjusted manually, when necessary. In the parental generation, rs13478518 located in exon 9 of *Tmem132d* displays opposite homozygosity in HAB mice (AA) vs. LAB mice (GG). Twenty four samples were genotyped twice, with identical outcome thus assuring high data quality and reproducibility.

### **Statistics**

Genomewide case-control analyses were conducted by applying the WG-Permer software (<http://wg-permer.org>) and R-2.5.0 (<http://cran.r-project.org>). Haplotypes were tested using PLINK (<http://pngu.mgh.harvard.edu/~purcell/plink/>). SNPs with deviation from Hardy-Weinberg-equilibrium as well as SNPs with a callrate below 98% (initial sample) or 90% (stage 2 sample) and SNPs with a minor allele frequency below 2.5% were not taken into further analysis. SNPs were tested for association with PDs using the allelic  $\chi^2$ -test and the Armitage test for trend respectively. As gender and age had no significant influence, they were not included as covariates. The level of significance

was set to 5% (family-wise error rate, FER), and p-values were corrected for multiple comparisons permutation-based using the Westfall-Young method. Power calculations were performed using CaTS (<http://www.sph.umich.edu/csg/abecasis/CaTS>). Population stratification was tested with

EIGENSTRAT as implemented in EIGENSOFT

(<http://genepath.med.harvard.edu/~reich/EIGENSTRAT.htm>).

Analyses for psychopathology-related phenotypes (e.g. severity of panic attacks) were performed using the generalized linear method with SPSS for Windows (Release 12, SPSS Inc., Chicago, Illinois 60606, USA) testing effects on the genotypic level. Gender was used as covariates for all analyses. For the analysis in the joint sample, we standardized different severity phenotypes (PD: PAS; UNICO: HAMAa; EDSP: D5 Score) using Z-standardization to compare the relative standings of items from distributions with different means and different standard deviations.

For the analysis of the linkage disequilibrium (LD) pattern and haplotype block delineation we used haplotypes estimated by Haploview (<http://www.broad.mit.edu/personal/jcbarret/haplo/>). Blocks were defined using the confidence interval method described by Gabriel <sup>29</sup> (**Figure 3**). The haplotype calculations were performed using the omnibus test to test for overall significance and the  $\chi^2$ -test for specific haplotypes. The haplotype counts are calculated for each copy of the chromosome. Therefore, the total number of haplotype counts adds to the double number of individuals. Microarray data normalization and evaluation was performed using the R-package BEADARRAY.<sup>30</sup> RT-qPCR experiments were analyzed using non-parametric and correlation tests (Kruskal-Wallis, Mann Whitney and Spearman tests using SPSS 12.0) with applying sequential Bonferroni correction.

Statistical analysis of association data in the HAB/LAB-based F2 panel was performed using WG-Permer ([www.mpipsykl.mpg.de/wg-permer](http://www.mpipsykl.mpg.de/wg-permer)). Trait values were rank-transformed to protect against artifacts. Analysis was done using a genotypic model, i.e. the three genotypic classes possible for each of the phenotypes were treated as a separate class each, and global test on equality of the three means was performed.

## RESULTS

### Quality control and population stratification

In stage 1, we genotyped 317,503 SNPs in 216 cases with PD vs. 222 matched healthy controls using the Illumina Sentrix HumanHap 300k Genotyping BeadChip. Cases and controls were recruited at the Max-Planck Institute of Psychiatry in Munich. All SNPs with a call rate of less than 98% (9,988 SNPs) and/or displaying deviation from Hardy-Weinberg equilibrium (HWE) at an error level of below  $10^{-5}$  (8,795 SNPs) were excluded from the analysis as well as all rare SNPs with a minor allele frequency (MAF) below 2.5% (1,058 SNPs). This resulted in 297,979 SNPs to be included in the case-control analysis. Of these SNPs, 15,472 (5.2%) showed a nominally significant deviation from HWE ( $p < 0.05$ ) which is almost identical to the expected number of false positive findings under the null hypothesis of no HWE deviations. The average case-wise call rate for the SNPs entering the analysis was 99.8%. 158 SNPs were genotyped twice in 140 individuals using the Sequenom mass-extension method or TaqMan and we observed 100% concordance between these methods and Illumina genotypes.

To test for possible effects of population stratification, we applied the method of genomic controls. We calculated  $\chi^2$  -values for the allelic case/control - test with all SNPs passing the quality control criteria mentioned above. The distribution of these  $\chi^2$  -values was compared with the theoretically expected  $\chi^2$  - distribution.  $\lambda$ , the ratio of the median of the test statistics and the median of the theoretical distribution is a measure for stratification (**Figure 1**). This ratio is 1.025 in this sample, which implies that no large effects of population stratification are present. Furthermore, we ran EIGENSTRAT, a method based on principal component analysis, which detects and corrects for population stratification.<sup>31</sup> No outliers were detected, which is another indicator that population stratification is not likely to be a confounder in our analysis.

### Case-control association in panic sample stage 1 and genotyping in panic sample stage 2

None of the nominal genomewide associations remained significant after correction for multiple testing using permutation-based corrections in the initial sample consisting of 216 cases and 222 control subjects (see **Figure 2** for the chromosomal distribution of all p-values).

The highest nominal case-control association was found for rs7309727 (allelic test:  $p=5.1e-7$ , Armitage test of trend:  $p=7.726e-7$ ) located in TMEM132D. We decided to select 64 SNPs with nominal p-values below or equal to  $p=1.0e-4$  in the first stage association and to analyze them in the independent stage 2 PD sample (225 cases and 240 controls). After genotyping, 57 of these SNPs passed the quality criteria in the second stage (call rate > 90%, MAF > 2.5%). 5 SNPs from stage 2 showed a nominal case-control association with PD (**Figure 2**). The highest associated SNP from stage 1 (rs7309727/TMEM132D) displayed a significant case-control association with PD in stage 2 (allelic test  $p=0.008$ , Armitage test  $p=0.006$ , **Table 2**). The T allele was found to be the risk allele for PD in both samples and the odds ratios (OR) were 2.2 (1.59 – 3.02) and 1.5 (1.12 – 2.03) in stage 1 and stage 2 samples, respectively (**Table 2**). The second best associated SNP in TMEM132D, rs11060369 (stage 1: allelic model  $p=0.0001$ , Armitage test  $p=0.0001$ ), showed a trend for association in the stage 2 sample in the allelic model ( $p=0.08$  two-sided,  $p=0.04$  one-sided in allelic model and Armitage test). Further nominally significant associations were found in allelic tests for rs7617530 (FHIT; stage 1 sample:  $p=0.0009$ ; stage 2 sample:  $p=0.007$ ), rs721022 (KPNA4 gene; stage 1 sample:  $p=0.00001$ ; stage 2 sample:  $p=0.007$ ) and rs12466450 (CRIM1 gene; stage 1 sample:  $p=0.0001$ ; stage 2 sample:  $p=0.008$ ).

We then re-analyzed the 2 TMEM132D SNPs and SNPs in FHIT, KPNA4, CRIM1 that independently replicated in stage 2 on a nominally significant level in the joint sample (stage 1-2) with 441 cases and 462 controls. For rs7309727, the significance of the association with PD in the joint sample of stage 1-2 increased and the obtained p-value ( $p=4.87e-8$ , allelic test) now remained significant after experiment-wide correction for multiple testing (corrected  $p=0.015$ ). For the second best SNP in TMEM132D, rs11060369, the p-value improved too ( $p=8.0e-05$ , OR 1.48, CI 1.22-1.8) but did not reach genomewide significance after correction for multiple testing. For rs7617530/FHIT, the nominal p-value decreased to  $p=1.0e-5$  in the combined sample (OR=1.5; CI: 1.2-1.9) but was not significant after correction for multiple testing. For the remaining SNPs, the p-values were actually less significant in the joint sample (rs12466450/CRIM1:  $p=0.003$ ; rs721022/KPNA4:  $p=0.008$ ). For these SNPs, the genotypes in stage 1 and 2 samples were in the same direction, however, due to differences in the ORs, the overall p-value in the combined sample increased. As only rs7309727 in TMEM132D

reached genomewide significance after correction for multiple testing, we focused on the 2 TMEM132D SNPs for stage 3 replication in a third independent panic and anxiety disorder case-control sample.

### **Stage 3 case-control association in comorbid anxiety sample**

In stage 3, we genotyped the two most strongly associated SNPs in the TMEM132D from the discovery sample in a third sample of patients (n=468) from Bonn (Germany) including PD (59.4%) and other anxiety disorders (41.6%) patients and controls (n=452) screened for the absence of depressive mood or anxiety symptoms (**Table 1**).<sup>19,20</sup> In contrast to the first two samples, the majority of these patients had also a primary diagnosis of other major psychiatric disorders (see **Table 1**). For rs7309727, there was a trend for association in stage 3 (p=0.076 one-sided, p=0.15 two-sided Armitage test). rs11060369 replicated in stage 3 with p=0.02 in Armitage test, p=0.01 in an allelic as well as Carrier C model (**Table 2**). When restricting the analysis to patients with only PD, allelic p values for the 2 SNPs were: rs7309727: p=0.21; rs11060369: p=0.02.

### **Association analysis of replicated SNPs in the joint case-control sample and haplotype analysis**

We then re-analyzed the two TMEM132D SNPs that independently replicated on a nominally significant level in the joint sample with 909 cases and 915 controls. In the joint sample from stages 1 to 3, the p-value for rs7309727 was p=1.36e-6 in the Armitage test and did not withstand the correction for multiple testing (OR=1.4, CI: 1.24-1.68). For rs11060369, the p-value further improved when stage 3 sample was included (p=7.63e-6, OR=1.4, CI: 1.19-1.57) but also did not reach experiment-wide significance (**Table 2**). The calculated attributable risk for rs7309727 in the joint sample was 17.9%, for rs11060369 22.7%.

Since both SNPs are in the same block of linkage disequilibrium, we tested the association of their two SNP haplotypes in the three samples. The two SNPs build 3 common haplotypes; CC, TA and AA (**Table 2**). In stage 1, the omnibus test for association of these haplotypes was highly significant (p=5.48e-7) and this association could be independently replicated both in stage 2 (p=0.01) and stage 3 (p=0.04) (p = 0.07 for PD subgroup only). In each of the three samples, haplotype TA was

overrepresented in cases (**Table 2**). In the joint sample of stages 1 to 3, the omnibus test for haplotype association reached the p-value of  $1.2 \times 10^{-7}$  (corrected 0.04, OR for best specific haplotype TA: OR=1.5 CI: 1.2-1.8).

### **Severity of anxiety symptoms:**

#### **Panic disorder:**

To further evaluate the putative role of the gene TMEM132D in PD and generally for pathological anxiety, we analyzed dimensional measures of anxiety severity in the stage 1 panic sample extended with additional 18 patients having social/simple phobia and concomitant syndromal panic attacks with all SNPs genotyped within the TMEM132D locus.

The TMEM132D gene is extending over 830kb on chromosome 12q24.32 - 12q24.33. We included all genotyped SNPs in the gene region plus the flanking 20kb for further analysis; this region was covered by 172 SNPs on the 300k Illumina BeadChip of which 170 turned out polymorphic (**Figure 3**). We tested all polymorphic SNPs for association with disease severity using the “Panic and Agoraphobia Scale” (PAS) total score reflecting the severity of the worst episode of panic symptoms (see the methods’ section for more detail). The strongest association was found for rs900256, a SNP located in intron 4 of the gene ( $p=0.0003$ ) (**Table 3**). Further testing revealed that this effect was mostly due to association with the severity of anticipatory anxiety (PASaa:  $p=0.0001$  allelic model). We observed an additive genetic effect with higher severity of anticipatory anxiety associated with the C-allele. Two more markers in close proximity (rs879560 [ $p=0.0006$  for anticipatory anxiety subscale, risk allele A] and rs10847832 [ $p=0.0007$ , risk allele A]) were also associated with anxiety severity (**Figure 3 and Table 3**). All three SNPs are in high linkage disequilibrium (rs900256 and rs879560:  $D'=1$ ,  $r^2=0.846$ ; rs900256 and rs10847832  $D'=0.855$ ,  $r^2=0.639$ ; rs879560 and rs10847832:  $D'=0.841$ ,  $r^2=0.524$ ). After correction for multiple testing for 170 analyzed SNPs, the association for rs900256 with anticipatory anxiety remained significant (Bonferroni corrected  $p=0.02$ ). There was no association with severity of depressive symptoms as measured by the Hamilton Depression Scale total score. Interestingly, the 3 SNPs associated with severity of anxiety symptoms were separated by two hotspots of recombination from rs7309727 and rs11060369, associated with PD in the case-control design.

**Unipolar depression:**

In order to more generally evaluate the significance of TMEM132D in anxiety phenotypes and psychopathology, we tested the 3 SNPs found to be related to severity of anticipatory anxiety in the PD sample also for association with the severity of anxiety symptoms in in-patients with unipolar depression (n=313). We replicated the association of two of these SNPs with anxiety severity in this sample (rs900256; p=0.001 carrier C model, rs879560; p=0.001 carrier A model). The alleles associated with the least anxiety symptoms were concordant between PD and unipolar depression patients (**Table 3** for rs900256 and rs879560). As in the PD sample, none of the SNPs was found associated with the severity of depressive symptoms or in the case-control design for unipolar depression.

In both samples, MPI panic and MPI depression, medication status at the time of assessment was available. Current antidepressant treatment had no significant effect on PASaa or HAMAa and did not change the strength of the genetic association with anxiety severity.

**Anticipatory anxiety in probands with panic attacks from the general population**

To provide more evidence about the role of TMEM132D in probands with life-time panic attacks but not necessarily with a diagnosed life-time PD, we genotyped rs900256 and rs879560 in individuals recruited by the prospective longitudinal Early-Developmental-Stages-of-Psychopathology study (EDSP) in Munich, MPI (n=140). Only individuals positive in the M-CIDI anxiety section, part D5 (anticipatory anxiety after having panic attacks, score 1-5) were included. Both SNPs replicated in the allelic model (rs900256: p=0.01; rs879560: p=0.01; see **Table 3**). In all three samples, allele C of rs900256 and allele A of rs879560 were associated with higher levels of anxiety.

**Association analysis of severity measures in the joint sample**

We then re-analyzed rs900256 and rs879560 in the joint sample (n=687) for the available anxiety severity scores (MPI panic: PAS anticipatory anxiety; Unipolar depression: HAMA emotional anxiety;

EDSP: D5 Score anticipatory anxiety after having a panic attack) using a Z-Score transformation of the individual rating scales with gender as covariate. All three scores assess dimensionally the severity of anticipatory anxiety and are thus comparable. Age had no significant influence and was therefore not included as covariate. For both SNPs the p-value decreased substantially (rs900256:  $p=1.4e-6$  Carrier C model; rs879560:  $p=5.0e-7$  Carrier A model), indicating that all samples contribute to this association (**Table 3**).

### **Effects of associated SNPs with the TMEM132D locus on TMEM132D gene expression**

To explore putative functional relevance of the SNPs associated with PD (rs7309727 and rs11060369) and anxiety severity (rs879560 and rs900256) we examined their correlation with TMEM132D mRNA expression in two publicly available datasets containing gene expression results from human lymphoblastoid cell lines of the HapMap project<sup>24</sup> and human postmortem cortex.<sup>25</sup> Analyzing the data from the 60 unrelated Caucasians (CEU) as well as all the combined sample of un-related individuals in the HapMap project ( $n=210$ ), we found no significant association of these SNPs with TMEM132D expression in lymphoblastoid cell lines. We next examined postmortem gene expression from frontal cortices of healthy controls of European descent ( $n=41$ ). The tags of SNPs associated with anxiety severity (rs4759607:  $p=0.013$ , rs4760030:  $p=0.015$  (genotypic model) which tag rs900256 with  $r$ -squared of 1.0) and rs879560 ( $p=0.018$ ) as well as rs11060369 ( $p=0.05$ ) showed significant association with TMEM132D mRNA expression. When examining the relevant carrier models (carrier A) for rs879560, carriers for the allele associated with higher anxiety levels also showed nominally higher TMEM132D expression (mean (SD) normalized TMEM132D expression =120.0 (5.3) vs. 104.0 (16.13),  $p=0.32$ ). In this sample, the protective homozygote genotypes were rare ( $n=4$ ), possibly explaining why this genetic model did not reach significance. Similar relationships were observed for rs4759607 and rs4760030. For rs11060369, the carrier A model which was the best model for association with PD also showed a significant association with gene expression,  $p=0.037$  (one-sided). In fact individuals with the risk genotype AA of rs11060369 ( $n=17$ ) displayed higher TMEM132D expression levels than individuals that are carriers of the protective C-allele ( $n=22$ ) (**Figure 4**). All results are described in the supplementary Table 1.

### ***Tmem132d* in an animal model of extremes in the genetic predisposition to anxiety**

In order to further validate a role of *Tmem132d* in the pathophysiology of PD, we used mice that were selectively bred to display extreme genetic predispositions to either high (HAB) or low (LAB) anxiety-related behavior, irrespective of gender.<sup>10,11,32</sup> Selection criterion was the elevated plus-maze (EPM), known to model elements of PD.<sup>33,34</sup> Both HAB and LAB lines were described to respond to the panicogenic drug FG-7142 with an increase in Fos expression in the cingulate cortex.<sup>35</sup> We analyzed mRNA expression of *Tmem132d* from a microarray experiment comparing gene expression levels between 6 male HAB and 6 male LAB mice in 5 brain regions (cingulate cortex, basolateral amygdala, central amygdala, dentate gyrus and hypothalamic paraventricular nucleus). Relative expression differences among brain regions were comparable to the profiles reported in the Allan Brain Atlas ([www.brain-map.org](http://www.brain-map.org)).

We observed nominally significant differences in *Tmem132d* expression in the cingulate cortex (upregulated 1.34 fold in HAB vs. LAB,  $p=0.004$ ) only. For all other brain regions the differences in *Tmem132d* expression between HAB and LAB animals were less than 1.13-fold. Microarray findings were confirmed by RT-qPCR analysis for *Tmem132d* in the cingulate cortex of the same animal model using female mice. The highest levels of *Tmem132d* expression were found with this method in HAB mice ( $n=10$ ), followed by unselected outbred CD1 controls ( $n=5$ ) and LAB mice ( $n=4$ ) (overall significance among the three groups:  $p=0.018$ ; with levels in HAB animals being almost 3.5 fold higher than in LAB animals,  $p=0.027$ ) (**Figure 5B**). *Tmem132d* expression in the cingulate cortex showed an inverse correlation with the time spent on the open arms of the EPM and is thus positively correlated with anxiety severity (overall significance among the three groups:  $p=0.003$ ) (**Figure 5A**). The functional impact of *Tmem132d* was further substantiated in a HABxLAB F2 panel of 510 male mice, where rs13478518 located in exon 9 of *Tmem132d* was found to be associated with anxiety-related behavior on the EPM ( $p<0.01$ ), with less anxious animals carrying the LAB-specific (i.e. GG) and more anxious ones the HAB-specific (i. e. AA) genotype (**Figure 5C**). Importantly, both depression-like behavior and locomotor activity failed to show an association (**Figure 5D-F**).

## DISCUSSION

This genomewide association study has detected evidence for association of PD with the gene TMEM132D in three independent German samples with a combined number of 909 cases and 915 controls. We found association of a two SNP haplotype in each of the three samples in the same direction, for a p-value of  $1.2 \times 10^{-7}$  in the combined sample with an odds ratio of 1.5 (CI 1.2-1.8) and thus within the range expected for complex psychiatric diseases (**Table 2**).<sup>36</sup> While this p-value did not reach genomewide significance as demanded by Dudbridge and Gusnanto (2008)<sup>37</sup> and thus the results have to be interpreted with caution, this genetic association with panic and anxiety disorders appears to be more robust than the ones recently reported from genomewide studies in major depression.<sup>38,39</sup> In particular, the additional evidence from associations with dimensional anxiety measures, human gene expression as well as data from an animal model of extremes in trait anxiety presented in this report provide strong support for a role of TMEM132D in anxiety-related disorders. As with all initial reports of genetic associations, further independent replications are needed to establish TMEM132D as a susceptibility gene for PD.

In a recent GWA study for PD in Japanese published by Otowa et al. (2009)<sup>40</sup>, two SNPs with a mean distance of 65 kb to the two best case-control markers presented in this paper showed nominal associations in this Japanese sample (rs1976829:  $p=0.003$ ; rs6486466:  $p=0.007$ ). While this is not a replication in the strict sense, it might be supportive evidence for a possible role of TMEM132D in PD.

Although the size of our discovery sample was modest, our study had adequate power to detect the reported effect size of OR 1.4 in the combined sample of 876 cases. Genetic effect sizes of this magnitude are probably the exception in complex psychiatric disorders<sup>41,42</sup> and we have thus very likely missed important genes of more modest effects in PD. In addition, the Illumina HumanHap300 Chip is providing good but not complete coverage of the European genome so that some susceptibility loci might have been missed due to insufficient marker density. Genetic association studies also cannot detect the cumulative contribution of rare alleles in a given gene which may also be relevant in complex psychiatric diseases.<sup>43-45</sup>

In genomewide association studies the advantage of an unbiased approach is counter-balanced by the need to correct for multiple testing. While conservative correction effectively reduces the number of false positives it may also mask true positives at the same time. As we cannot specify any universal threshold for statistical significance of true findings for PD, we decided to select the most strongly associated markers for replication in stage 2, even if these associations are not significant after correction for multiple testing in stage 1.<sup>42,46</sup> Following this strategy and then re-testing the top SNPs in two additional samples, we could confirm a haplotype association with a p-value of  $1.2 \times 10^{-7}$  in the combined sample despite the lack of genomewide significance in the discovery sample.<sup>47</sup> All three samples differ in regard to the comorbidity of panic and anxiety patients and selection criteria for controls. This may be a reason for lower ORs in stage 2 and 3 compared to the discovery sample. In the initial sample, only PD patients without current comorbidity with major psychiatric disorders were analyzed against “super-healthy” controls, whereas in the second sample, panic patients were compared to blood donor controls and in the third sample comorbid panic and anxiety patients were compared to controls screened with a brief questionnaire. In fact, in sample 3 the group of patients with PD (n=258) had a high comorbidity rate with MD (62.9%), bipolar disorders (24.1%) and schizophrenic disorder (13%). In contrast, in the group of phobia patients, a lower number of patients with psychiatric comorbidity was present (comorbidity rate 60%), so that these patients might be phenotypically closer to the patients in stages 1 and 2 where most patients had an anxiety disorder only, or other psychiatric disorders secondary to the anxiety disorder. This might also explain why we see less strong associations when restricting the analysis in sample 3 to PD only.

Two SNPs in this gene were associated with anticipatory anxiety in patients with PD as well as the severity of anxiety symptoms in patients with major depression or panic attacks but not the severity of depressive symptoms (**Table 3**). This points towards the possibility that these genetic effects are specific for the severity of experienced anxiety but not restricted to a certain disorder. However, while the same risk allele is associated in all three samples, the 2 anxiety samples show an additive genetic effect while the depression sample shows a heterozygote disadvantage effect. This discrepancy might result through slight differences in LD with the actual causal variant in the 3 samples or different underlying environmental or genetic factors in depression vs. anxiety. While in our statistical analysis

medication status did not change the strength of the genetic association with anxiety severity, we can not fully exclude that the high percentage of patients with current antidepressant medication in the MD group influences the genetic effects on the anxiety phenotype. Finally, replication of the association in the depression cohort is needed to exclude false positive results.

These SNPs likely tag a second functional locus in addition to the one identified by rs7309727 and rs11060369, as they are separated from this SNP by two haplotype block boundaries, indicating hotspots of recombination. Single mutations at several distinct loci within a gene, no matter whether on the same or a different LD block, causing the same or similar phenotypes have been described for a number of monogenic diseases.<sup>48</sup> In addition, the replication of two independent loci in the same gene with two related phenotypes (PD case-control and severity of anxiety symptoms) also strengthens the evidence that TMEM132D might indeed be relevant for pathological neural processes involved in the regulation of anxiety and fear. We hypothesize that the associations of the SNPs in TMEM132D with anxiety severity across the different psychiatric samples reflects an effect on the degree of anxiety symptoms experienced in the presence of another psychiatric disorder but not on baseline anxiousness. rs900256, rs879560 and rs10847832 would thus be moderators of anxiety symptoms in patients with current psychiatric disorders. rs7309727 and rs11060369, on the other hand, would determine disease status (panic disorder yes or no) but not influence the severity of the disorder once it has manifested. We found no significant interaction effects between severity SNPs and case-control SNPs in any of the samples on either case-control association or associations with anxiety severity. This further supports the hypothesis that these two loci influence different types of anxiety-related phenotypes. The two genetic loci, the SNPs associated with anxiety severity and the SNPs associated case-control, are not in LD and could represent distinct functional variants within the gene. Of course, further studies in larger samples and especially in cohorts with other psychiatric disorders will be necessary to verify this hypothesis.

The two associated loci are located in intron 3 and 4 of TMEM132D respectively. The linkage disequilibrium structure of both regions suggests that the potential functional variants tagged by these associations do not likely lie in exonic or classic 5' regulatory regions. Regulatory regions have,

however, also been described for introns.<sup>49,50</sup> The fact that SNPs in both loci are independently associated with TMEM132D gene expression in human frontal cortex, support the possibility that they are tagging functional intronic regulatory variants and that they are indeed related to TMEM132D function. However, because of the low number of individuals with gene expression data in the frontal cortex in the Myer's et al data, and especially low numbers in the group of homozygote risk allele carriers (see supplementary table 1), these associations have to be interpreted with caution.

We then examined and extended the hypothesis that TMEM132D might indeed be relevant for pathological neural processes involved in the regulation of anxiety and fear in a complementary inter-species approach. Using an established animal model of extremes in the genetic predisposition to anxiety<sup>10,11,32</sup> we could show that *Tmem132d* mRNA expression in the cingulate cortex is positively correlated with anxiety-related behavior. This observation was consistent in microarray and RT-qPCR experiments as well as in male and female HAB, CD1 and LAB animals, suggesting a gender-independent relationship of *Tmem132d* with anxiety-related behavior (**Figure 5**). The correlation of higher TMEM132D expression with extreme anxiety-related behaviors in this animal model is consistent with the finding that the risk genotype AA of rs11060369, overrepresented in PD patients, was also associated with increased TMEM132D expression in frontal cortex. Importantly, these anxiety-dependent differences in this animal model were brain region-specific. Only the cingulate cortex but not the basolateral amygdala, central amygdala, dentate gyrus and hypothalamic paraventricular nucleus showed an upregulation of *Tmem132d* in HAB animals in our microarray experiment. The cingulate cortex is closely connected to the amygdala, the brain region central to the generation of fear and anxiety and its activation seems to modulate the response of the amygdala to fearful stimuli and expression of fear in humans.<sup>51-53</sup> A series of functional imaging studies have implicated activity changes in the anterior cingulate cortex not only in fear but also in pathological anxiety states in humans such as phobic fear, PD, generalized anxiety disorder, social anxiety disorder and post traumatic stress disorder studies.<sup>54-57</sup> Given the fact that anxiety/fear-related brain circuits seem to be strongly conserved across species, these results could suggest that an altered expression of *Tmem132d* in the cingulate cortex may contribute to an altered activation profile of this brain region in the presence of anxiogenic stimuli and thus to a predisposition to pathological states of anxiety.

In addition to differences in expression, an exonic *Tmem132d* SNP was found to co-segregate with anxiety-related behavior in an F2 panel independent of both depression-like behavior and locomotor activity (**Figure 5C-F**), thus suggesting an evolutionary conserved, causal involvement of this gene in anxiety-related phenomena. The specific association to anxiety but not depression-related behavior again parallels the above-described findings in humans.

The molecular function of the gene product, also called transmembrane protein 132D, KIAA1944 and MOLT, is still unclear. TMEM132D is predicted as a single pass type I membrane protein. The predicted 1,099-amino acid protein has a calculated molecular mass of about 130 kD and contains an N-terminal hydrophobic signal peptide, 7 predicted N-glycosylation sites, 2 predicted O-glycosylation sites, a number of phosphorylation sites, and a C-terminal transmembrane domain ([www.ncbi.nlm.nih.gov/sites/entrez/db=OMIM](http://www.ncbi.nlm.nih.gov/sites/entrez/db=OMIM)). Immunohistochemical studies showed a 5:1 ratio of *Tmem132d* expression in white matter compared to gray matter in the cerebral cortex.<sup>58</sup> *Tmem132d* was also detected in corpus callosum and in white matter in the spinal cord and optic nerve. Using cultured rat oligodendrocyte lineage cells and stage-specific markers, Nagase et al. (2001), showed that *Tmem132d* was expressed by mature oligodendrocytes but not by oligodendrocyte precursor cells, concluding that oligodendrocytes start expressing TMEM132D during the course of maturation and that this protein could be involved in the neural interconnection and also signalling.<sup>59</sup> Its high expression in white matter and involvement in oligodendrocyte maturation may be relevant for the efficient connection of the cingulate cortex to other fear-related brain regions.

In conclusion, this genomewide association study of PD has detected a moderate but reproducible association with a two-SNP haplotype in TMEM132D. In addition, an independent locus within this gene may be associated with the severity of anxiety symptoms in anxiety, affective disorders, possibly suggesting a more general role in neural processes involved in the regulation of experienced fear and anxiety. This is supported and extended by the fact that the expression of this gene was correlated with the level of anxiety-related behavior in HAB/LAB mice and that a freely segregating F2 panel revealed an association between a *Tmem132d* SNP and locomotion-independent anxiety-related behavior. Finally, SNPs in both human loci are likely tagging functional variants as they associate

with TMEM132D gene expression in human cortex. The results of this study demonstrate for the first time the identification of an evolutionary conserved susceptibility gene for anxiety-related phenotypes using unbiased genomewide analyses in an inter-species approach.

#### **ACKNOWLEDGEMENTS**

The authors would like to thank G. Ernst-Jansen, G. Gajewsky, J. Huber, E. Kappelmann, S. Sauer, S. Damast, M. Koedel, M. Asmus and A. Sangl for their excellent technical support. We also thank Rene Breuer from Central Institute of Mental Health from Mannheim for excellent management of the blood and phenotypic database. Katharina Domschke and Jürgen Deckert were supported by the Deutsche Forschungsgemeinschaft (SFB-TRR-58 C2 and Z2). We thank NGFN for a grant enabling the genotyping of PopGen subjects within the second round of funding. We thank further NGFN MoodS and the Max Planck Excellence Foundation for grant support.

#### **Conflict of interest statement:**

The authors declare that they have no conflicts of interest.

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Table 1:

Demographic data of the genotyped case-control samples stage 1-3.

	<b>Stage 1</b>		<b>Stage 2</b>		<b>Stage 3</b>	
	cases	controls	cases	controls	cases	controls
N	216	222	225	240	468	452
sex	male 33.2%	male 32.9%	male 40.4%	male 40.2%	male 33.3%	male 50.9%
	female 66.8%	female 67.1%	female 59.6%	female 59.8%	female 66.7%	female 49.1%
age (SD)	38.9 (12.0)	38.8 (11.7)	35.8 (10.7)	38.6 (12.1)	43.3 (13.3)	47.2 (15.5)
diagnosis	PDA 83.3%	none	PDA 67.5%	none	PDA 39 %, PD 20.4%	none
	PD 16.7%		PD 32.5%		Social Phobia 12.8%	
					Specific Phobia 16.1%	
					Other Anxiety 11.7%	
					<u>Comorbidity</u> : MDE 51.6%	
					BP 21.8%, SchiD 10.6%	

BP: bipolar disorder; MDE: depressive disorder; PDA: panic disorder with agoraphobia; PD: panic disorder without agoraphobia; SchiD: schizophrenic disorder; SD: standard deviation.

**Table 2:**

Genotype frequency distribution and case-control p-values for rs7309727 and rs11060369 using Armitage trend test in the Stage 1-3 samples, in the joint sample and haplotypes for rs7309727/rs11060369.

<b>rs7309727</b>	<b>CC (n / %)</b>	<b>CT (n / %)</b>	<b>TT (n / %)</b>	<b>HWE</b>	<b>p-value case-control OR (CI)</b>
<b>Stage 1</b>					
controls	154 / 69.4%	59 / 26.6%	9 / 4.1%	0.25	p = 7.726e-07
cases	100 / 46.3%	94 / 43.5%	22 / 10.2%	1.00	OR (CI): 2.2 (1.59-3.02)
<b>Stage 2</b>					
controls	144 / 60.0%	87 / 36.3%	9 / 3.7%	0.45	p = 0.006
cases	114 / 50.7%	88 / 39.1%	23 / 10.2%	0.34	OR (CI): 1.5 (1.12-2.03)
<b>Stage 3</b>					
controls	263 / 58.3%	163 / 36.1%	25 / 5.6%	1.0	p = 0.153
cases	250 / 53.4%	189 / 40.4%	29 / 6.2%	0.4	OR (CI): 1.2 (0.94-1.44)
<b>Joint analysis</b>					
controls	561 / 61.4%	309 / 33.8%	43 / 4.7%	0.92	p = 1.36e-6
cases	463 / 51.0%	371 / 40.9%	74 / 8.1%	1.0	OR (CI): 1.4 (1.24-1.68)
<b>rs11060369</b>	<b>AA (n / %)</b>	<b>AC (n / %)</b>	<b>CC (n / %)</b>	<b>HWE</b>	<b>p-value case-control OR (CI)</b>
<b>Stage 1</b>					
controls	78 / 35.1%	102 / 45.9%	42 / 18.9%	0.41	p = 0.0001
cases	103 / 47.7%	99 / 45.8%	14 / 6.5%	0.14	OR (CI): 1.74 (1.31-2.33)
<b>Stage 2</b>					
controls	92 / 38.2%	118 / 49.0%	30 / 12.9%	0.59	p = 0.08
cases	103 / 46.0%	99 / 44.2%	22 / 9.8%	0.88	OR (CI): 1.28 (0.97-1.69)
<b>Stage 3</b>					
controls	168 / 37.2%	221 / 48.9%	63 / 13.9%	0.55	p = 0.02
cases	209 / 44.7%	208 / 44.4%	51 / 10.9%	1.0	OR (CI): 1.26 (1.04-1.53)
<b>Joint analysis</b>					
controls	338 / 36.9%	441 / 48.2%	136 / 14.9%	0.73	7.63e-06
cases	415 / 45.7%	406 / 44.7%	87 / 9.6%	0.45	OR (CI): 1.4 (1.19-1.57)
<b>Haplotypes</b>					
<b>rs7309727/rs11060369</b>	<b>CC</b>	<b>TA</b>	<b>CA</b>	<b>p-value (omnibus test)</b>	
<b>Stage 1</b>					
controls (n%)	186 / 41.9%	77 / 17.1%	181 / 41.0%	5.48e-7	
cases (n%)	127 / 29.2%	138 / 31.9%	167 / 38.9%		
ChiSquare test	p = 0.0001	p = 5.1e-7	p = 0.52	0.01	
OR (CI)	1.7 (1.1-2.5)	2.3 (1.5-3.7)	1.1 (0.8-1.7)		
<b>Stage 2</b>					
controls (n%)	179 / 37.9%	105 / 22.1%	196 / 40.0%	0.04	
cases (n%)	143 / 31.1%	134 / 30.7%	171 / 38.2%		
ChiSquare test	p = 0.07	p = 0.003	p = 0.39	1.2e-7 (corrected experiment-wide 0.04)	
OR (CI)	1.3 (0.9-1.9)	1.6 (1.0-2.5)	1.1 (0.8-1.7)		
<b>Stage 3</b>					
controls (n%)	347 / 37.9%	213 / 24.2%	342 / 37.9%	1.2e-7 (corrected experiment-wide 0.04)	
cases (n%)	309 / 33.1%	249 / 26.9%	374 / 40.0%		
ChiSquare test	p = 0.016	p = 0.11	p = 0.34	1.0 (0.8-1.2)	
OR (CI)	1.4 (1.0-1.8)	1.2 (0.9-1.6)	1.1 (0.8-1.4)		
<b>Joint sample</b>					
controls (n%)	712 / 39.2%	395 / 21.1%	719 / 39.6%	1.2e-7 (corrected experiment-wide 0.04)	
cases (n%)	579 / 31.1%	521 / 29.8%	712 / 39.1%		
ChiSquare test	p = 7.4e-6	p = 4.9e-7	p = 0.9	1.0 (0.8-1.2)	
OR (CI)	1.4 (1.1-1.7)	1.5 (1.2-1.8)	1.0 (0.8-1.2)		

**Table 3:**

Genotype distribution for rs900256 and rs879560 for the anxiety severity phenotypes in the MPI anxiety sample, depression sample and EDSP sample.

<b>rs900256</b>	<b>CC</b>	<b>CT</b>	<b>TT</b>	<b>p-value (model)</b>
<b>MPI anxiety (n/%)</b>	52 / 22.2%	125 / 53.4%	57 / 24.4%	
Anticipatory anxiety (SD)	6.4 (1.2)	5.9 (1.8)	5.2 (2.1)	0.0004 / 0.0001 / 0.0006
HMD (SD)	14.4 (6.4)	13.9 (7.0)	13.2 (6.6)	0.55 / 0.31 / 0.30 (genotyp/allelic/carrier C)
<b>MPI depression (n/%)</b>	77 / 24.6%	170 / 54.3%	66 / 21.1%	
HAMAa (SD)	14.4 (4.2)	15.6 (4.3)	13.4 (4.1)	0.0007 / 0.24 / 0.001
HMD (SD)	26.4 (6.2)	27.1 (6.3)	26.0 (5.4)	0.54 / 0.89 / 0.42 (genotyp/allelic/carrier C)
<b>MPI EDSP (n/%)</b>	38 / 27.1%	67 / 47.9 %	35 / 25.0%	
D5 Score (SD)	2.0 (1.0)	1.6 (0.97)	1.5 (0.78)	0.04 / 0.01 / 0.12 (genotyp/allelic/carrier C)
<b>Joint sample (n/%)</b>	167 / 24.3%	363 / 52.8%	157 / 22.9%	
Z-Score	0.15 (0.92)	0.09 (1.00)	-0.31 (1.00)	4.7e-6 / 4.7e-5 / 1.4e-6 (genotyp/allelic/carrier C)
<b>rs879560</b>	<b>AA</b>	<b>AG</b>	<b>GG</b>	
<b>MPI panic (n/%)</b>	43 / 18.4%	134 / 57.3%	57 / 24.3%	
Anticipatory anxiety (SD)	6.5 (1.1)	5.9 (1.8)	5.2 (2.1)	0.0009 / 0.0006 / 0.001
HMD (SD)	14.3 (6.4)	14.0 (7.1)	13.3 (6.1)	0.54 / 0.31 / 0.31 (genotyp/allelic/carrier A)
<b>MPI depression (n/%)</b>	76 / 24.3%	165 / 52.7%	72 / 23.0%	
HAMAa (SD)	14.3 (4.2)	15.7 (4.3)	13.4 (3.9)	0.0003 / 0.21 / 0.001
HMD (SD)	26.8 (6.5)	27.5 (6.5)	26.1 (5.4)	0.65 / 0.99 / 0.68 (genotyp/allelic/carrier A)
<b>MPI EDSP (n/%)</b>	36 / 25.7%	65 / 46.4%	39 / 27.9%	
D5 Score (SD)	2.0 (0.98)	1.75 (1.0)	1.46 (0.76)	0.05 / 0.01 / 0.03 (genotyp/allelic/carrier A)
<b>Joint sample (n/%)</b>	155 / 22.6%	365 / 53.1%	167 / 24.3%	
Z-Score	0.13 (0.93)	0.12 (1.01)	-0.32 (0.97)	3.3e-6 / 7.3e-5 / 5.0e-7 (genotyp/allelic/carrier A)

D5-Score: M-CIDI section D, part 5, anticipatory anxiety after having panic attacks; EDSP sample:

Early-Developmental-Stages-of-Psychopathology study; HAMAa: Hamilton Anxiety score, subscore emotional anxiety; HMD: Hamilton Depression score; MPI: Max-Planck Institute for Psychiatry; SD: standard deviation.

**Figure legends:****Figure 1. Extent of population stratification in the MPI sample.**

The distribution of  $\chi^2$ -values expected under the null hypothesis versus those observed. The  $\chi^2$ -values were obtained in the analysis of the MPI sample, using the allelic test, the correction factor found is nearly 1 ( $\lambda=1.0245$ ). Most values adhere almost perfectly to the diagonal with the expected values without correction for lambda, indicating that a possible systematic error due to population stratification even before the correction is small.

**Figure 2. Genomewide association study results for panic disorder susceptibility loci.**

Results from the first stage analysis using 216 panic cases versus 222 matched controls. The x-axis represents genomic position, the y-axis shows  $-\log^{10}(p)$ . 4 SNPs with nominal significance in stage 2 are highlighted in bold.

**Figure 3. Summary of the case-control and psychopathology-related results in the gene TMEM132D in the MPI panic sample.**

The x-axis represent the position on chromosome 12 with the position of TMEM132d (exons as vertical lines) and the linkage disequilibrium (LD) structure of this region according to the HapMap phase II data in CEPBs with  $D'$  as measure of LD. On the Y-axis p-values are displayed as  $-\log_{10}(P_{\text{nom}})$ : case-control panic: triangles; severity of anticipatory anxiety: squares.

**Figure 4. Expression profile of rs11060369 in the human frontal cortex (data from Myers et al., 2007).****Figure 5. *Tmem132d* in an animal model of extremes in the genetic predisposition to anxiety.**

A) Anxiety-related behavior (% of time spent on the open arms of the elevated plus-maze, EPM) and B) relative expression values of *Tmem132d* by RT-qPCR in the cingulate cortex of male (A) and female (B) HAB (high anxiety-related behavior, n=10) mice, CD1 controls (n=5) and LAB (low

anxiety-related behavior, n=4) mice. For correlation of individual EPM (A) and expression (B) data:  $r=-0.65$ ;  $p=0.003$ .

C-F) Association between phenotypic indices of male HABxLAB F2 mice and *Tmem132d* SNP-related genotypes of AA (HAB-typical homozygotes, n=114), AG (heterozygotes, n=271) or GG (LAB-typical homozygotes, n=125). C) Anxiety-related behavior: the SNP rs13478518 co-segregates with % time spent in the open arms of the EPM; D, E) Locomotor activity: no association of rs13478518 with closed arm entries (EPM) and total distance traveled (open field, OF); F) Depression-like behavior: no association of rs13478518 with total immobility time (tail-suspension test, TST). Data are expressed as mean + SEM. \*  $p<0.05$ ; \*\*  $p<0.01$ ; \*\*\*  $p<0.001$ .

**Supplementary Table 1:** the statistical analysis of post-mortem TMEM132D gene expression in frontal cortices of healthy controls (N = 41) using the probe GI\_27484731 (Myers et al., 2007)

Figure 1

Erhardt et al., 2009

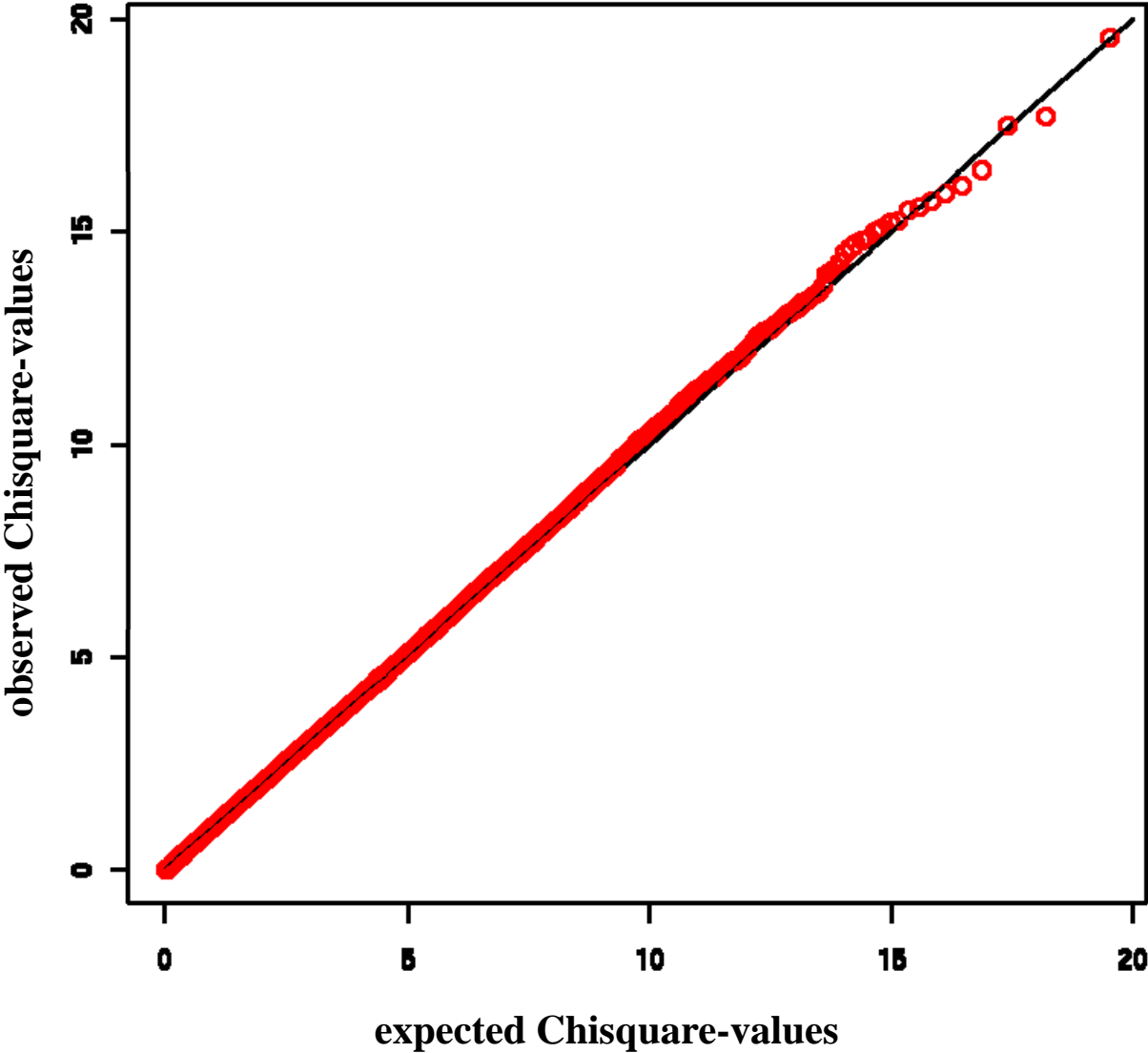


Figure 2

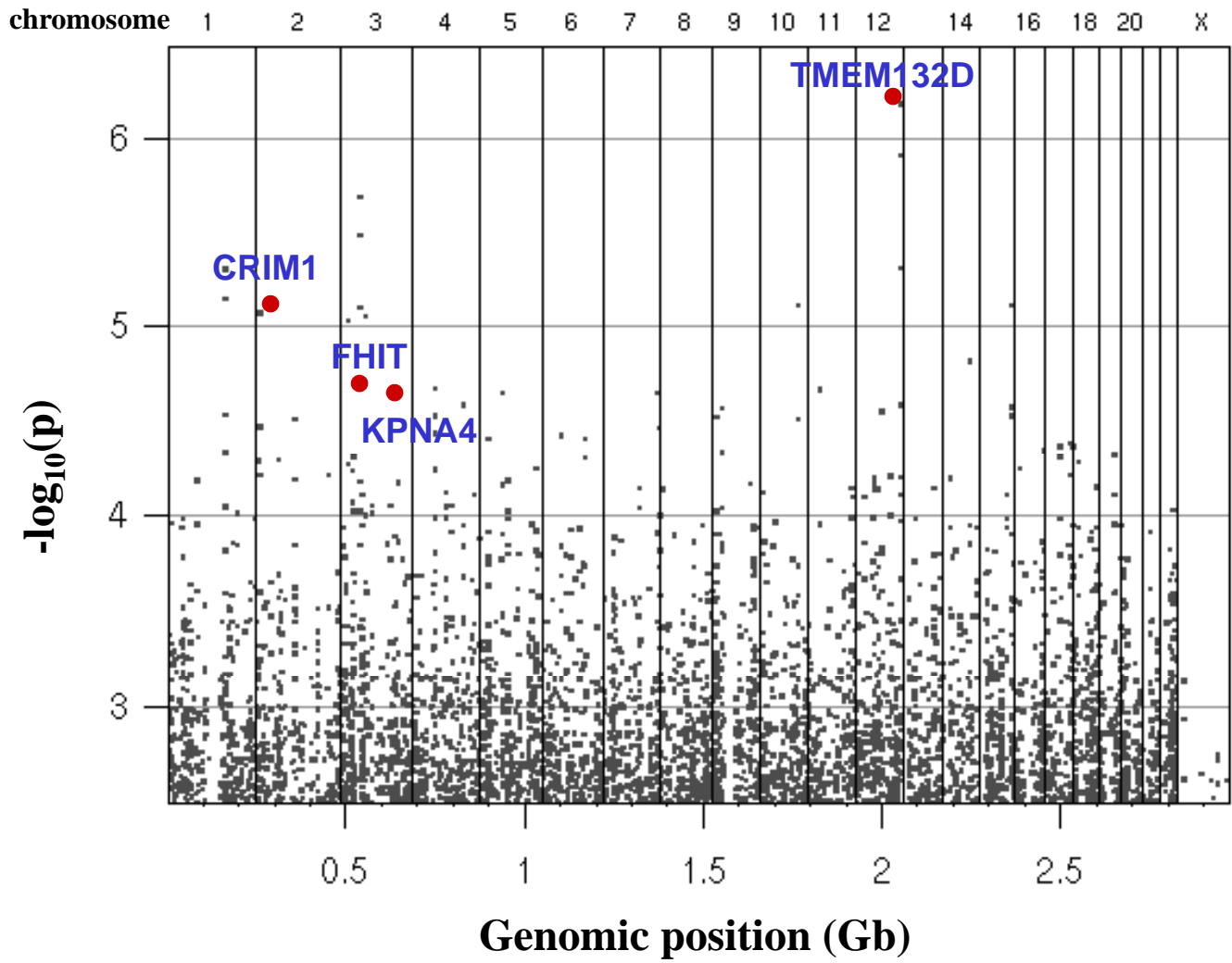
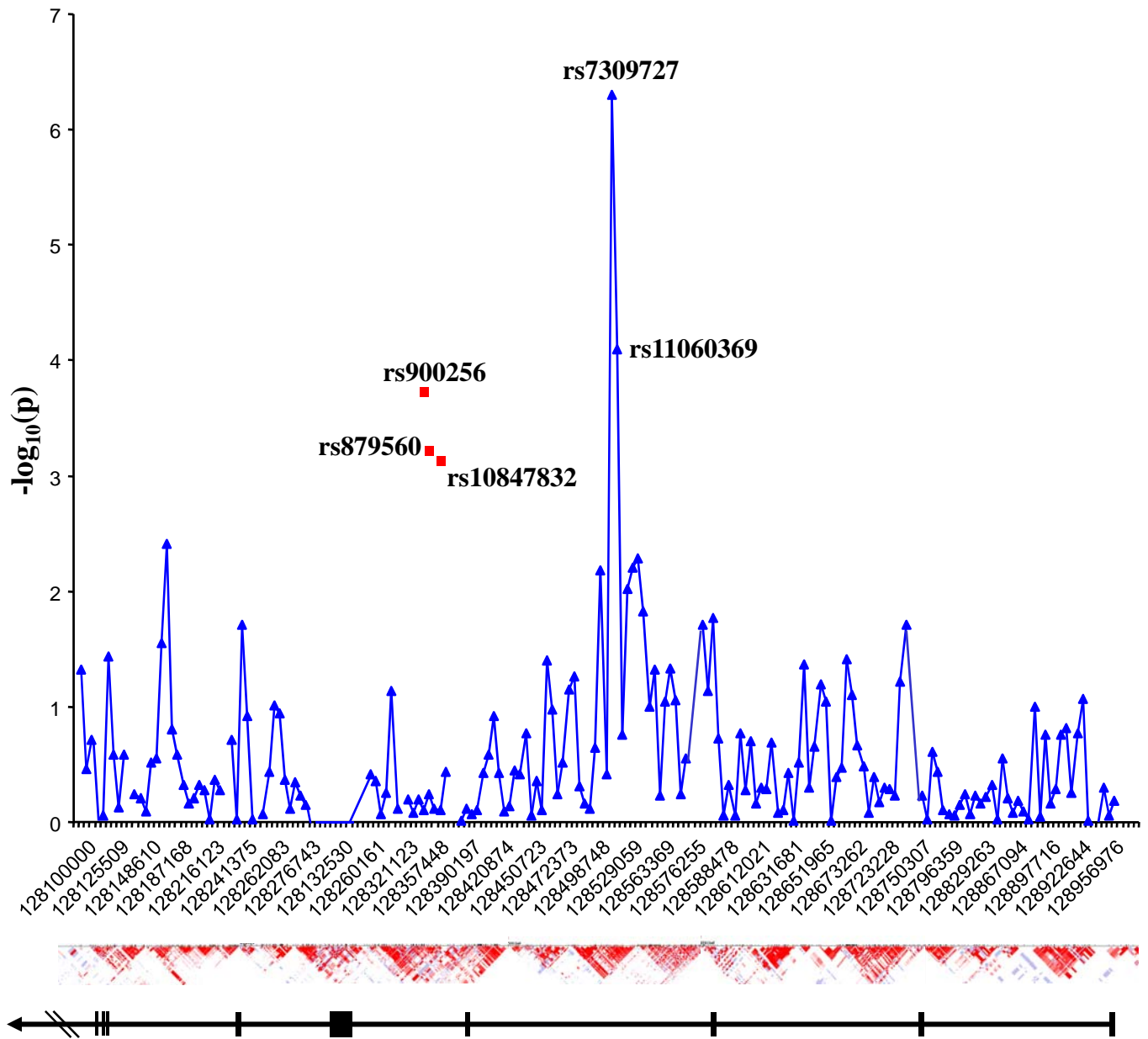


Figure 3



**Figure 4**

Erhardt et al., 2009

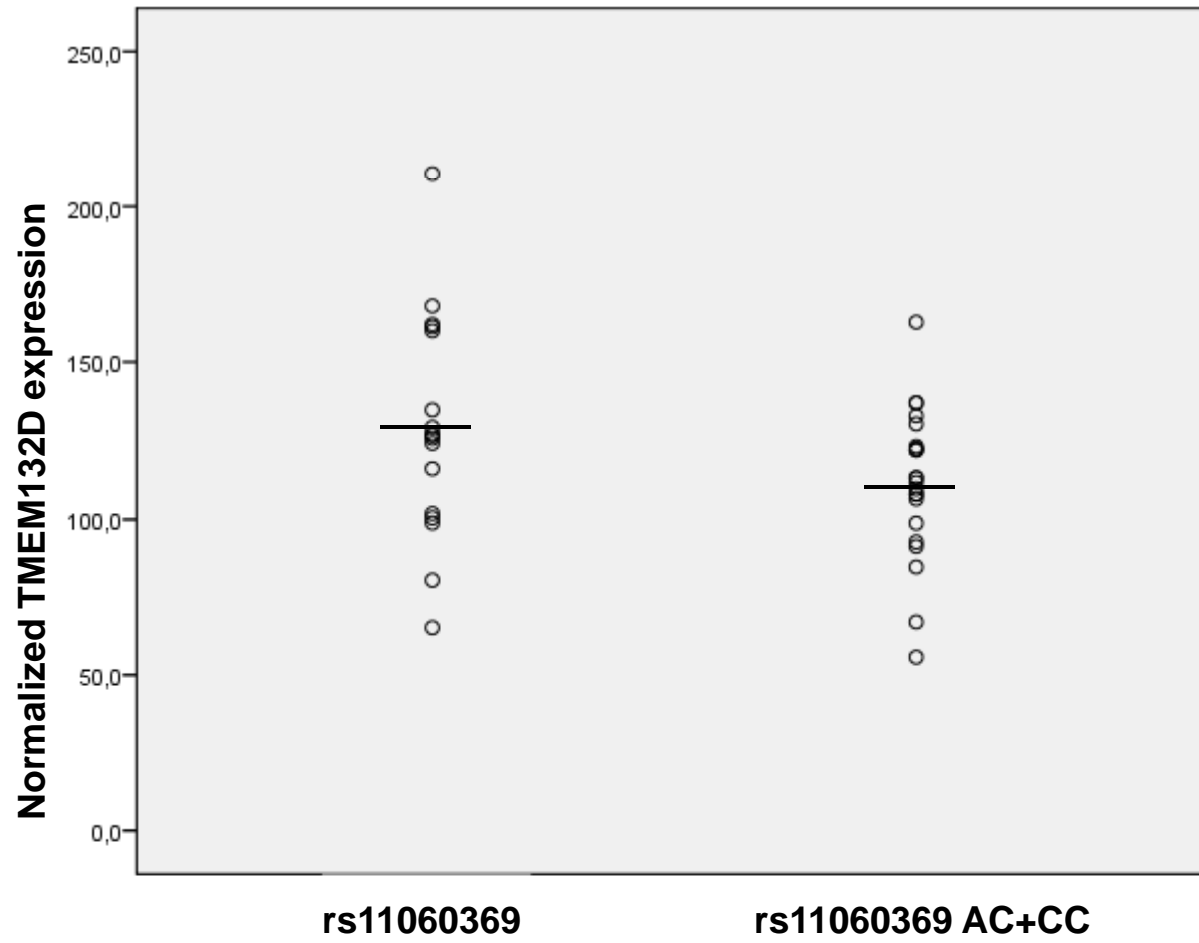


Figure 5

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