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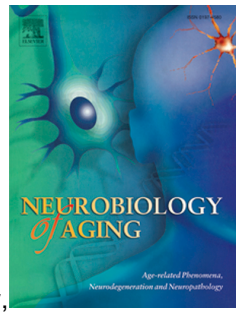

Male-specific epistasis between *WWC1* and *TLN2* genes is associated with Alzheimer's disease

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Research Article

Male-specific epistasis between *WWC1* and *TLN2* genes is associated with Alzheimer's disease.

Elena S. Gusareva[a*], Jean-Claude Twizere[b], Kristel Sleegers[c,d], Pierre Dourlen[e,f], Jose F. Abisambra[g,h,i,j], Shelby Meier[g,h], Ryan Cloyd[g], Blaine Weiss[g], Bart Dermaut[e,f], Kyrylo Bessonov[a], Sven J. van der Lee[k], Minerva M. Carrasquillo[l], Yuriko Katsumata[m], Majid Cherkaoui[b], Bob Asselbergh[c,d], M. Arfan Ikram[n,o], Richard Mayeux[p], Lindsay A. Farrer[q], Jonathan L. Haines[r], Margaret A. Pericak-Vance[s], Gerard D. Schellenberg[t], on behalf of Genetic and Environmental Risk in Alzheimer's Disease 1 consortium (GERAD1), on behalf of Alzheimer's Disease Genetics Consortium (ADGC), on behalf of The European Alzheimer Disease Initiative Investigators (EADI1 Consortium), Rebecca Sims[u], Julie Williams[u], Philippe Amouyel[f], Cornelia M van Duijn[k], Nilüfer Ertekin-Taner[l,v], Christine Van Broeckhoven[c,d], Franck Dequiedt[b], David W. Fardo[m,w], Jean-Charles Lambert[f], Kristel Van Steen[a,x,y]

[a]*Medical Genomics Research Unit, GIGA-R, University of Liège, Belgium*

[b]*Molecular Biology of Diseases Research Unit, GIGA-R, University of Liège, Belgium*

[c]*Neurodegenerative Brain Diseases group, Center for Molecular Neurology, VIB, Antwerp, Belgium*

[d]*Institute Born-Bunge and University of Antwerp, Antwerp, Belgium*

[e]*U1167-RID-AGE, Facteurs de risque et déterminants moléculaires des maladies liées au vieillissementa, Universite de Lille Nord de France, Lille, France*

[f]*NSERM U1167, Institut Pasteur de Lille, Universite de Lille Nord de France, Lille, France*

[g]*Sanders-Brown Center on Aging, University of Kentucky, College of Medicine, KY, USA*

[h]*Department of Physiology, University of Kentucky, College of Medicine, KY, USA*

[i]*Epilepsy Center, University of Kentucky, College of Medicine, KY, USA*

[j]*Spinal Cord & Brain Injury Research Center, University of Kentucky, College of Medicine, KY, USA*

[k]*Department of Epidemiology, Erasmus University Medical center, Rotterdam, the Netherlands*

[l]*Mayo Clinic Florida, Department of Neuroscience, Jacksonville, FL, USA*

[m]*Department of Biostatistics, College of Public Health, University of Kentucky, KY, USA*

[n]*Department of Neurology, Erasmus University Medical center, Rotterdam, the Netherlands*

[o]*Department of Radiology, Erasmus University Medical center, Rotterdam, the Netherlands*

[p]*Gertrude H. Sergievsky Center, Department of Neurology, and Taub Institute on Alzheimer's Disease and the Aging Brain, Columbia University, NY, USA*

[q]*Departments of Biostatistics, Medicine (Genetics Program), Ophthalmology, Neurology, and Epidemiology, Boston University, MA, USA*

[r]*Department of Epidemiology and Biostatistics, Case Western Reserve University, OH, USA*

[s]*The John P. Hussman Institute for Human Genomics and Dr. John T. Macdonald Foundation Department of Human Genetics, University of Miami, FL, USA*

[t]*Department of Pathology and Laboratory Medicine, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA, USA*

A list of GERAD1 and EADI1 members and affiliations appears in the ***Note S1***

[u]*Medical Research Council Centre for Neuropsychiatric Genetics and Genomics, Institute of Psychological Medicine and Clinical Neurosciences, Cardiff University School of Medicine, Cardiff, UK*

[v]*Mayo Clinic Florida, Department of Neurology, Jacksonville, FL, USA*

[w]*Sanders-Brown Center on Aging, University of Kentucky, KY, USA*

[x]*Walloon Excellence in Life sciences and BIOtechnology (WELBIO), Belgium*

[y]*Department of Human Genetics, KU Leuven, Leuven, Belgium*

Corresponding author: Elena S. Gusareva, Present Address: SCELSE, 60 Nanyang Drive, SBS-01N-27, Singapore 637551, Tel: +65 65923603, Fax: +65 63167349. E-mail address: gusareva.elena@gmail.com

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52 **Abbreviations**

53 AD – Alzheimer's disease

54 WWC1 – WW and C2 domain containing 1, aka KIBRA

55 TLN2 – talin 2

56 APOE – apolipoprotein E

57 EADI1 – European Alzheimer Disease Initiative Investigators

58 GERAD1 – Genetic and Environmental Risk for Alzheimer's disease consortium

59 RS – Rotterdam Study

60 ADGC – Alzheimer's Disease Genetic Consortium

61 BOOST – BOolean Operation-based Screening and Testing method

62 MB-MDR – Model-Based Multifactor Dimensionality Reduction method

63 MAF – minor allele frequency

Abstract

Systematic epistasis analyses in multifactorial disorders are an important step to better characterize complex genetic risk structures. We conducted a hypothesis-free sex-stratified genome-wide screening for epistasis contributing to Alzheimer's disease (AD) susceptibility. . We identified a statistical epistasis signal between the SNPs rs3733980 and rs7175766 that was associated with Alzheimer's disease in males (genome-wide significant $p_{\text{Bonferroni-corrected}}$=0.0165). This signal pointed towards the genes *WWC1* (WW and C2 domain containing 1, aka *KIBRA*; 5q34) and *TLN2* (talin 2; 15q22.2). Gene-based meta-analysis in three independent consortium datasets confirmed the identified interaction: the most significant ($p_{\text{meta-Bonferroni-corrected}}$=9.02*$10^{-3}$) was for the SNP-pair rs1477307 and rs4077746. In functional studies, WWC1 and TLN2: co-expressed in the temporal cortex brain tissue of Alzheimer's disease subjects (β=0.17, 95% CI 0.04 to 0.30, p=0.01); modulated Tau toxicity in *Drosophila* eye experiments; co-localized in brain tissue cells, N2a neuroblastoma, and HeLa cell lines; and co-immunoprecipitated both in brain tissue and HEK293 cells. Our finding points towards new AD-related pathways and provides clues towards novel medical targets for the cure of AD.

Keywords

Alzheimer's disease, epistasis, gene-gene interaction, protein-protein interaction, *WWC1*, *TLN2*

1. Introduction

Alzheimer's disease (AD) is a progressive, irreversible neurodegenerative disorder characterized by the development of amyloid plaques and neurofibrillary tangles, the loss of connections between neurons, and nerve cell death. AD is highly heritable and genetically heterogeneous with 58-79% of risk attributed to genetic factors (Gatz et al., 2006; Sims and Williams, 2016). Although genome-wide association studies (GWAS) have strongly improved our knowledge of AD genetics (Ridge et al., 2013), genetic risk factors explain no more than 30% of heritability (Cuyvers and Sleegers, 2016). In this contribution we focus on late-onset AD, the most common form of the disease with onset age >65 years. The most established genetic factor for AD, apolipoprotein E gene (*APOE*, 19q13), exhibits allelic heterogeneity - *APOE*'s *ε4* allele is a risk enhancer, while the *ε2* allele is protective (Bertram et al., 2007).

AD presents notable sexual dimorphism (Mielke et al., 2014). Records exist of sex differences in brain, such as in brain anatomy, age-related declines in brain volume and brain glucose metabolism (Carter et al., 2012), and sex hormones influencing AD progression (Musicco, 2009). Risk associated with the *APOE-e4* allele is stronger in females than in males, and loss of chromosome Y have been associated with increased AD risk in males (Dumanski et al., 2016). These data support complex interplay between sex and genetic background regarding AD predisposition.

Gene regulatory and biochemical networks create dependencies among genes that are realized as gene-gene interactions (epistasis) (Templeton, 2000). Although epistasis has been well studied in model organisms using biological experiments (Miko, 2008), hypothesis-free discovery of biological epistasis via statistical methods remains challenging in humans. This is in part due to the conceptual discrepancy between statistical and biological epistasis (Moore, 2005), the utility of over-simplified population-level models to capture complex individual phenomena, insufficient power, and the gross multiple testing burden inherent in genome-wide epistasis screening. Therefore, most evidence for epistasis in AD is hypothesis-driven, using prior biological or statistical knowledge (Ebbert et al., 2015). The same holds for sex-specific searches for co-involvement of multiple genetic loci in AD (Medway et al., 2014).

Gusareva et al. published the first replicable interaction associated with AD using a genome-wide exhaustive screening approach that combines strengths over different analytic approaches (Gusareva and Van Steen, 2014), identified a statistical interaction between *KHDRBS2* (rs6455128) and *CRYL1* (rs7989332), and exhibited downstream functional consequences (Gusareva et al., 2014). Here, we used the same European Alzheimer Disease Initiative Investigators (EADI1) consortium cohort (Lambert et al., 2009) (2259/6017 AD cases/controls) and an adapted hypothesis-free genome-wide exhaustive epistasis screening protocol to identify sex-specific interactions with AD. We identified AD-associated male-specific statistical interaction between variants of the genes *WWC1* (WW and C2 domain containing 1 or kidney and brain expressed protein, aka *KIBRA*; locus 15q22.2) and *TLN2* (talin 2, locus 15q22.2). This novel statistical epistasis signal was replicated in two out of three independent consortium datasets via gene-based replication strategy (Gusareva and Van Steen, 2014). Extensive biological validation studies (subcellular co-localization and immunoprecipitation analyses, transcriptome analysis, experiments in model organisms (*Drosophila Melanogaster*), as well as *in silico* protein docking and molecular dynamics assessments) further helped elucidate the epistatic relationship.

2. Methods

2.1 Study populations

The discovery cohort consisted of a sample of 2259 late-onset AD patients and 6017 controls from 3 cities in France (Bordeaux, Dijon and Montpellier), as part of EADI1. Follow-up statistical analyses used data from three AD consortia: 1) the Genetic and Environmental Risk for Alzheimer's disease consortium (GERAD1) including cohorts from Germany, UK, and the USA (Harold et al., 2009); 2) the Rotterdam Study (RS), a prospective cohort study that started in 1990 in Rotterdam (the Netherlands) (Hofman et al., 2013) and 3) the Alzheimer's Disease Genetic Consortium (ADGC) that collects genetic data from over 30 studies in the US (Naj et al., 2011). Data collection quality control procedures have been described in

131 the corresponding references. Only subjects with complete information on sex and age were included in
132 the analyses. Sex-specific sample size distributions and age characteristics are given in the Table S1.

133

134 *2.2 Genotyping*

135 The EADI1 and RS samples were genotyped by Illumina Human 610-Quad BeadChip, (Hofman et al.,
136 2013; Lambert et al., 2009) the GERAD1 samples by Illumina 610-quad chip and by Illumina
137 HumanHap550 Beadchip(Harold et al., 2009), the ADGC subjects by Illumina or Affymetrix high-density
138 SNP microarrays (Naj et al., 2011). Applied genotype filtering procedure as described in the Note S2
139 leaving 312,064 SNPs for epistasis analyses with EADI1. Replication cohorts used only directly
140 genotyped SNPs.

141

142 *2.3 Statistical discovery and replication analysis*

143 Following guidelines in Gusareva et al. (Gusareva and Van Steen, 2014), we tested for all pairwise
144 statistical interactions between SNPs in association to AD in sex-stratified samples within EADI1. Two
145 different analytic techniques both parametric (customized version of the BOolean Operation-based
146 Screening and Testing (BOOST) (Wan et al., 2010) with stringent Bonferroni correction) and non-
147 parametric (Model-Based Multifactor Dimensionality Reduction (MB-MDR) (Cattaert et al., 2011; Van
148 Lishout et al., 2013) that uses permutation-based gammaMAXT algorithm for multiple testing correction
149 (Lishout et al., 2015)) were adopted in this study with default options (Note S3). Statistical epistasis
150 signals at the genome-wide significance level of 0.05 were followed up with a logistic regression analyses
151 adjusting for age at time of subject examination and the first 4 SNP-based principal components (to adjust
152 for confounding by shared genetic ancestry). Evidence of interaction was based on a likelihood-ratio test
153 statistic with 4 degrees of freedom to reflect two SNPs with 3 genotypes each (in the absence of missing

multi-locus genotypes). Main effect single-SNP associations were assessed via Cochrane-Armitage trend test in SVS Version 7.5 software (Golden Helix, Inc.).

For replication analysis, we selected 68 and 98 SNPs assigned to *WWC1* (5q34: 167651670 - 167829334 bp) and *TLN2* (15q22.2: 60726802 - 60920733 bp), respectively, according to NCBI B36 genome assembly (SNP list is provided in Table S2). We did not consider SNPs from any regulatory regions outside *WWC1* and *TLN2* genes. Thus, all the SNPs falling into the boundaries of *WWC1* and *TLN2* genes and typed in all the study cohorts (discovery EADI1 and the 3 replication cohorts: GERAD1, RS, and ADGC) were exhaustively tested for two-way intergenic interactive association with AD, in males and females separately. We used logistic regression adjusted for age and genetic population stratification as before. The number of independent tests (Nyholt, 2004) was 1564 (of 6664 total). All obtained *p*-values (not corrected for multiple testing $p_{nominal}$) for EADI1, GERAD1, RS, and ADGC were meta-analyzed using Fisher's combined *p*-value (Fisher, 1948) and Stouffer's Z score (Stouffer et al., 1949) methods, giving rise to meta-analysis *p*-values (p_{meta}). Details on the applied significance criteria are described in the Note 4.

2.4 Functional analysis and biological validation

We used transcriptome analysis to assess co-expression of *WWC1* and *TLN2* in temporal cortex and cerebellum human brain regions with data from the brain expression GWA study (eGWAS) (Allen et al., 2012; Zou et al., 2012) (Note 5). The laboratory fruit fly *Drosophila* melanogaster was used to further explore the role of *WWC1* and *TLN2* in model organisms (Note S6). In addition, formalin-fixed temporal cortexes of male AD patients were used to perform brain immunohistochemistry (Note S7). The latter was performed in 2 independent labs to robustly establish reproducibility. To assess sub-cellular localization of WWC1 and TLN2, we performed immunofluorescence and confocal microscopy analyses (Note S8). We also investigated the presence of WWC1 and TLN2 in the same complex via immunoprecipitation

analysis (Note S9). Molecular mechanisms of interaction between WWC1 and TLN2 were modeled via protein docking (Note S10) and molecular dynamics *in silico* experiments (Note S11).

The entire analysis protocol is described in Fig. 1.

3. Results

3.1 Synergy between variants of WWC1 and TLN2 in association to AD

Both parametric (BOOST) and non-parametric (MB-MDR) analyses highlighted epistasis between the SNPs rs3733980 and rs7175766 (MAFs=0.365, 0.307 in EADI1, respectively) as genome-wide significant in males (BOOST: $p_{\text{Bonferroni-corrected}}$=0.018, MB-MDR: $p_{\text{permutation-based}}$=0.005). Case/control distributions within the 9 multi-locus genotype combinations and MB-MDR "high risk"/"low risk" labelling are in the Table S3. Only rs3733980 also showed a main effect (p_{nominal}=0.015, trend test), which would not withstand stringent multiple testing correction. The identified epistasis signal remained statistically significant in a logistic regression model accounting for age and the first 4 PCs ($p_{\text{Bonferroni-corrected}}$=0.0165). The *APOE* gene did not confound the identified interaction, since we found no dependence between the *APOE ε4* AD-risk allele and the 9-level categorical SNP pair for these SNPs, χ_8^2). No female-specific epistasis was identified (BOOST, MB-MDR p>0.05).

3.2 Statistical replication of epistasis between WWC1 and TLN2

We considered all pairwise intergenic interactions between the directly-genotyped 68 SNPs of *WWC1* and 98 SNPS of *TLN2* (Table S2) for follow-up replication analysis in both sexes with the GERAD1, RS and ADGC datasets. In males, the SNP-pair rs3733980 and rs7175766 was significant in a single study (EADI1: $p_{\text{Bonferroni-corrected}}=5.29*10^{-10}$). Rs7175766 appeared 4 times in the top 10 male-specific meta-analysis results but did not show any marginal association with AD (p_{nominal}=0.546, trend test). Interaction

between rs1477307 and rs4077746 was found in 3 study populations (EADI1: $p_{nominal}$=0.040, RS: $p_{nominal}$=9.37*10^{-4} and ADGC: $p_{nominal}$=5.06*10^{-5}, but not in GERAD1: $p_{nominal}$=0.544; Fisher's combined $p_{meta\text{-}Bonferroni\text{-}corrected}$=2.74*10^{-3} and Stouffer's Z score $p_{meta\text{-}Bonferroni\text{-}corrected}$=9.02*10^{-3}; Table S4). In females, similar meta-analysis gave no replicable epistasis signals (Table S5).

3.3 Functional analysis and biological validation

Transcriptome analysis revealed significant positive association between expression levels of *WWC1* (probe ID - ILMN_1658619) and *TLN2* (probe ID - ILMN_1700042) in temporal cortex brain samples from autopsied AD subjects (β=0.17, p=0.01) and from combined autopsied AD and non-AD subjects (β=0.20, p=0.0003). These associations were mostly driven by females (temporal cortex from autopsied AD females: β=0.28, p=0.005, combined autopsied AD and non-AD females β=0.20, p=0.016) but were not prominent in males. This association was only marginally significant for autopsied non-AD subjects (β=0.19, p=0.05). In the cerebellar tissue, no significant associations between expression levels of *WWC1* and *TLN2* gens were observed (Table S6).

We also tested whether *WWC1* and *TLN2* could modulate AD physiopathology in human Tau (2N4R)-expressing *Drosophila*, an *in vivo* model of AD (review (Gistelinck et al., 2012)). *Kibra*, ortholog of *WWC1* (Fig. 2A, 2B and 2C), and *rhea*, ortholog of *TLN2* (Fig. 2A, 2D and 2E), were tested as modifiers of *Tau toxicity* in *Drosophila* eye. In *Drosophila*, *kibra* belongs to the growth controlling Hippo pathway. Gain (loss) of *kibra* results in smaller (bigger) eyes (Baumgartner et al., 2010), which we also observed (Fig. 2A and B). Expression of human Tau (2N4R) in the eye with the *GMR* driver resulted in smaller rough eyes. The eye size was partially restored in *kibra*$^{2/+}$ haploinsufficient background, upon RNAi-mediated knockdown of *kibra* (Fig. 2B and 2C) and in *rhea*$^{1/+}$ haploinsufficient background (Fig. 2D and 2E). Coexpression of *kibra* with Tau resulted in lethality and the only escapers that we obtained had smaller eyes. For *kibra* knockdown and *kibra* overexpression, the effect may be additive as in both

conditions without Tau expression, fly eyes are respectively bigger and smaller (Fig. 2A and B). For *kibra* haploinsufficiencies, only 1 out of 4 independent null mutations restored the eye size precluding us to firmly conclude that *kibra* interacts with Tau in *Drosophila* eye. The result in the $rhea^{1/+}$ haploinsufficient background (Fig. 2D and 2E) suggested that *rhea* interacted functionally with human Tau in *Drosophila* eye.

Immunohistochemistry of the brain of a male autopsied AD patient indicated strong expression of WWC1 in the soma of neuronal cells throughout the temporal lobe of the cerebral cortex (Fig. 3). In these neurons, WWC1 presented in the cytoplasm with presumed membrane and/or cytoskeleton associations and strong neuritic accumulations in some cells. TLN2 also presented in the cytoplasm of neuronal cells, although immunoreactivity was low. In addition to the weak neuronal signal, a strong TLN2 signal was detected in the endothelial cells of blood vessels.

We also performed co-immunofluorescent staining analyses of human Braak I and Braak VI brains (Braak and Braak, 1991) (Fig. 4). After performing quantitative pixel intensity spatial correlation analysis (extracting Pearson's, Manders', and Costes' parameters (autothreshold and randomization) (Bolte and Cordelieres, 2006)), we determined that TLN2 (Talin2) and WWC1 (aka KIBRA) co-localized in all cases. Interestingly, WWC1 staining appeared to be more cellular in Braak I compared to Braak VI tissue, where the staining appeared stronger and more widely distributed.

In complement, we confirmed co-localization of WWC1 and TLN2 in HeLa cell lines and in mouse N2a neuroblastoma cells. When overexpressed in HeLa cells, *WWC1* displayed diffuse cytoplasm localization and small perinuclear rings (Fig. 5, Flag-WWC1), and TLN2-GFP displayed cytoplasmic focal adhesion localization with elongated fibrillar adhesions through the cell body (Fig. 5, TLN2-GFP), consistent with previous studies (Kremerskothen et al., 2003; Praekelt et al., 2012). Co-expression of both WWC1 and TLN2 dramatically changed TLN2 localization. In the presence of WWC1, TLN2-GFP appeared concentrated in cytoplasmic foci (Fig. 5, compare GFP staining for TLN2 and WWC1+TLN2) surrounded by Flag-WWC1 rings (Fig. 5, WWC1+TLN2-GFP, merge image). In N2a cells, WWC1 and TLN2 were

found to co-localize in cytoplasm and in filopodia-like protrusions (Fig. S1). However, different co-localization patterns observed in N2a cells may be due to different levels of the proteins expressions.

Furthermore, immunoprecipitation analysis (IP) both in human brain samples and in HEK293 cells indicated the presence of WWC1 and TLN2 in the same protein complex. The levels of the two proteins were variable in all conditions and brain regions queried (Braak I and Braak VI brains (Braak and Braak, 1991), Fig. 6A (upper panel)). WWC1 co-immunoprecipitated with the anti-TLN2 antibody (Fig. 6A (lower panel)); as expected, TLN2 bands were evident in the western blot. Interestingly, when the WWC1 antibody was used, TLN22 bands were absent (Fig. 6A (lower panel). These data suggest that the anti-WWC1 antibody could competitively disrupt the TLN2 and WWC1 interaction. In HEK293 cells, TLN2-GFP specifically co-purified with Flag-WWC1 when both proteins were overexpressed together (Fig. 6B).

To model molecular mechanisms of interaction between WWC1 and TLN2 we performed protein docking and molecular dynamics (MD) *in silico* experiments. We determined the top 10 ranked WWC1/TLN2 poses (Fig. S2) via ClusPro 2.0 docking server (Comeau et al., 2004a, b; Kozakov et al., 2006). Poses 2 and 7 showed the most favorable conditions for complex formation as their average MM/PBSA protein-ligand binding free energies (dG_{bind}) were amongst the most negative showing the lowest dispersion over the course of the 50 ns aqueous simulations. In all 50 ns MD simulations, WWC1 and TLN2 remained physically associated in a complex throughout the entire course of simulation. The average dG_{bind} remained negative for all 10 poses (dG_{bind} ranged from -16 to -227 kJ/mol indicating the size of the binding affinity between the two proteins; Table S7 and Fig. S3).

4. Discussion

This is the first contribution showing (sex-specific) biological epistasis in AD between genes identified via exhaustive genomic epistasis analysis: *WWC1* (WW and C2 domain containing 1 or kidney and brain expressed protein, aka *KIBRA*) and *TLN2* (talin 2). *WWC1* is expressed in brain regions responsible for

learning and memory (hippocampus and cortex) and is involved in maintaining of synaptic plasticity (Vogt-Eisele et al., 2014). *TLN2* expression is restricted to heart, skeletal muscle and brain (synapses and focal adhesions) (Di Paolo et al., 2002). It plays an important role in the assembly of actin filaments (particularly affecting actin dynamics and clathrin-mediated endocytosis at neuronal synapses (Morgan et al., 2004)) and in spreading and migration of various cell types. *WWC1* has already been associated with memory-related disorders including AD (Burgess et al., 2011; Corneveaux et al., 2010; Papassotiropoulos et al., 2006; Rodriguez-Rodriguez et al., 2009), while *TLN2* has not. However, in our study *rhea* (ortholog of *TLN2* in *Drosophila*) modulated Tau toxicity in *Drosophila* and thus may be involved in AD pathology. Interestingly, recent studies identified several other components of the cell adhesion pathway as modifiers of Tau toxicity in *Drosophila* (Dourlen et al., 2016; Shulman et al., 2014). Studying the mechanisms of the identified epistatic interaction, we performed comprehensive functional biological experiments. WWC1 and TLN2 were co-expressed in the temporal cortex brain tissue (responsible for learning and memory) of AD subjects, co-localized in both brain tissue cells, in neuroblastoma N2a and HeLa cell lines, and co-immunoprecipitated both in brain tissue and HEK293 cells. The physical interaction between WWC1 and TLN2 was also supported by *in silico* experiments where the binding affinity between the two proteins was pretty strong with favorable conditions for forming a stable protein complex.

We may speculate on the involvement of WWC1 and TLN2 in common signaling pathways connected to signal transduction via synapses that are impaired when dementia symptoms and AD progress. Since overexpression of WWC1 was previously associated with AD (Burgess et al., 2011), we speculate that impairment expression of WWC1 and/or TLN2 proteins may destabilize actin filaments. Additional work is required to further describe a functional interplay between WWC1 and TLN2 and to explain why we observed the interaction at an individual level for both sexes, whereas we could detect association with AD only in males at a population level (despite of the theoretical power loss for epistasis detection in a sample stratum of males). A few explanations are possible and should be investigated in detail: the influence of sex hormones on the epistasis manifestation, the involvement of a third interacting component

(i.e., an interacting gene) linked to the sex chromosomes, other types of sex-specific variant(s) in *WWC1* and *TLN2*, among others. Regardless, our findings provide impetus for an in-depth search of AD-related mutation(s) in *WWC1* and *TLN2* genes to better explore and grasp biological mechanisms underlying the identified sex-specific epistasis signals. Targeted next-generation sequencing of the interacting genes may facilitate the identification of new functional mutations (either common or rare) that play a role in protein structure, stability, solubility, folding, and affinity of interaction with ligand(s), to name a few.

There is still a big divide between statistical epistasis and biological epistasis. The ambition in detecting statistical epistasis is to close this gap by improved analysis protocols and to formulate guidelines towards the interpretation of statistical findings in the context of epistasis. The field has evolved a lot over the last decade, in this sense. This does not change the fact that indeed, the power of a genome-wide epistasis screening (GWAI analysis) using a single study is much smaller than the power of a corresponding main effects GWA analysis using the same data (Gauderman, 2002). Our experience with large-scale epistasis studies is consistent with this, usually only giving rise to 1 or 2 reliable statistical findings (that is, findings for which we can rule out numerical instability issues or strong main effects overtaking the joint effects of the loci involved). Regardless, results dating back from already suggested that biological inference from statistical models is not an utopia (Moore, 2005).

5. Conclusion

In this research we aimed to identify novel gene/protein targets to pave the way towards novel biochemical pathways related to AD via SNP panels as a starting point. By following a rigorous analytic genome-wide epistasis detection protocol (Gusareva and Van Steen, 2014), which minimizes false positive findings and enhances functional relevance, the statistically replicable epistasis was identified. A series of biological experiments indicated novel protein-protein interaction between WWC1 and TLN2 that can potentially be a medical target for the cure of AD. To our knowledge, this is the first report in AD

where a hypothesis-free screening led to evidence for replicable statistical interaction and where functional studies were performed beyond the transcriptome.

Disclosure statement

The authors have no actual or potential conflicts of interest.

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Founding sources are listed in the Note S12.

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Figure legends

Fig. 1. Analysis protocol including genome-wide association interaction (analytical block) and biological validation of epistasis (experimental block).

Fig. 2. Genetic interaction between *kibra, rhea* and human Tau in the eye of *Drosophila.* A. Table presenting the homology of *WWC1* and *TLN2* with their *Drosophila* orthologs. B. and C. Image and size quantification of fly eyes expressing the 2N4R Tau isoform (*GMR>Tau*) in loss-of-function (in blue) and gain-of-function (GOF, in green) *kibra* conditions (scale bar 0.1 mm). The *GMR>* images correspond to the same *kibra* conditions without Tau expression. Numbers above the x axis in the graphs indicate the number of eyes that were quantified. Knockdown (overexpression) of *kibra* rescued partially (enhanced) Tau toxicity in the eye (C. right graph). This was likely an additive effect of the modulation of *kibra* with Tau as knockdown (overexpression) of *kibra* alone increased (decreased) the size of the eyes (C. left graph). However one haploinsufficient condition, $kibra^{2/+}$, partially rescued Tau toxicity (C. right graph) without affecting the eye on its own (C. left graph). D. and E. Image and size quantification of fly eyes expressing the 2N4R Tau isoform (*GMR>Tau*) in loss-of-function (in blue) *rhea* conditions (scale bar 0.1 mm). Expression of Tau in the haploinsufficient $rhea^{1/+}$ background resulted in bigger eyes (E. right graph) whereas haploinsufficient $rhea^{1/+}$ flies have similar eye size than control (E. left graph), suggesting a genetic interaction between Tau and *rhea*.

Fig. 3. Presence and localization of WWC1 and TLN2 in the temporal cortex of an AD patient. A. Single immunostainings with chromogenic detection reveals in neuronal cytoplasm a moderate to strong WWC1 staining and low TLN2 expression. B. Fluorescence double immunostaining confirms the presence of WWC1 and TLN2 in neuronal cells. Strong neuritic WWC1 accumulations are highlighted with arrows, blood vessel endothelial cells with high TLN2 signal are marked with arrowheads. Scale bar = 50µm.

Fig. 4. TLN2 and WWC1 (aka KIBRA) co-localize in AD and control brains. Representative images of healthy (Braak I, A-C) and late stage AD (Braak VI, D-F) brains that were immunofluorescently labeled with anti-Talin2 (green) and anti-KIBRA (red) antibodies. Co-localization analysis was performed on positive immunofluorescent signals from multi-z-stack confocal microscopy images. Braak I (A-C) and VI (D-F) brains showed positive co-localization between both signals (C and F). DAPI (blue) was used to reveal cell nuclei. G-I) Representative images of brain sections incubated with only secondary, but not primary, antibodies to reveal non-specific staining. Three Braak I and three Braak VI brains were imaged. A total of 9 sets of confocal z-stacked images were obtained for each condition (Braak I and VI).

Fig. 5. WWC1 (aka KIBRA) and TLN2 co-localize in HeLa cells. HeLa cells were transfected with expressing vectors for TLN2-GFP and/or Flag-WWC1. Cells on glass coverslips were fixed, permeabilized and labeled with an anti-Flag M2 antibody followed by Alexa633-conjugated secondary antibody and Dapi nuclear staining. Images were analyzed using a confocal microscope.

Fig. 6. WWC1 and TLN2 present in the same protein complex. A (upper panel). Representative western blot showing varying levels of TLN2 and WWC1 in superior medial temporal gyrus (SMTG) and hippocampal (HC) homogenates from Braak I and VI brains. A (lower panel). Representative western blots of co-IP showing that WWC1 associates with TLN2. TLN2, however, did not co-immunoprecipitate when anti-WWC1 antibodies were used. Ø represents brain homogenates that were not incubated with primary antibodies (only secondary). Ages and sex of each sample is shown. B. HEK293 cells were transfected with expressing vectors for TLN2-GFP and/or Flag-WWC1 as indicated. Cell lysates were immunoprecipitated using anti-Flag M2 antibody followed by SDS-PAGE and western blot using an anti-GFP antibody. Five percent of the amount of each lysate was used as positive control for protein expression.

ANALYTICAL BLOCK

Discovery cohort EADI1: 3150 males (788 cases), 5110 females (1455 cases)

582,982 SNPs

1. Samples and markers quality control: HWE test in controls ($P > 1*10^{-4}$), call rate > 98%, marker allele frequency (MAF > 0.05)

474,020 SNPs

2. LD pruning: Window size 50 bp, window increment 1 bp, LD r^2 threshold 0.75

312,064 SNPs

3. Exhaustive genome-wide screening for pair-wise SNP interactions in males and females: BOOST, MB-MDR and regression modeling in R

Replication cohorts: GERAD1 3929 males (988 cases), RS 2376 males (264 cases),
ADGC 6149 males (2584 cases)

68 SNPs - *WWC1,* 98 SNPs - *TLN2*

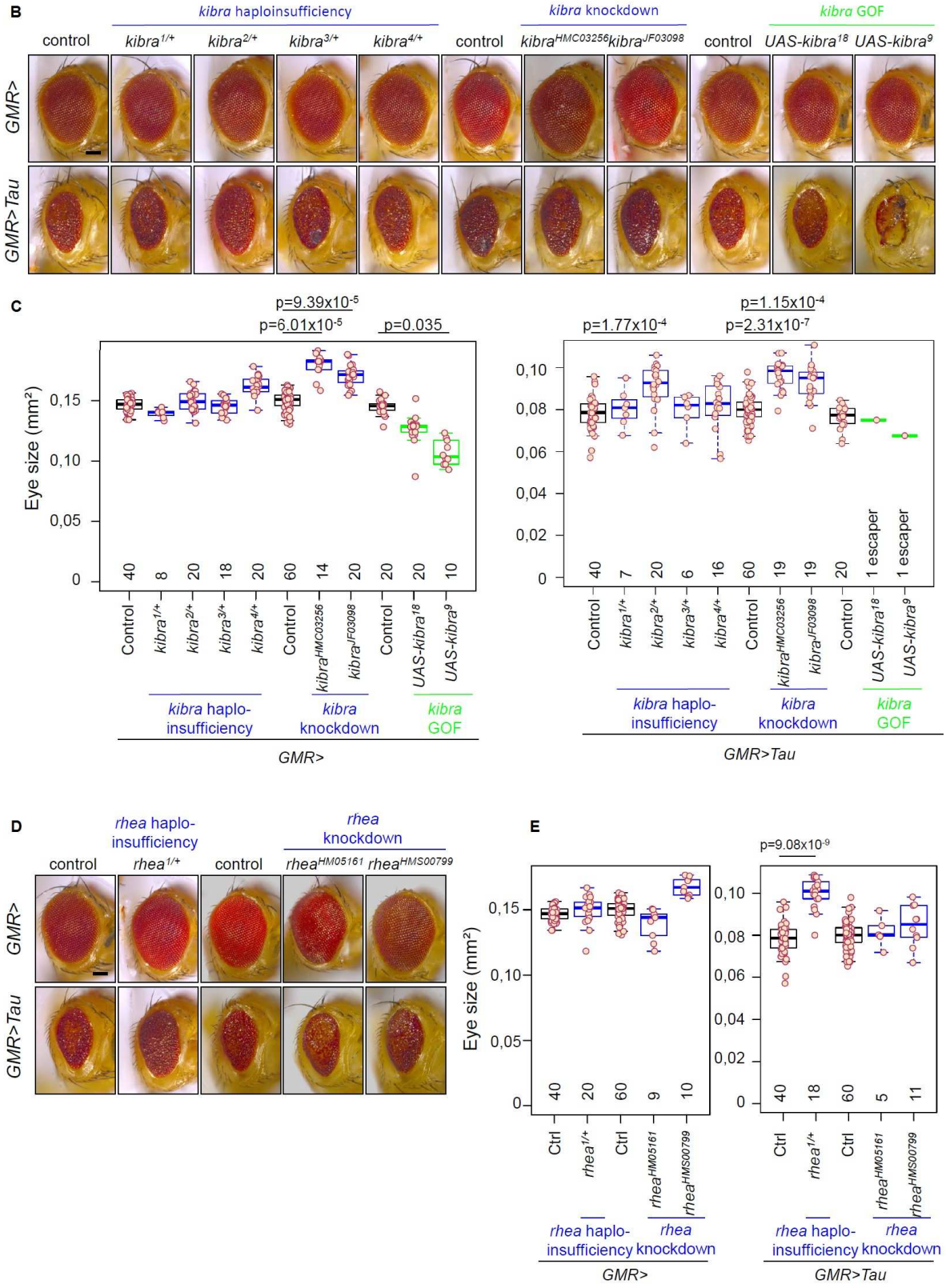
4. "Gene-based" replication analysis and meta-analysis

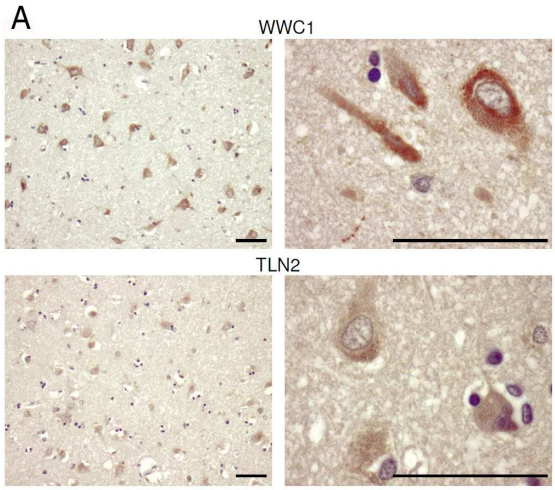
EXPERIMENTAL BLOCK

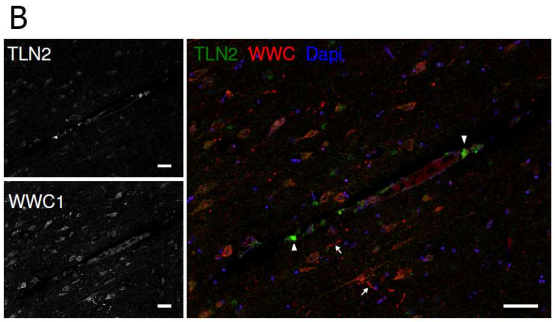
5. Biological validation of statistical epistasis (series of functional analyses):
Transcriptome analysis to assess co-expression of *WWC1* and *TLN2* in brain tissues of AD and non-AD subjects
Experiments in model organisms (i.e., Tau toxicity in the *Drosophila* eye) to test whether *WWC1* and *TLN2* can modulate AD physiopathology
Immunofluorescence and confocal microscopy to confirm presence of WWC1 and TLN2 in human brain cells and to assess their co-localization in common cellular compartments
Immunoprecipitation analysis to confirm physical interaction between WWC1 and TLN2 in a real biological system
Protein docking and molecular dynamics analysis to get more inside into mechanisms of the physical interaction between WWC1 and TLN2

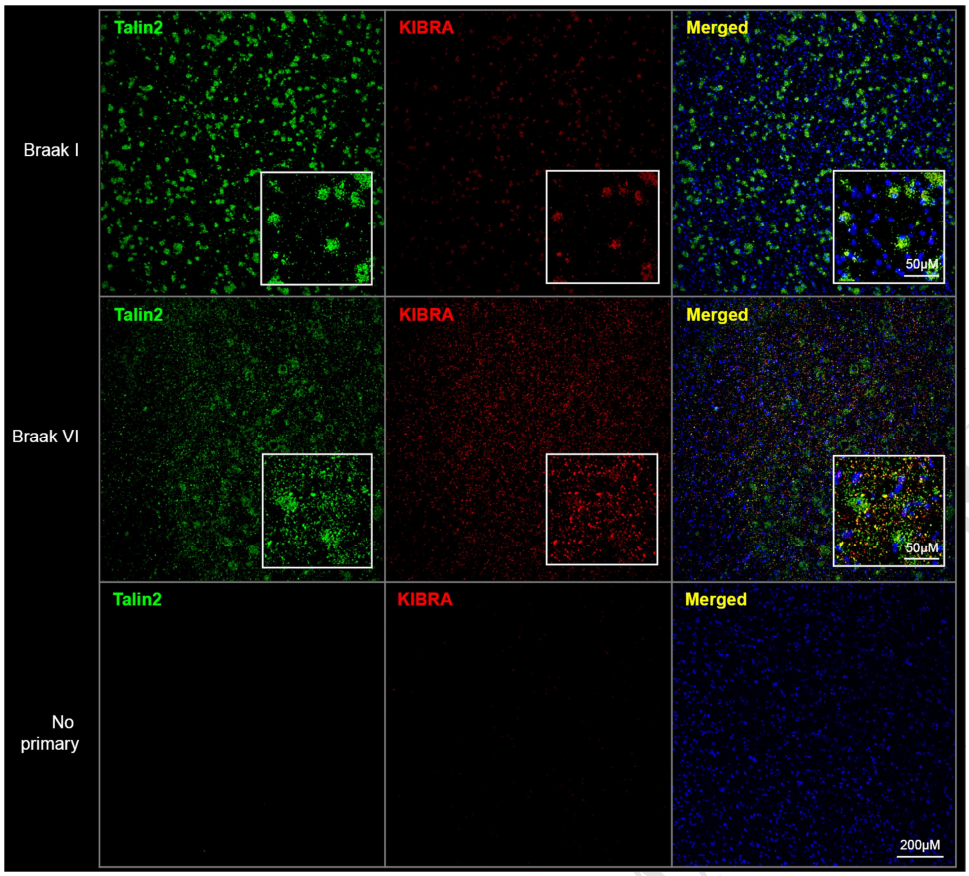
A

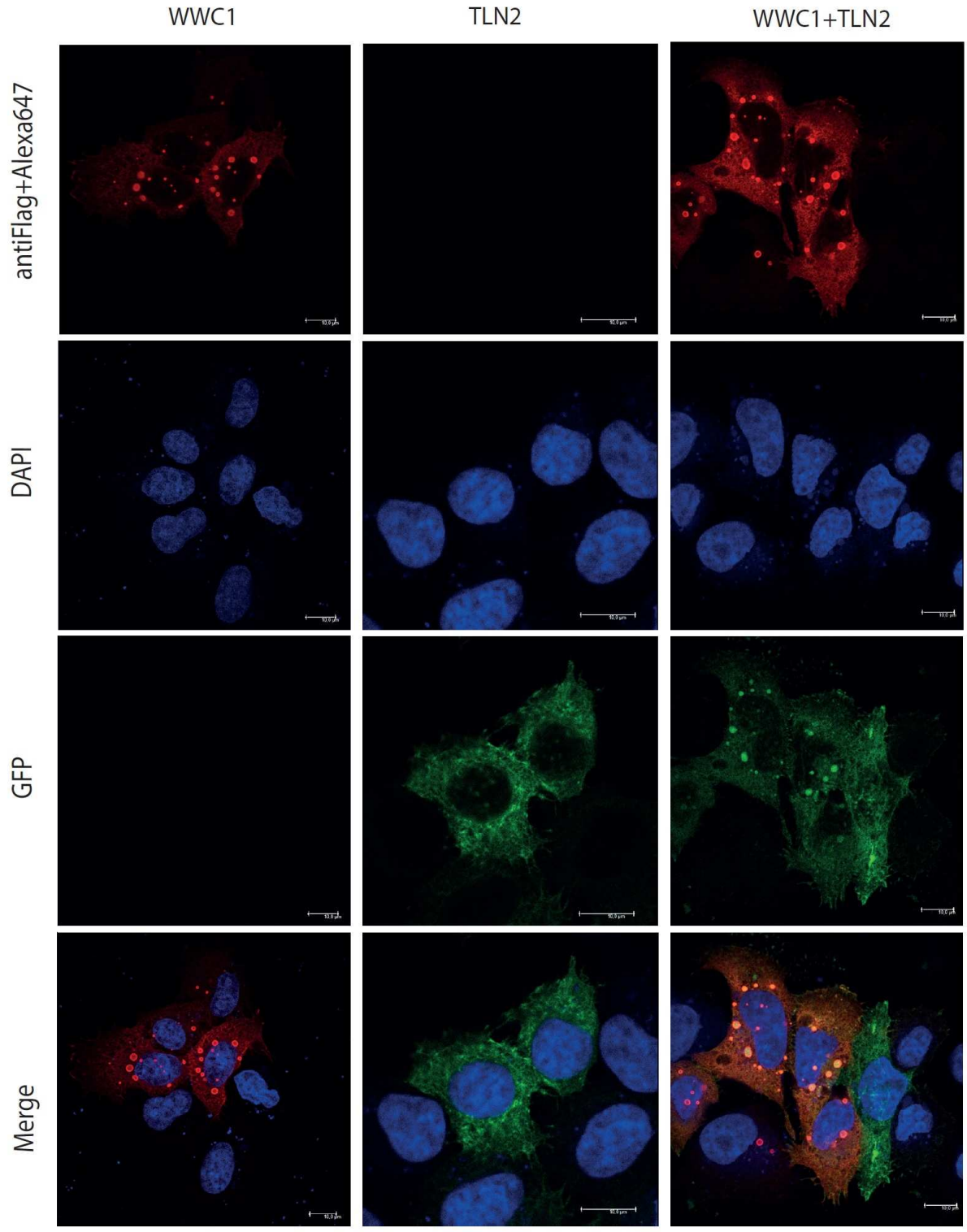
Human gene	Fly gene	DIOPT score	Identity (%)	Similarity (%)
WWC1	*kibra*	8	34	52
TLN2	*rhea*	9	47	67

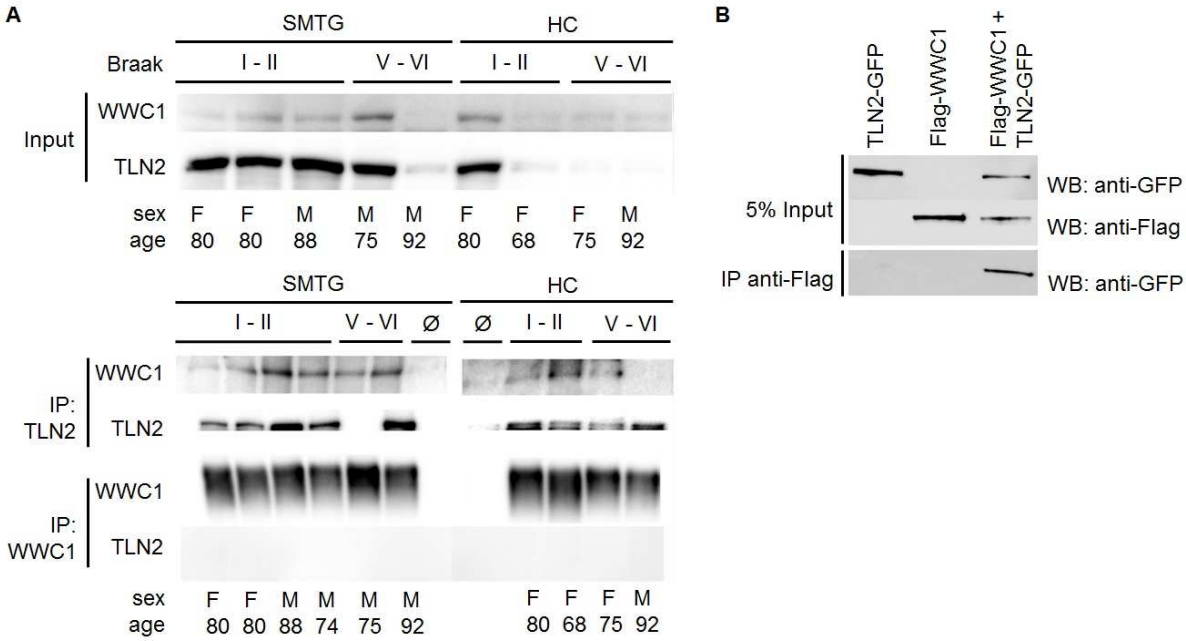









WWC1
TLN2
WWC1+TLN2
antiFlag+Alexa647
DAPI
GFP
Merge



Highlights

1. Exhaustive hypothesis-free genome-wide screening for epistasis was conducted.
2. Replicable statistical interaction between *WWC1* and *TLN2* genes was identified.
3. A series of biological experiments verified novel protein-protein interaction between WWC1 and TLN2.

Data statement

Genome-wide genotype data of the EADI1 consortium are available at https://www.cng.fr/alzheimer/ and https://beaune.cng.fr/alz_results/. Data collections of the ADGC are deposited in NIAGADS and can be assessed at https://www.niagads.org/resources/related-projects/alzheimers-disease-genetics-consortium-adgc-collection. Genotype data of the GERAD1 and RS were provided upon request according to consortium regulations. Because of restrictions based on privacy regulations and informed consent of the participants, data cannot be made freely available in a public repository. The transcriptome data are available at http://dx.doi.org/10.7303/syn3157225 and http://dx.doi.org/10.7303/syn3157249.