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The neuronal sortilin-related receptor SORL1 is genetically associated with Alzheimer disease

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The recycling of the amyloid precursor protein (APP) from the cell surface via the endocytic pathways plays a key role in the generation of amyloid β peptide (Aβ) in Alzheimer disease. We report here that inherited variants in the SORL1 neuronal sorting receptor are associated with late-onset Alzheimer disease. These variants, which occur in at least two different clusters of intronic sequences within the *SORL1* gene (also known as *LR11* or *SORLA*) may regulate tissue-specific expression of SORL1. We also show that SORL1 directs trafficking of APP into recycling pathways and that when SORL1 is underexpressed, APP is sorted into Aβ-generating compartments. These data suggest that inherited or acquired changes in SORL1 expression or function are mechanistically involved in causing Alzheimer disease.

The accumulation of Aβ peptide, a neurotoxic proteolytic derivative of APP, is a central event in the pathogenesis of Alzheimer disease[1]. Accumulation of Aβ in the brain is associated with disease-causing inherited variants in the *APP*[2], presenilin 1 (*PSEN1*)[3] presenilin 2 (*PSEN2*)[4] and apolipoprotein E (*APOE*) genes[5,6]. The generation of Aβ occurs in several subcellular compartments, but a principal location is during the re-entry and recycling of APP from the cell surface via the endocytic pathway (**Fig. 1a**)[7–11]. We reasoned that inherited variants in these pathways might modulate APP processing and thereby affect risk for Alzheimer disease. This concept is supported by prior reports that (i) the expression of several candidate proteins within these pathways (such as SORL1 (ref. 12) and VPS35 (ref. 13)) is reduced in brain tissue from individuals with Alzheimer disease and (ii) reductions in the expression of some of these proteins are associated with increased Aβ production[13–15]. However, it is unclear whether these changes are causal or simply reactive to Alzheimer disease.

To address this question, we investigated genetic associations between Alzheimer disease and SNPs in selected members of the vacuolar protein sorting (VPS) gene family, including *VPS35* (16q12); *VPS26A* (10q21); sortilin *SORT1* (1p21–p13); sortilin-related VPS10-containing receptors *SORCS1* (10q23–q25), *SORCS2* (4p16) and

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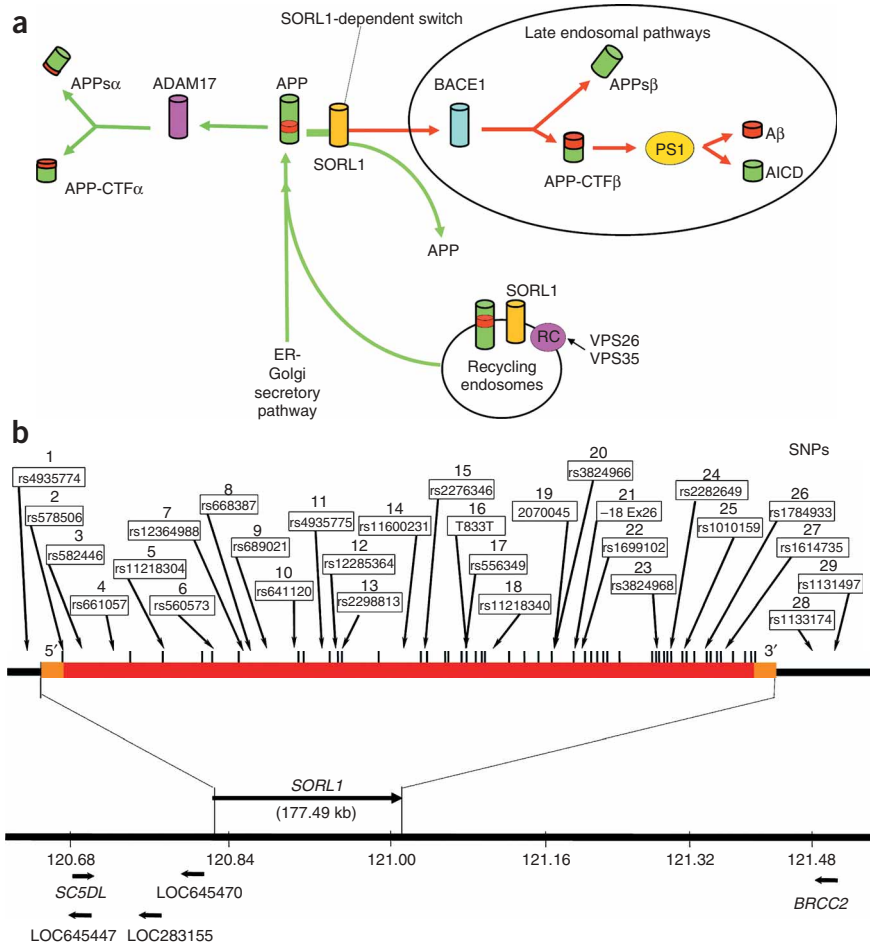

Figure 1 Putative cell biological role and genetic architecture of SORL1. (**a**) Diagram of APP processing pathways. APP holoprotein is synthesized in the endoplasmic reticulum (ER) and Golgi. Proteolytic cleavage through the Aβ peptide domain by ADAM17 and other α-secretase enzymes generates N-terminal soluble APPsα and membrane-bound APP α C-terminal fragments (APP-CTFα) fragments. Sequential cleavage by BACE1 (β-secretase) generates N-terminal APPsβ and membrane bound APP-CTFβ fragments. The latter undergoes presenilin-dependent γ-secretase cleavage to generate Aβ and amyloid intracellular domain (AICD). SORL1 binds both APP holoprotein (see **Fig. 2**) and VPS35 (not shown) and acts as a sorting receptor for APP holoprotein. Absence of SORL1 switches APP holoprotein away from the retromer recycling pathway and instead directs APP into the β-secretase cleavage pathway, increasing APPsβ production (**Fig. 3c**) and then into the γ-secretase cleavage pathway to generate Aβ (see **Fig. 3b**). Blockade of the retromer complex (RC) by inhibiting retromer complex proteins such as VPS26A (**Fig. 3d**) or VPS35 has a similar effect, also increasing APPsβ and Aβ production. (**b**) Genomic map of *SORL1* showing the location of SNPs genotyped in this study. Orange bars represent the 5′ UTR and 3′ UTR, red bar represents intragenic regions and black vertical bars represent each of the 48 exons. SNPs 1, 28 and 29 are located in extragenic intervals.

SORCS3 (10q23-q25); and the sortilin-related receptor, low-density lipoprotein receptor class A repeat–containing protein *SORL1* (11q23–q24). We tested several SNPs from these genes in six independent data sets that have sufficient power to detect modest gene effects ($\lambda_s = 1.5$). We collected these data sets with restricted ancestral origins in order to minimize the confounding effects of allelic heterogeneity[16,17]. Indeed, two of these six data sets (Caribbean Hispanic FAD and Israeli Arab), were drawn from population isolates with a limited number of founders[18,19].

We divided these six data sets into a 'discovery cohort' composed of families with late-onset familial Alzheimer disease (FAD) and a 'replication cohort' composed of discordant sibships and collections of individuals with Alzheimer disease and normal controls matched for age, gender and ethnic origin. We analyzed the FAD pedigrees in the discovery cohort (124 north European FAD families[20,21] and 228 Caribbean Hispanic FAD families[22]; **Supplementary Table 1 online**) with conservative family-based association (FBAT) methods, which are less sensitive to population stratification. We then reinvestigated positive results from the discovery cohort in the replication cohort (**Supplementary Table 1**). This replication cohort contained (i) northern European individuals from a case-control study (178 individuals with sporadic Alzheimer disease and 242 controls of self-identified Caucasian European ancestry)[20], (ii) MIRAGE Caucasian sibships (276 Caucasian sibships from the MIRAGE Study)[23,24], (iii) MIRAGE African American sibships (238 African American sibships from the MIRAGE Study)[23,24] and (iv) Israeli Arab affected individuals and controls (all 111 individuals with Alzheimer disease and 114 normal controls were from the Wadi Ara population study)[19,25].

We also obtained fully independent replication from a large data set composed of three cohorts of Americans of self-identified European Caucasian ancestries that were separately ascertained, genotyped and analyzed statistically at the Mayo Clinic (1,405 individuals with Alzheimer disease and 2,124 controls; **Supplementary Table 1**).

RESULTS

SNPs in *SORL1* are associated with late-onset Alzheimer disease

We initially screened at least two SNPs in the intragenic sequences of the *SORL1*, *VPS26A*, *VPS35*, *SORCS1*, *SORCS3*, *SORCS2* and *SORT1* genes for association with Alzheimer disease in the two independent FAD 'discovery data sets'. We did not observe any allelic associations with *VPS26A*, *VPS35*, *SORCS3* or *SORT1* (**Supplementary Tables 2** and **3** online). However, one SNP in *SORCS1* (rs7082289: $P = 0.013$), one SNP in *SORCS2* (rs7694823: $P = 0.015$) and two SNPs in *SORL1* showed nominally significant association in at least one of the FAD data sets (rs2298813: $P = 0.012$; rs2070045: $P = 0.031$).

To validate these initial results, we investigated a second series of SNPs from the *SORCS1*, *SORCS2* and *SORL1* genes in the two FAD discovery data sets (**Table 1** and **Fig. 1b**). We did not detect any association with the additional SNPs in *SORCS1* (a total of nine SNPs) or in *SORCS2* (a total of six SNPs) (**Supplementary Tables 2** and **3**). However, six SNPs clustered in two distinct regions of the *SORL1* gene were significantly associated with Alzheimer disease in at least one discovery data set and also in at least one replication data set (**Table 2** and **Supplementary Table 4** online). Notably, at five of these SNPs, the alleles associated with Alzheimer disease were identical in both the discovery and replication data sets (**Table 2** and **Supplementary**

Table 1 ***SORL1*** **SNPs used in this study**

Marker number	dbSNP rs number	Alleles	Orientation/strand	Physical map location (bp)	Distance from previous marker (in bp)	SNP type
1	rs4935774	A/G	rev/T	120826964	—	Upstream of 5′ UTR
2	rs578506	C/G	fwd/B	120828687	1,723	Intron
3	rs582446	A/G	fwd/T	120833069	4,382	Intron
4	rs661057	C/T	fwd/B	120834164	1,095	Intron
5	rs11218304	C/T	rev/B	120854321	20,157	Intron
6	rs560573	A/T	fwd/B	120866094	11,773	Intron
7	rs12364988	A/G	rev/T	120872836	6,742	H269H
8	rs668387	C/T	rev/B	120873131	295	Intron
9	rs689021	A/G	rev/T	120876330	3,199	Intron
10	rs641120	C/T	fwd/B	120886175	9,845	Intron
11	rs4935775	C/A	rev/T	120894712	8,537	Intron
12	rs12285364	C/T	fwd/B	120898436	3,724	Intron
13	rs2298813	A/G	fwd/T	120898894	458	T528A
14	rs11600231	C/T	fwd/B	120911918	13,024	Intron
15	rs2276346	G/T	fwd/B	120919686	7,768	Intron
16	SORL1-T833T	A/T	fwd/T	120931165	11,479	T833T
17	rs556349	G/T	rev/B	120931417	252	Intron
18	rs11218340	A/T	fwd/B	120936564	5,147	Intron
19	rs2070045	G/T	fwd/B	120953300	16,736	S1187S
20	rs3824966	C/G	fwd/T	120953393	93	Intron
21	SORL1-18ex26	C/G	fwd/T	120959359	5,966	(–18) 5′ of exon 26
22	rs1699102	C/T	fwd/B	120962172	2,813	N1246N
23	rs3824968	A/T	rev/T	120981132	18,960	A1584A
24	rs2282649	C/T	fwd/B	120984168	3,036	Intron
25	rs1010159	C/T	rev/B	120988611	4,443	Intron
26	rs1784933	A/G	fwd/T	120994626	6,015	Intron
27	rs1614735	C/A	rev/T	120998211	3,585	Intron
28	rs1133174	A/G	fwd/T	121006965	8,754	Downstream of 3′ UTR
29	rs1131497	C/G	fwd/B	121007955	990	Downstream of 3′ UTR

Marker intervals are calculated on the basis of NCBI locations. SNPs are referred to in this paper by sequential numbers (marker number) reflecting their relative physical map positions. Orientation or strand information was obtained from NCBI: 'fwd/T' refers to forward or top strand; 'rev/B' refers to reverse or bottom strand. The linkage disequilibrium maps for these SNPs are given in **Supplementary Table 3.**

Table 4). Thus, at the 5′ end of *SORL1*, Alzheimer disease was associated with the C, G and C alleles at SNPs 8, 9 and 10, respectively, in the Caribbean Hispanic FAD (P = 0.013, 0.017 and 0.021, respectively), Israeli Arab case-control (P = 0.002, 0.007 and 0.005, respectively) and north European case-control data sets (P = 0.021, 0.040 and 0.067, respectively; **Table 2** and **Supplementary Table 4**). Similarly, at the 3′ end of *SORL1*, Alzheimer disease was associated with the G and T alleles at SNPs 19 and 23, respectively, in the north European FAD (P = 0.031 and 0.0031, respectively) and north European case-control data sets (P = 0.00082 and 0.00073, respectively; **Table 2** and **Supplementary Table 4**). *Post hoc* statistical adjustment for *APOE* genotype, age and gender did not alter the conclusions that (i) there were allelic associations between Alzheimer disease and two clusters of SNPs in distinct regions of *SORL1* in different data sets and (ii) that these associations replicated in multiple independent data sets.

We performed haplotypic analyses using a sliding window method[26] with a window size of three contiguous SNPs, confirming the single-SNP analyses by demonstrating replicated haplotypic associations in two regions of *SORL1* (**Table 3** and **Supplementary Table 5** online). Thus, at the 5′ end of *SORL1*, the CGC haplotype at SNPs 8, 9 and 10 was associated with Alzheimer disease in the Caribbean Hispanic FAD (global P = 0.0098, haplotype P = 0.0053, haplotype frequency estimated by FBAT = 0.638 versus 0.583 in unrelated controls), the Israeli Arab case-control (global P = 0.023, haplotype P = 0.0085, frequency = 0.661 in cases versus 0.539 in controls) and the north European case-control data set (haplotype P = 0.045, frequency = 0.638 in cases versus 0.566 in controls; **Table 3** and **Supplementary Table 5**). In the Israeli Arab data set, the overlapping GCC haplotype at SNPs 9, 10 and 11 showed even greater evidence for association (global P = 0.0080; haplotype P = 0.0047). As might be expected, SNPs 8, 9 and 10 also possessed a protective haplotype. Thus, the TAT haplotype at SNPs 8, 9 and 10 was associated with decreased risk of Alzheimer disease in these data sets (Hispanic FAD: haplotype P = 0.0086; haplotype frequency estimated by FBAT = 0.317 versus 0.394 in unrelated controls; Israeli Arab case-control: frequency = 0.301 in affected individuals versus 0.434 in controls, and haplotype P = 0.0037; north European Caucasian case-control: frequency = 0.351 in affected individuals versus 0.417 in controls, and haplotype P = 0.068).

We observed a second cluster of replicated haplotypic associations at the 3′ end of *SORL1* in the north European data sets. Thus, the overlapping haplotypes of CTT at SNPs 22–24 and TTC at SNPs 23–25 were associated with Alzheimer disease in the north European FAD and north European case-control data sets ($0.001 < \text{haplotype } P < 0.02$; **Table 3** and **Supplementary Table 5**). This region of *SORL1* also showed significant haplotypic associations in the MIRAGE African American sibships. However, the haplotypic associations at SNPs 23–25 in the MIRAGE African American sibships were with different haplotypes (global P = 0.0043; disease-associated 'ACT' haplotype-P = 0.0025, frequency = 0.513; protective 'ACC' haplotype P = 0.0044, frequency = 0.403; **Table 3** and **Supplementary Table 5**). The conclusion that there are at least two distinct regions of *SORL1* that are associated with Alzheimer disease in different populations was supported when we examined shorter or longer haplotypes (**Supplementary Tables 6–9** online).

To provide a completely independent confirmation of the association between Alzheimer disease and *SORL1*, we genotyped SNPs 4, 5, 8, 9, 12, 19 and 22–25 and analyzed them at an independent facility in three series of American affected individuals and controls of European ancestry ascertained at the Mayo Clinic (n = 1,405 late-onset Alzheimer disease cases and 2,124 controls; **Supplementary Table 1**)[27,28]. The north European Caucasians and the Mayo data sets have slightly different allele frequencies and haplotype structures and may therefore have slightly different ancestral origins. Nevertheless, we observed significant associations at SNPs 4, 12, 19 and 23–25 in the overall Mayo data set (single-SNP: $0.009 \leq P \leq 0.046$). Two of the three sub-data sets individually generated highly significant results ($0.001 < P < 0.007$) for one or more of these SNPs (**Table 4**). Notably, the alleles and haplotypes at SNPs 19 and 22–25 that were associated with increased risk for Alzheimer disease in the Mayo data

Table 2 Single-SNP association results

		Discovery data sets								Replication data sets							
		North European families				Caribbean Hispanic families				Israeli Arab case-control				North European case-control			
SNP	Minor allele	MAF	Info families	*P*	Risk allele	MAF	Info families	*P*	Risk allele	MAF	*P*	OR (95% c.i.)	Risk allele	MAF	*P*	OR (95% c.i.)	Risk allele
8	**T**	0.429	51	0.515		**0.388**	**80**	**0.013**	**C**	**0.399**	**0.002**	**1.84 (1.25–2.71)**	**C**	**0.392**	**0.021**	**1.46 (1.06–2.01)**	**C**
9	**A**	0.429	54	0.708		**0.379**	**78**	**0.017**	**G**	**0.397**	**0.0071**	**1.70 (1.15–2.50)**	**G**	**0.398**	**0.040**	**1.37 (1.01–1.86)**	**G**
10	**T**	0.429	55	0.565		**0.374**	**79**	**0.021**	**C**	**0.385**	**0.0051**	**1.74 (1.18–2.56)**	**C**	0.390	0.067	1.32 (0.98–1.77)	C
17	T	0.277	51	0.0057[a]	T	0.488	91	0.170		0.384	0.042	1.49 (1.01–2.18)	G	0.321	0.205	1.24 (0.89–1.72)	
19	**G**	**0.207**	**42**	**0.031**	**G**	0.245	84	0.617		0.236	0.499	1.16 (0.75–1.80)		**0.285**	**0.00082**	**1.79 (1.27–2.53)**	**G**
23	**T**	**0.264**	**53**	**0.0031[a]**	**T**	0.288	78	0.513		0.345	0.672	1.09 (0.74–1.61)		**0.125**	**0.00073**	**2.16 (1.37–3.40)**	**T**

After adjustment for multiple testing with an FDR level of 0.1, the cutoff *P* values for significant association are 0.024 in the north European family data set and 0.003 in the Hispanic family data set. MAF, minor allele frequency; OR, odds ratio; *P*, single-SNP χ^2 *P* value; Info families, number of informative families. **Supplementary Table 3** also shows allele and genotype frequencies. The alleles putatively associated with Alzheimer disease are depicted only for SNPs generating nominal *P* values ≤ 0.10. Separate confirmatory analyses were performed in four independent 'replication data sets' (north European and Israeli Arab case-control data sets, and MIRAGE Caucasian Americans and African-American sib pairs). Corrections for multiple testing were not applied in these replication analyses. Boldface indicates identical alleles that are nominally associated with disease in at least two independent data sets. The *P* values in the case-control cohort are for allelic association. Alleles putatively associated with Alzheimer disease are depicted only for those SNPs generating nominal *P* values < 0.10. Complete data are contained in **Supplementary Table 4**.
[a]Association was significant after correction for multiple testing.

sets (boldface in **Tables 4** and **5**) were the same as those associated with increased risk for Alzheimer disease in both the north European FAD data set and in the north European case-control data set (boldface in **Tables 2** and **3**). When we considered all of the Caucasian case-control samples together ($n = 1{,}583$ Alzheimer disease cases and 2,366 controls), the associations remained robust (single-SNP: $0.002 \leq P \leq 0.04$, with three SNPs giving $P < 0.008$). Notably, both the Mayo data set and the overall Caucasian case-control data set also detected association with SNP 4 ($P = 0.009$ and $P = 0.002$, respectively), a result not evident in the individual data sets.

Cell biology of SORL1

The SNPs and haplotypes identified here are unlikely to be the actual causal variants. We sequenced the exons and immediate intron-exon boundaries in carriers of the disease-associated haplotypes at SNPs 8–10 or SNPs 22–24, and we investigated *SORL1* splice forms recovered by RT-PCR. However, we did not identify any pathogenic sequence variants enriched in individuals with Alzheimer disease (**Supplementary Table 10** online). The possibility that the observed associations with SNPs inside *SORL1* might reflect pathogenic variants outside *SORL1* can be excluded because none of the SNPs flanking the 5′ and 3′ ends of *SORL1* showed association with Alzheimer disease. It is therefore likely that the observed associations with SNPs reflect the presence of pathogenic variants within the intronic sequences of *SORL1* near SNPs 8–10 and 22–24. We speculate that these putative intronic *SORL1* variants might modulate the cell type–specific transcription or translation of *SORL1* in carriers of the Alzheimer disease–associated haplotypes. This hypothesis would be supported by the recent observation of reduced expression of SORL1 in neurons but not glia of some individuals with sporadic Alzheimer disease[12].

Direct exploration of this hypothesis is difficult. First, the variations in SORL1 expression in Alzheimer disease brain have been cell type specific, with SORL1 expression depressed in neurons but not glia[12]. Second, there are only limited brain tissue samples from individuals where *SORL1* SNP marker phase (and thus haplotypes) are known. Nevertheless, tentative support for the hypothesis that Alzheimer disease–associated haplotypes in *SORL1* may be associated with reduced *SORL1* transcription is provided by quantitative real-time PCR studies of SORL1 expression in lymphoblasts from carriers of the CTT Alzheimer disease haplotype at SNPs 22–24. Sufficient numbers of samples were not available to test the effects of SNPs 8–10. These experiments demonstrated that *SORL1* was expressed in Alzheimer disease haplotype carriers at less than half the levels observed in obligate carriers of non–Alzheimer disease haplotypes (10,324 ± 8,215 arbitrary units in carriers versus 23,650 ± 17,999 in non-carriers (mean ± s.d.), normalized to β-actin mRNA; $P < 0.05$, two-tailed Mann-Whitney U-test; $n = 8$ independent samples; $n = 3$ replications). However, it is also of note that univariate regression analyses showed that *SORL1* haplotype status accounted for only ~14% of this variance ($P = 0.08$). This latter result implies that other genetic and nongenetic factors can also modulate SORL1 expression and, perhaps, therefore, risk for Alzheimer disease.

The observation that specific genetic variants in *SORL1* are associated with Alzheimer disease and that these same variants may be accompanied by reduced SORL1 expression is significant for the following reasons. First, these observations lead to the conclusion that the previously reported reductions in SORL1 expression in neurons in sporadic Alzheimer disease are likely to be causal rather than simply reactive. This notion is supported by that fact that *SORL1* expression is not altered in other types of Alzheimer disease with known etiology (for example, FAD with mutant *PSEN1*)[12,29]. Second,

Table 3 Haplotypes for all three-SNP windows that have a global *P* value for association with Alzheimer disease of $P \leq 0.05$ in at least one data set

						Discovery data sets									
						North European families					Caribbean Hispanic families				
SNP#			HAP			Hap frequency	Info families	*Z* score	Hap *P* value	Global sim *P*	Hap frequency	Info families	*Z* score	Hap *P* value	Global sim *P*
8	**9**	**10**	**C**	**G**	**C**	0.579	52	–0.392	0.695	0.152	**0.638**	**75**	**2.786**	**0.0053**	**0.0098**
8	9	10	T	A	T	0.395	52	0.893	0.372		0.317	72	–2.628	0.0086	
9	**10**	**11**	**G**	**C**	**C**	0.404	57	–1.259	0.208	0.553	0.217	62	–1.202	0.229	**0.022**
9	10	11	A	T	A	0.380	57	0.860	0.390		0.319	83	–2.006	0.045	
9	10	11	G	C	A	0.178	42	0.465	0.642		0.428	79	2.557	**0.011**	
22	**23**	**24**	**C**	**T**	**T**	**0.188**	**45**	**2.779**	**0.0054**	**0.018**	0.205	66	–0.103	0.918	0.554
22	23	24	C	A	T	0.003	2	*	*		0.008	3	*	*	
22	23	24	T	T	T	0.014	4	*	*		0.019	7	*	*	
23	24	25	A	C	T	0.735	58	–1.742	0.082	**0.041**	0.551	80	1.351	0.177	0.824
23	**24**	**25**	**T**	**T**	**C**	**0.196**	**47**	**1.878**	**0.060**		0.220	70	–0.811	0.417	
23	24	25	A	C	C	0.037	13	0.475	0.635		0.183	51	–0.608	0.543	
23	24	25	A	T	C	0.004	2	*	*		0.008	3	*	*	

						Replication data sets														
						Israeli Arab case-control					North European case-control					MIRAGE African-Americans Sibs				
SNP#			HAP			Control frequency	Case frequency	*Z* score	Hap *P* value	Global sim *P*	Control frequency	Case frequency	*Z* score	Hap *P* value	Global sim *P*	Hap frequency	Info families	*Z* score	Hap *P* value	Global sim *P*
8	**9**	**10**	**C**	**G**	**C**	**0.539**	**0.661**	**2.633**	**0.0085**	**0.023**	**0.566**	**0.638**	**2.001**	**0.045**	0.154	0.473	50	–0.439	0.661	0.727
8	9	10	T	A	T	0.434	0.301	–2.901	0.0037		0.417	0.351	–1.826	0.068		0.076	26	0.510	0.610	
9	**10**	**11**	**G**	**C**	**C**	**0.282**	**0.407**	**2.828**	**0.0047**	**0.0080**	**0.385**	**0.455**	**1.972**	**0.049**	0.223	0.071	23	–0.550	0.582	0.682
9	10	11	A	T	A	0.410	0.247	–3.390	0.0007		0.393	0.323	–1.904	0.057		0.112	23	0.280	0.780	
9	10	11	G	C	A	0.256	0.251	–0.258	0.796		0.184	0.191	0.337	0.736		0.399	55	0.148	0.883	
22	**23**	**24**	**C**	**T**	**T**	0.250	0.227	–0.579	0.563	0.286	**0.069**	**0.134**	**2.795**	**0.0052**	**0.00065**	0.035	11	0.589	0.556	0.314
22	23	24	C	A	T	*	*	*	*		0.170	0.082	–3.096	**0.0020**		0.001	1	*	*	
22	23	24	T	T	T	0.015	0.041	1.387	0.165		0.011	0.033	2.272	**0.023**		0.007	3	*	*	
23	24	25	A	C	T	0.535	0.563	0.668	0.504	0.547	0.667	0.674	0.129	0.897	**0.00035**	**0.513**	**44**	**3.029**	**0.0025**	**0.0043**
23	**24**	**25**	**T**	**T**	**C**	0.265	0.259	–0.135	0.892		**0.085**	**0.167**	**3.268**	**0.0011**		0.047	18	–0.252	0.801	
23	24	25	A	C	C	0.103	0.103	–0.057	0.955		0.066	0.046	–1.080	0.280		0.403	53	–2.852	0.0044	
23	24	25	A	T	C	*	*	*	*		0.168	0.097	–2.508	**0.012**		0.003	1	*	*	

All *P* values of $P \leq 0.05$ are in boldface. Haplotypes that show increased risk for Alzheimer disease in at least two independent data sets are shown in boldface. Haplotypes that show reduced risk in at least two independent data sets are underlined. Complete data for all SNPs is contained in **Supplementary Table 5**. Hap *P* value, haplotype *P* value (Haplo.stats v1.1.1); Global sim *P*, permutation-based global haplotypic *P* value reported by FBAT (hbat -p option) or Haplo.stats; *, sample size too small to generate meaningful result; Info families, number of informative families.

Table 4 Single-SNP association results for independent replication dataset from Mayo Clinic

		Mayo Jacksonville series				Mayo Rochester series				AUT series			
SNP#	Minor allele	MAF	Risk allele	OR (95% c.i.)	*P*	MAF	Risk allele	OR (95% c.i.)	*P*	MAF	Risk allele	OR (95% c.i.)	*P*
4	**C**	0.418		1.10 (0.92–1.31)	0.306	0.455		1.05 (0.90–1.23)	0.504	0.424		1.19 (0.98–1.45)	0.076
5	C	0.413		0.99 (0.83–1.18)	0.876	0.397		1.08 (0.92–1.26)	0.373	0.397		1.11 (0.91–1.35)	0.301
8	T	0.431		1.13 (0.94–1.34)	0.187	0.440		0.97 (0.83–1.13)	0.692	0.431		1.20 (0.98–1.45)	0.071
9	A	0.436		1.12 (0.94–1.34)	0.202	0.443		0.96 (0.82–1.13)	0.640	0.448		1.10 (0.97–1.34)	0.313
12	**T**	0.044		1.14 (0.74–1.75)	0.548	0.050		1.10 (0.77–1.55)	0.606	**0.052**	**T**	**1.98 (1.26–3.12)**	**0.003**
19	**G**	0.224		1.14 (0.93–1.41)	0.210	0.242		1.19 (1.00–1.42)	0.055	0.230		1.15 (0.92–1.44)	0.225
22	C	0.321		1.20 (1.00–1.45)	0.052	0.349		1.07 (0.91–1.26)	0.413	0.331		1.13 (0.92–1.39)	0.227
23	**T**	**0.296**	**T**	**1.31 (1.08–1.59)**	**0.006**	0.321		1.09 (0.93–1.29)	0.287	0.302		1.12 (0.91–1.37)	0.294
24	**T**	**0.278**	**T**	**1.31 (1.08–1.59)**	**0.007**	0.299		1.06 (0.89–1.25)	0.513	0.281		1.15 (0.93–1.42)	0.199
25	**C**	**0.333**	**C**	**1.35 (1.12–1.63)**	**0.001**	0.355		1.03 (0.88–1.21)	0.712	0.340		1.15 (0.94–1.41)	0.163

		Combined Mayo series				Combined Caucasian data sets			
SNP#	Minor allele	MAF	Risk allele	OR (95% c.i.)	*P*	MAF	Risk allele	OR (95% c.i.)	*P*
4	**C**	**0.437**	**T**	**1.14 (1.03–1.25)**	**0.009**	**0.431**	**T**	**1.16 (1.05–1.27)**	**0.002**
5	C	0.402		1.06 (0.96–1.17)	0.227	0.403		1.09 (0.99–1.19)	0.081
8	T	0.436		1.08 (0.98–1.19)	0.113	**0.432**	**C**	**1.11 (1.01–1.22)**	**0.027**
9	A	0.442		1.05 (0.95–1.16)	0.322	0.438		1.08 (0.98–1.18)	0.109
12	**T**	**0.049**	**T**	**1.25 (1.00–1.56)**	**0.046**	0.049		1.20 (0.97–1.48)	0.087
19	**G**	**0.234**	**G**	**1.13 (1.01–1.26)**	**0.038**	**0.238**	**G**	**1.18 (1.06–1.31)**	**0.0023**
22	C	0.336		1.09 (0.98–1.20)	0.108	0.334		1.08 (0.98–1.19)	0.119
23	**T**	**0.309**	**T**	**1.12 (1.01–1.24)**	**0.031**	**0.292**	**T**	**1.15 (1.04–1.27)**	**0.0075**
24	**T**	**0.289**	**T**	**1.12 (1.00–1.24)**	**0.042**	**0.286**	**T**	**1.11 (1.00–1.23)**	**0.040**
25	**C**	**0.345**	**C**	**1.12 (1.01–1.24)**	**0.026**	**0.343**	**C**	**1.11 (1.01–1.22)**	**0.033**

Independent confirmation of the association of Alzheimer disease with *SORL1* in Caucasians was obtained by genotyping 10 SNPs (4, 5, 8, 9, 12, 19, and 22–25) in three additional series of American Caucasians from the Mayo Clinic[27,28]. For single-SNP tests, the χ^2 test was used, and corrections for multiple testing were not applied in these directed replication analyses. SNPs that show increased risk for Alzheimer disease are in boldface. The same alleles were associated with Alzheimer disease in the Mayo Clinic data sets and in the north European case-control data set.

these observations raise the question of how changes in SORL1 expression or function might affect risk for Alzheimer disease. To explore this question, we undertook cell biological experiments, demonstrating that SORL1 directly binds APP and differentially regulates its sorting into endocytic or recycling pathways (**Fig. 1a**).

Coimmunoprecipitation experiments in native HEK cells demonstrated that endogenous SORL1 physically interacts with the endogenous APP holoprotein (**Fig. 2**) and with VPS35 (which drives cargo selection in the retromer via VPS10-containing proteins like SORL1 (ref. 30 and data not shown). SORL1, however, does not bind to APP C-terminal fragments produced by α-, β- or γ-secretase cleavage (**Fig. 2**). These protein-protein interactions are specific because SORL1 does not bind to other type 1 membrane proteins (for example, BACE1 (ref. 31) and **Fig. 3**) or to VPS26 (which links VPS35 to the other structural elements of the retromer (ref. 30 and data not shown).

The interaction between SORL1, VPS35 and APP holoprotein provides a mechanism by which SORL1 can regulate differential sorting of APP into the retromer recycling pathway or into the late endosomal pathway (where APP undergoes β- and γ-secretase cleavage to generate Aβ). In agreement with this hypothesis, overexpression of SORL1, which would be predicted to divert APP holoprotein into the retromer recycling pathway, results in decreased Aβ production (82% of control, $P < 0.05$, $n = 5$ replications; **Fig. 3a**). Conversely, short interfering RNA (siRNA) suppression of SORL1 expression, which we speculate might mimic the effects of Alzheimer disease–associated variants in *SORL1*, results in deflection of APP holoprotein away from the retromer recycling pathway and into the late endosome-lysosome pathway. As would be predicted, siRNA suppression of SORL1 leads to (i) overproduction of the soluble N-terminal ectodomain of APP (APPsβ) generated by BACE1 cleavage of APP holoprotein (149.45% ± 9.66 of control (mean ± s.e.m.), $P < 0.0001$, $n = 5$ replications; **Fig. 3c**) and (ii) overproduction of Aβ by the subsequent γ-secretase cleavage of the APP C-terminal stub generated by BACE1 (Aβ40, 189% of control; Aβ42, 202% of control, $P < 0.001$; three independent siRNA oligonucleotides with five replications each; **Fig. 3b**). Our conclusion that SORL1 regulates sorting of APP into the retromer-recycling pathway is supported by the observation of identical effects after suppression of the retromer proteins VPS26A (Aβ40, 186% of control value; Aβ42, 183% of control value, $P < 0.001$, $n = 5$ replications; **Fig. 3d**) or VPS35 (ref. 13). These results and conclusions are in very good agreement with independent reports that appeared during preparation of this manuscript[14,15].

DISCUSSION

Taken together, our results suggest that genetic and possibly environmentally specified changes in SORL1 expression or function are causally linked to the pathogenesis of Alzheimer disease and have a modest effect on risk for this disease. The precise identity of the genetic effectors in *SORL1* remains to be determined. However, the results described here imply that (i) there are several different Alzheimer disease–associated allelic variants in distinct regions of the *SORL1* gene in different populations; (ii) these variants are

Table 5 Haplotype results for three-SNP windows for SNPs 22–25 in Mayo Clinic replication data sets

						Mayo Jacksonville series					Mayo Rochester series					AUT series				
SNP#			HAP			Control freq	Case freq	*Z* score	Hap *P* value	Global sim *P*	Control freq	Case freq	*Z* score	Hap *P* value	Global sim *P*	Control freq	Case freq	*Z* score	Hap *P* value	Global sim *P*
22	**23**	**24**	**C**	**T**	**T**	**0.235**	**0.285**	**2.641**	**0.0083**	**0.015**	0.279	0.296	0.926	0.354	0.220	0.248	0.271	1.051	0.293	0.435
22	23	24	T	A	C	0.690	0.644	–2.248	0.025		0.641	0.624	–0.920	0.358		0.666	0.628	–1.566	0.117	
22	23	24	C	A	C	0.039	0.034	–0.732	0.464		0.041	0.036	–0.644	0.520		0.038	0.050	1.146	0.252	
22	23	24	C	T	C	0.021	0.019	–0.390	0.697		0.022	0.026	0.745	0.456		0.028	0.022	–0.731	0.465	
22	23	24	T	T	T	0.008	0.017	1.708	0.088		0.014	0.010	–0.926	0.354		0.015	0.018	0.627	0.530	
22	23	24	C	A	T	*	*	*	*		*	*	*	*		*	*	*	*	
23	**24**	**25**	**T**	**T**	**C**	**0.242**	**0.300**	**2.959**	**0.0031**	**0.0070**	0.293	0.305	0.681	0.496	0.386	0.263	0.288	1.115	0.265	0.432
23	24	25	A	C	T	0.700	0.631	–3.261	0.0011		0.646	0.629	–0.913	0.362		0.668	0.633	–1.445	0.148	
23	24	25	A	C	C	0.029	0.046	1.911	0.056		0.036	0.031	–0.752	0.452		0.036	0.046	0.936	0.349	
23	24	25	T	C	C	0.022	0.017	–0.767	0.443		0.019	0.026	1.121	0.262		0.022	0.021	–0.208	0.835	
23	24	25	A	T	C	*	*	*	*		*	*	*	*		*	*	*	*	

						Combined Mayo series					Combined Caucasian data sets				
SNP#			HAP			Control freq	Case freq	*Z* score	Hap *P* value	Global sim *P*	Control freq	Case freq	*Z* score	Hap *P* value	Global sim *P*
22	**23**	**24**	**C**	**T**	**T**	**0.263**	**0.284**	**1.964**	**0.050**	**0.332**	**0.247**	**0.270**	**2.258**	**0.024**	**0.0051**
22	23	24	T	A	C	0.657	0.633	–2.063	0.039		0.658	0.635	–2.101	0.036	
22	23	24	C	A	C	0.040	0.039	–0.178	0.859		0.042	0.043	0.005	0.996	
22	23	24	C	T	C	0.023	0.022	–0.171	0.864		0.022	0.022	0.004	0.997	
22	23	24	T	T	T	0.013	0.015	0.846	0.397		0.013	0.017	1.538	0.124	
22	23	24	C	A	T	*	*	*	*		**0.017**	**0.010**	**–2.315**	**0.021**	
23	**24**	**25**	**T**	**T**	**C**	**0.275**	**0.298**	**2.039**	**0.041**	**0.099**	**0.259**	**0.285**	**2.504**	**0.012**	**0.017**
23	24	25	A	C	T	0.662	0.631	–2.670	0.0076		0.663	0.636	–2.443	0.015	
23	24	25	A	C	C	0.035	0.041	1.361	0.174		0.037	0.042	0.873	0.383	
23	24	25	T	C	C	0.020	0.021	0.118	0.906		0.019	0.020	0.341	0.733	
23	24	25	A	T	C	*	*	*	*		0.017	0.011	–2.208	**0.027**	

Although the Mayo Clinic data sets and north European data sets have slightly different haplotype structures, the CTT haplotype at SNP 22–24 and the overlapping TTC haplotype at SNPs 23–25 (in boldface) show increased risk for Alzheimer disease in the Mayo Clinic and north European Caucasians (in boldface in **Table 3**). Haplotypes with reduced risk of Alzheimer disease are underlined but are different from those in the north European data sets, suggesting the potential existence of several protective alleles in this region. MAF, minor allele frequency; OR, odds ratio; *P*, single-SNP χ^2 *P* value; Hap *P* value, haplotype *P* value (Haplo.stats v1.1.1); Global sim *P*, permutation-based global haplotypic *P* value reported by FBAT (hbat -p option) or Haplo.stats; *, sample size too small to generate meaningful result.

likely to be in intronic regulatory sequences that might govern cell type–specific or tissue-specific expression of *SORL1* and (iii) these variants affect this risk by altering the physiological role of SORL1 in the processing of APP holoprotein.

In sharp contrast to *APOE* (where *APOE* ε4 is associated with Alzheimer disease in most data sets[32]), no single *SORL1* SNP or haplotype is associated with increased risk for Alzheimer disease in all six data sets, and some data sets fail to show any association with *SORL1*. However, four points mitigate concerns that the association between *SORL1* and Alzheimer disease is spurious. First, the association was initially identified using conservative family-based association tests, which are less sensitive to confounding due to population stratification[33]. Second, at each set of SNP clusters, the same alleles and haplotypes were associated with increased risk for Alzheimer disease in at least three unrelated data sets, some of which were drawn from different ancestral origins. Third, the discovery of association with different SNPs in different populations does not indicate a spurious result. The association of disease with a single allele in all data sets (that is, an *APOE* ε4–like association) is not a universal observation for either complex or monogenic diseases[17]. Thus, the

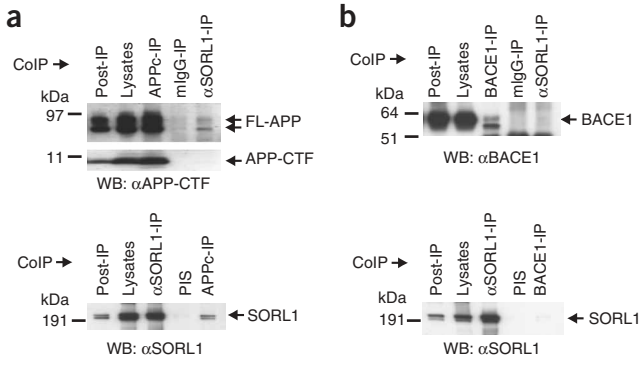

Figure 2 SORL1 specifically interacts with APP holoprotein but not with its proteolytic derivatives. (**a**) Small quantities of endogenous APP holoprotein can be immunoprecipitated with endogenous SORL1, but APP C-terminal fragments cannot (APP-CTFs, generated by α- or β-secretase) (top). Conversely, small quantities of endogenous SORL1 can be coprecipitated with endogenous APP holoprotein (bottom). (**b**) SORL1 does not interact with BACE1 (β-secretase). Coimmunoprecipitations (coIP) with antibodies to overexpressed BACE1-V5 do not capture SORL1 (bottom). Conversely, SORL1-directed antibodies do not coimmunoprecipitate BACE1 (top) even though BACE1 also traffics through the endosome to the Golgi pathway.

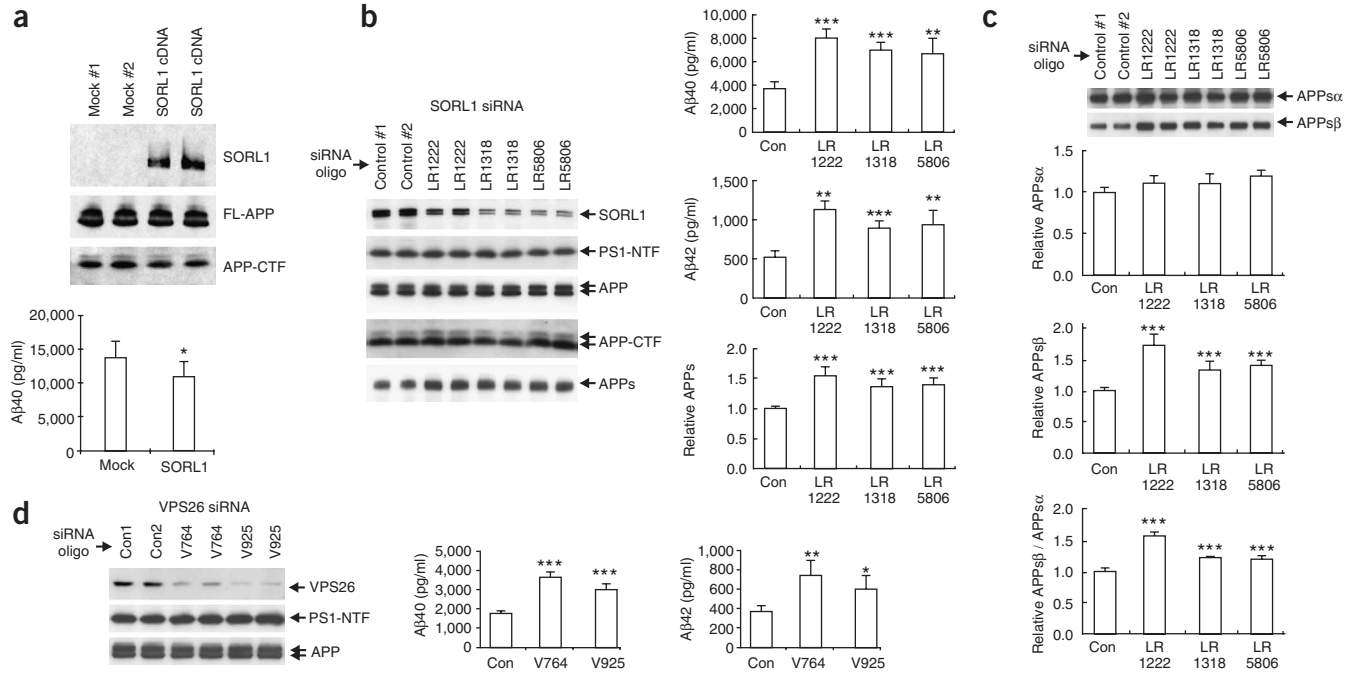

Figure 3 SORL1 modulates APP trafficking into the endocytic β- and γ-secretase proteolytic pathways. (**a**) Overexpression of SORL1 reduces Aβ40 (and Aβ42; data not shown) secretion ($P < 0.05$). Top, representative protein blot for SORL1 and APP in HEK293 cells stably expressing APP_{Swe} and transiently transfected with empty vector (mock) or SORL1 ($n = 2$ independent transfections). Bottom, ELISA assays of secreted Aβ40 (and Aβ42; data not shown) after SORL1 overexpression. Error bars, s.d. $^*P < 0.05$ ($n = 2$ replications). FL-APP, full-length APP. (**b**) Suppression of SORL1 expression with three independent siRNA primers (LR1222, LR1318, and LR5806) did not alter the expression levels or maturation of APP, APP-C83 CTFs or presenilin 1 (left) but significantly increased Aβ40, Aβ42 and APPs secretion (right; $^{**}P < 0.005$, $^{***}P < 0.001$, $n = 5$ replications, three siRNA oligomers). (**c**) Anti-SORL1 siRNA significantly increases APPsβ in the medium but does not affect APPsα. Top, protein blots of conditioned medium from cells treated with nonsense siRNA oligonucleotides (control #1 and control #2) or with anti-SORL1 siRNA oligonucleotides probed with 2H3 antibody to APPsα or SW192 antibody to APPsβ ($n = 5$ replications). Bar graphs are normalized to controls. $^{**}P < 0.0001$, $n = 5$ replications. (**d**) Suppression of VPS26 did not alter APP or presenilin 1 maturation (center and lower panels) but did increase Aβ40 and Aβ42 secretion (right panel; $^*P < 0.005$, $^{**}P < 0.001$, $n = 5$ replications, two siRNA oligomers). The control primer had no such effect. All statistical significance values were obtained with two-tailed *t*-tests.

occurrence of pathogenic mutations across multiple domains of disease genes (allelic heterogeneity) and the absence of these variants in some data sets (locus heterogeneity) are frequently observed in both monogenic and complex traits[34,35]. Fourth, the absence of significant associations in two data sets (MIRAGE Caucasian sibships and the Mayo Rochester data set) does not negate the findings from the other data sets. There are several potential explanations for the failure to detect a significant association in these two data sets. These potential explanations include (i) insufficient power to reliably detect the association in all series; (ii) locus heterogeneity (that is, non-*SORL1* causes might have been overrepresented and *SORL1*-associated causes underrepresented in some data sets) or (iii) allelic heterogeneity (that is, the association may have been obscured if the biologically active *SORL1* alleles had occurred on multiple SNP backgrounds in some data sets). The probable existence of allelic heterogeneity has important implications for replication studies. Such studies will need to assess a battery of SNPs focused on data sets with as homogeneous a genetic background as possible.

Our results also resolve the conundrum concerning the significance of reduced expression of SORL1 and several other genes potentially involved in APP trafficking in brain tissue from individuals with Alzheimer disease. Our results argue that the reduction in *SORL1* expression in affected brain tissue is likely to be a primary and pathogenic event, whereas the reduction in VPS35 expression is likely to be a secondary event.

Finally, our data demonstrate that SORL1 has a key physiological role in the differential sorting of APP holoprotein. In the presence of SORL1, APP holoprotein is recovered via the retromer. In the absence of SORL1, APP is released into late endosomal pathways, where it is subjected to β-secretase cleavage, and subsequently γ-secretase cleavage, which generate Aβ (**Fig. 1a**).

METHODS

Subjects. Informed consent was obtained from all participants using procedures approved by institutional review boards at each of the clinical research centers collecting human subjects. The clinical diagnosis of 'probable' or 'possible' Alzheimer disease was defined according to the National Institute of Neurological and Communication Disorders and Stroke–Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) diagnosis criteria at clinics specializing in memory disorders. Clinical characteristics of the north European, MIRAGE, Caribbean Hispanic FAD, Israeli Arab and Mayo Caucasian American data sets are summarized in **Supplementary Table 1** (refs. 19,20,22–25,27,28). The north European case-control set is drawn from the same populations as the north European FAD data set[20,22]. The three Mayo data sets were drawn from Caucasian affected individuals and controls assessed in clinical series at the Rochester and Jacksonville Mayo Clinics or from Caucasian brains in which the presence or absence of Alzheimer disease was determined neuropathologically by autopsy ('AUT' in **Supplementary Table 1**).

Genetic analyses. Genotyping was performed using the GenomeLab SNPstream System, and primer sets were as in **Supplementary Table 3**

(Beckman Coulter). We genotyped 100 DNA samples twice for every SNP marker (the concordance rate was >99%). APOE was genotyped as described[5]. Genotyping of the Mayo samples was performed on an ABI 7900 instrument using TaqMan chemistry with primers and probes designed by Applied Biosystems. The entire ORF of the *SORL1* gene was sequenced in 12 individuals with sporadic Alzheimer disease, 12 individuals with familial Alzheimer disease and two normal controls selected from the north European and Caribbean Hispanic data sets (**Supplementary Tables 3** and **10**).

Alternatively spliced transcripts were sought by conventional RT-PCR in eight overlapping fragments using total RNA isolated from frontal cortex (16 normal controls and 17 individuals with sporadic Alzheimer disease from the Canadian Brain Tissue Bank and the New York Brain Bank; **Supplementary Table 3**).

Statistical analyses. SNP marker data were assessed for deviations from Hardy-Weinberg equilibrium (using Pedstats software) and for mendelian inheritance errors (using Pedcheck software). Single-point family-based association was assessed with FBAT v1.5.5 (ref. 36), using an additive genetic model with the null hypothesis of no linkage and no association. Allele frequencies were estimated by FBAT using the EM algorithm. APOE ε4 carrier status was included in the analyses using PBAT v2.6 (refs. 37–40). The χ^2 test (or the Fisher's exact test) was used to assess genotypic and allelic associations between Alzheimer disease. Multivariate logistic regression analysis was performed to adjust for APOE ε4, sex and age-at-onset or age-at-examination.

Statistical significance and multiple testing corrections. The Benjamini-corrected false discovery rate (FDR)[41] was used with a cutoff level of 0.1 to correct for multiple testing. The *P* values presented are nominal *P* values. The cutoff *P* values for significance in each data set are shown in the table legends.

Linkage disequilibrium. LD structure was examined using Haploview. Haplotype blocks were defined using the confidence intervals algorithm. The default settings were used in these analyses, which create 95% confidence bounds on D' to define SNP pairs in strong LD.

Haplotype analyses. Haplotype analyses were carried out with a sliding window of three contiguous SNPs using FBAT for family data and Haplo.stats v1.1.1 for case-control data[16,26,42–44]. The analyses were repeated using sliding windows of two, four, five and six SNPs.

Expression plasmids and cDNA constructs for human SORL1. The cDNA clones encoding APP K670N/M671L Swedish mutation (APP_{Swe}) and BACE1 (V5-tagged at the C terminus) were as described previously[45,46].

Cell culture and transfection. The HEK293 cell line stably expressing APP_{Swe} was as described[47]. Transient transfection of BACE1 cDNA was performed using LipofectAMINE 2000 (Invitrogen).

RNA interference. siRNA oligonucleotides were designed using the online siRNA Design Tool (Dharmacon Research). The siRNAs for SORL1 are in **Supplementary Table 3**. The siCONTROL Non-Targeting siRNAs #1 and #2 (Dharmacon Research) were used as a negative control.

Transfections were performed using LipofectAMINE 2000 according to the manufacturer's recommendations. In case of consecutive transfections, cells were split after 24 h and then retransfected 24 h later. After culturing for an additional 24 h, the conditioned medium was collected for the Aβ assay, and the cells were harvested for protein blotting.

Antibodies, immunoprecipitation and protein blotting. Antibodies were as follows: mouse monoclonal anti-human LR11/SORL1gp250 (BD Transduction Laboratories) and 5-4-30-19-2 (from H.B.); rabbit antibody to the C terminus of SORL1 (from W.H.); rabbit polyclonal antibody to PS1-NTF (Ab14, from S. Gandy, Temple University); mouse monoclonal anti-myc (Invitrogen); rabbit polyclonal antibody to the C terminus of APP (Sigma) and anti-BACE1 (EE-17, Sigma). Proteins were immunoprecipitated in 1% digitonin[48], subjected to protein blot and visualized by ECL (Amersham).

Aβ, APPsα and APPsβ assays. Aβ40 and Aβ42 peptide levels were measured by sandwich ELISA[49]. APPs, APPsα and APPsβ were measured by protein blotting using antibodies 22C11 (Chemicon), 2H3 and SW192 (Elan Pharmaceuticals), respectively. Differences were assessed by two-tailed Student's *t*-test.

Quantitative RT-PCR. PCR primer pairs targeting SORL1 exon 23 were as in **Supplementary Table 3**. Total RNA (5 μg) was reverse transcribed using a random hexamer. Real-time PCR was performed in a 384-well format using an ABI Prism 7900HT instrument and the Sybr Green detection method. Samples were analyzed in triplicate, and mean expression levels corresponding to *SORL1* mRNA expression were normalized to β-actin mRNA levels.

URLs. Haploview is available at http://www.broad.mit.edu/mpg/haploview/index.php.

Note: Supplementary information is available on the Nature Genetics website.

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AUTHOR CONTRIBUTIONS

The authors' roles are described in **Supplementary Table 11** online.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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