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S. Michal Jazwinski
Tulane University School of Medicine

Sangkyu Kim
Tulane University School of Medicine

Jianliang Dai
Tulane University School of Medicine

Li Li
LSU Health Sciences Center - New Orleans

Xiuhua Bi
Tulane University School of Medicine

See next page for additional authors

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HRAS1 and *LASS1* with *APOE* are associated with human longevity and healthy aging

S. Michal Jazwinski,[1] Sangkyu Kim,[1,*] Jianliang Dai,[1,2,*] Li Li,[3] Xiuhua Bi,[1] James C. Jiang,[1] Jonathan Arnold,[2] Mark A. Batzer,[4] Jerilyn A. Walker,[4] David A. Welsh,[5] Christina M. Lefante,[6] Julia Volaufova,[6] Leann Myers,[7] L. Joseph Su,[6,†] Dorothy B. Hausman,[8] Michael V. Miceli,[1] Eric Ravussin,[9] Leonard W. Poon,[10] Katie E. Cherry[11] and Michael A. Welsch[12] for the Georgia Centenarian Study and the Louisiana Healthy Aging Study

[1]Tulane Center for Aging and Department of Medicine, Tulane University Health Sciences Center, New Orleans, LA 70112, USA
[2]Genetics Department, University of Georgia, Athens, GA 30602, USA
[3]Neuroscience Center, Louisiana State University Health Sciences Center, New Orleans, LA 70112, USA
[4]Department of Biological Sciences, Louisiana State University, Baton Rouge, LA 70803, USA
[5]Department of Medicine, Louisiana State University Health Sciences Center, New Orleans, LA 70112, USA
[6]School of Public Health, Louisiana State University Health Sciences Center, New Orleans, LA 70112, USA
[7]Department of Biostatistics, School of Public Health and Tropical Medicine, Tulane University Health Sciences Center, New Orleans, LA 70112, USA
[8]Department of Foods and Nutrition, University of Georgia, Athens, GA 30602, USA
[9]Pennington Biomedical Research Center, Baton Rouge, LA 70808, USA
[10]Institute of Gerontology, University of Georgia, Athens, GA 30602, USA
[11]Department of Psychology, Louisiana State University, Baton Rouge, LA 70803, USA
[12]Department of Kinesiology, Louisiana State University, Baton Rouge, LA 70803, USA

Summary

The search for longevity-determining genes in human has largely neglected the operation of genetic interactions. We have identified a novel combination of common variants of three genes that has a marked association with human lifespan and healthy aging. Subjects were recruited and stratified according to their genetically inferred ethnic affiliation to account for population structure. Haplotype analysis was performed in three candidate genes, and the haplotype combinations were tested for association with exceptional longevity. An *HRAS1* haplotype enhanced the effect of an *APOE* haplotype on exceptional survival, and a *LASS1* haplotype further augmented its magnitude. These results were replicated in a second population. A profile of healthy aging was developed using a deficit accumulation index, which showed that this combination of gene variants is associated with healthy aging. The variation in *LASS1* is functional, causing enhanced expression of the gene, and it contributes to healthy aging and greater survival in the tenth decade of life. Thus, rare gene variants need not be invoked to explain complex traits such as aging; instead rare congruence of common gene variants readily fulfills this role. The interaction between the three genes described here suggests new models for cellular and molecular mechanisms underlying exceptional survival and healthy aging that involve lipotoxicity.

Key words: haplotypes; healthy aging profile; lipotoxicity; longevity genes; population stratification.

Introduction

Exceptional longevity has genetic and environmental components, but in humans they have often been difficult to identify (Christensen *et al.*, 2006). Most of the research on the genetics of human aging has focused on the genetic risk factors associated with age-related diseases and disorders, with *APOE* being one prime candidate (Christensen *et al.*, 2006). This is not surprising given the impact of variation in this gene on cardiovascular and neuropathological aspects of aging (Strittmatter *et al.*, 1993; Schächter *et al.*, 1994; Song *et al.*, 2004; Christensen *et al.*, 2006). Studies in model systems have also recently guided the search for genetic determinants of human longevity, pointing to several pathways and processes contributing to enhanced lifespan (Jazwinski, 1996; Finch & Ruvkun, 2001; Kenyon, 2005). However, this approach has largely been limited to components of the insulin/IGF-1 pathway (Hong *et al.*, 2008; Suh *et al.*, 2008; Willcox *et al.*, 2008). We decided to expand on this approach by examining the Ras and ceramide signaling pathways. The involvement of Ras signaling in lifespan determination has been adduced in studies in yeast (Chen *et al.*, 1990; Sun *et al.*, 1994) and in mice (Migliaccio *et al.*, 1999), while the role of ceramide signaling emerges from research in yeast (D'mello *et al.*, 1994; Jazwinski & Conzelmann, 2002; Guillas *et al.*, 2003) and in *Caenorhabditis*

Correspondence
S. Michal Jazwinski, PhD, Department of Medicine, Tulane University Health Sciences Center, 1430 Tulane Ave., SL-12, New Orleans, LA 70112, USA. Tel.: 504 988 8253; fax: 504 988 8835; e-mail: sjazwins@tulane.edu
*Contributed equally.
†Present address: Modifiable Risk Factors Branch, National Cancer Institute, Rockville, MD 20892, USA.

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elegans (Tedesco *et al.*, 2008; Mehta *et al.*, 2009; Menuz *et al.*, 2009).

The *APOE ε4* allele is a risk factor for early mortality, while the alleles *ε2* and *ε3* are enriched in exceptionally old individuals in some studies (Christensen *et al.*, 2006). Apolipoprotein E (APOE) is important in chylomicron and very low density lipoprotein (VLDL) metabolism (Mahley & Rall, 2000). Thus, it is one of the factors responsible for maintenance of circulating lipid homeostasis. This balance can break down with age, and it is clearly disrupted in some individuals resulting in an increase in the risk for cardiovascular disease and other disorders (Mahley & Rall, 2000). The rise in circulating cholesterol and triglycerides results in vascular damage and can be a cause of lipotoxicity, a potential trigger of insulin resistance and diabetes (Kusminski *et al.*, 2009). This lipotoxicity may trigger responses that are mediated by HRAS1, a small G-protein involved in signal transduction (Ramos, 1999). Furthermore, some of the pathways that are induced are modulated by ceramide, a product of the ceramide synthase LASS1 (Jazwinski & Conzelmann, 2002; Ogretmen & Hannun, 2004). Ceramide synthase responds to growth and to stress signals via Tor complex TORC2 and calcineurin, respectively (Dickson, 2008). Lipotoxicity stimulates ceramide synthesis (Kusminski *et al.*, 2009), and the resulting ceramide can signal apoptosis, resulting in the removal of damaged cells (Koybasi *et al.*, 2004). It can also facilitate the transfer of cholesterol from cells to high density lipoprotein (HDL) (Witting *et al.*, 2003). Therefore, we postulate that the *HRAS1* and *LASS1* genes interact genetically with the *APOE* gene to reduce age-related increase in lipotoxic events, and that a combination of variants or haplotypes in these three genes is associated with exceptional survival.

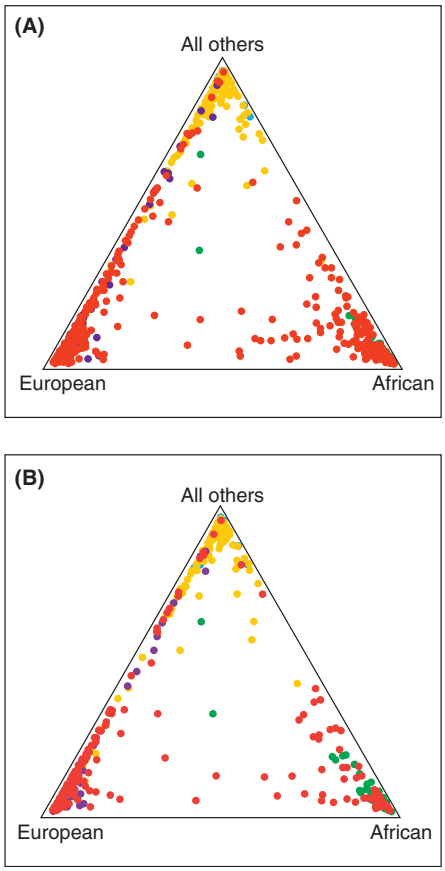

Fig. 1 Stratification of the populations by ethnic affiliation. Alu genotypes and Structure analysis were employed to assign subjects to two major strata, European and African, in the Georgia (A) and Louisiana (B) populations. The Structure analysis was carried out in two batches for Louisiana consisting of 434 and 435 samples to speed computation, the latter of which is shown, while all 650 samples from Georgia were analyzed in one batch. Dots: red (Georgia or Louisiana samples), green (African control samples), purple (European control samples), light blue (Asian control samples), orange (Indian control samples). Indian refers to the Indian subcontinent. Not all samples are evident owing to superposition of dots.

Results

Gene combination associated with exceptional longevity

We have initiated a genetic analysis of exceptional longevity in the Georgia and Louisiana populations. Our attention in this study was exclusively directed to *APOE*, *HRAS1*, and *LASS1* in a candidate gene approach. As noted earlier, variants of *APOE* are associated with longevity. This pattern is also found in the Georgia and Louisiana populations, for centenarians and nonagenarians respectively (Table S1). Both the Georgia and Louisiana populations are admixed with the two major strata being of European and African origin. To address this problem, we stratified each population by ethnic affiliation using an identical-by-descent (IBD) genetic system (Fig. 1). We placed the subjects in each population into two major groups of African and European origin. Together, these two groups constituted 81% and 83% of subjects of 20–59 years old and 72% and 77% of centenarians and nonagenarians in the Georgia and Louisiana samples, respectively. The ability to stratify is markedly enhanced by the Alu system used here, which can trace genealogy within the primate lineage. The mean correct prediction rates with the 100 Alu used here are 95–99% (Bamshad *et al.*, 2003). After stratification the association of *APOE* with longevity persisted (Table S2). However, no such association was detected for *HRAS1* or *LASS1* here (Table S3).

To test the hypothesis that the genetic interaction postulated above is associated with longevity, we genotyped the Georgia sample at the three relevant genomic regions. None of the 15 SNP examined showed significance for departure from Hardy–Weinberg proportions in either the African or European-origin populations in Georgia. (The same was found separately for the Louisiana population.) We searched for combinations of haplotypes in the three genes (15 SNP) in the Georgia population present at $\sim$ 0.01 or greater frequency, using expectation maximization. We then tested them for association with exceptional longevity (Table S4). Such associations were detected in the subpopulation of European origin in Georgia, stratified with an assignment probability of 0.9 (Table 1), but not in the subpopulation of African origin. They involve the *ε3* containing *APOE*

Table 1 Association of haplotypes with exceptional longevity and healthy aging

Population	Haplotype	Case (*N*; frequency) Control (*N*; frequency)	OR (CI)	Exact *P* (adjusted *P*)
Georgia	*APOE·HRAS1·LASS1* (CCT)	≥ 98 years old (147; 0.073) 20–59 years old (188; 0.010)	7.68 (2.55–23.10)	4.1×10^{-5} (2.88×10^{-4})
Georgia	*APOE·HRAS1·LASS1* (CGT)	≥ 98 years old (147; 0.191) 20–59 years old (188; 0.084)	2.56 (1.61–4.08)	7.8×10^{-5} (4.66×10^{-4})
Louisiana	*APOE·HRAS1·LASS1* (CCT)	≥ 90 years old (204; 0.105) 20–59 years old (251; 0.039)	2.93 (1.68–5.09)	9.5×10^{-5} (5.68×10^{-4})
Louisiana	*APOE·HRAS1·LASS1* (CGT)	≥ 90 years old (204; 0.175) 20–59 years old (251; 0.067)	2.97 (1.92–4.58)	6.91×10^{-7} (5×10^{-6})
Georgia	*APOE·HRAS1·LASS1* (CCT)	≥ 98 -year-old female (128; 0.099) 20–59 -year-old female (115; 0.009)	12.52 (2.93–53.57)	1×10^{-5} (2×10^{-5})
Georgia	*APOE·HRAS1·LASS1* (CGT)	≥ 98 -year-old male (19; 0.251) 20–59 -year-old male (73; 0.026)	12.40 (3.55–43.29)	9×10^{-6} (9×10^{-6})
Louisiana	*APOE·HRAS1·LASS1* (CCT)	≥ 90- year-old female (125; 0.092) 20–59- year-old female (163; 0.032)	3.12 (1.47–6.63)	1.97×10^{-3} (3.93×10^{-3})
Louisiana	*APOE·HRAS1·LASS1* (CGT)	≥ 90 -year-old male (79; 0.279) 20–59- year-old male (88; 0.074)	4.86 (2.50–9.45)	1×10^{-6} (2×10^{-6})
Louisiana	*APOE·HRAS1·LASS1* (CCT)	≥ 90 -year-old healthy (68; 0.023) ≥ 90 -year-old unhealthy (68; 0.076)	0.29 (0.08–1.04)	*5.11×10^{-2} (5.11×10^{-2})
Louisiana	*APOE·HRAS1·LASS1* (CGT)	≥ 90 -year-old healthy (68; 0.295) ≥ 90- year-old unhealthy (68; 0.026)	15.48 (5.08–47.15)	1.06×10^{-9} (2.11×10^{-9})

European subpopulations (0.9 assignment probability) were examined. The haplotypes are ATTTC and CGCGCGT for *APOE* and *HRAS1*, respectively. Healthy and unhealthy were classified as described in Experimental procedures.
OR, odds ratio; CI, 95% confidence interval.
*Not significant.

haplotype (ATTTC) and the *HRAS1* (CGCGCGT) haplotype in combination with the *LASS1* (CCT) or (CGT) haplotypes, in which the SNP are listed starting with the furthest upstream SNP for each gene. The odds ratios (OR) of 7.68 and 2.56 were obtained for the *APOE*, *HRAS1*, and *LASS1* (CCT) or (CGT) haplotype combinations, respectively. The stratification of the population at the 0.9 assignment probability level was sufficient for this analysis, because no significant differences were observed in the frequencies of the ethnic affiliation markers between the control and centenarian groups in the European subpopulation.

To determine whether these associations are present in the Louisiana population, we carried out the same analyses independently. The results obtained in the Georgia sample replicated in the Louisiana sample (Table 1). The same haplotype combinations were associated with exceptional longevity in the subpopulation of European origin in Louisiana, stratified with an assignment probability of 0.9, and no significant association was found in the African-origin subpopulation. The OR were 2.93 and 2.97 for the *APOE*, *HRAS1*, and *LASS1* (CCT) or (CGT) haplotype combinations, respectively. The lower OR of the *LASS1* (CCT)-containing haplotype combination in the Louisiana population is likely due to the greater demographic selection for centenarians than for nonagenarians in the Georgia and Louisiana samples, respectively, but this does not appear to apply to the *LASS1* (CGT).

The lack of an association with longevity in the African-American subpopulation from both Georgia and Louisiana could be attributed to the relatively lower numbers of subjects from this group. However, it is likely that this may be an intrinsic difference in the subpopulation. Analysis of the frequency of the haplotypes in the individual genes supports the notion that there is an intrinsic difference between the European and African subpopulations in both Georgia and Louisiana (Table 2). The major difference is in the *APOE* haplotype frequency, with a smaller difference in *LASS1*. The European populations in Georgia and Louisiana also differ, as an examination of the haplotype blocks in the three genes suggests (Fig. S1).

Table 2 Haplotype frequencies in the general population

Haplotype	*APOE*	*HRAS1*	*LASS1* (CCT)	*LASS1* (CGT)
European				
Georgia (*N* = 220)	0.237	0.634	0.289	0.504
Louisiana (*N* = 293)	0.237	0.664	0.253	0.561
African				
Georgia (*N* = 145)	0.041	0.633	0.200	0.333
Louisiana (*N* = 52)	0.08	0.60	0.15	0.28

A Structure assignment probability of 0.8 was used for this analysis to provide a conservative evaluation of any differences. The 20- to 59 -year-old age groups were used here, as representative of the general population. *APOE* (ATTTC) and *HRAS1* (CGCGCGT) haplotypes are shown.

Haplotype frequencies differ with age

To ascertain the potential role of the three-gene interaction in promoting exceptional survival, we examined the differences in various age groups in the frequency of the two haplotype combinations identified above in the European subpopulation. The results are shown for the combinations containing the *LASS1*

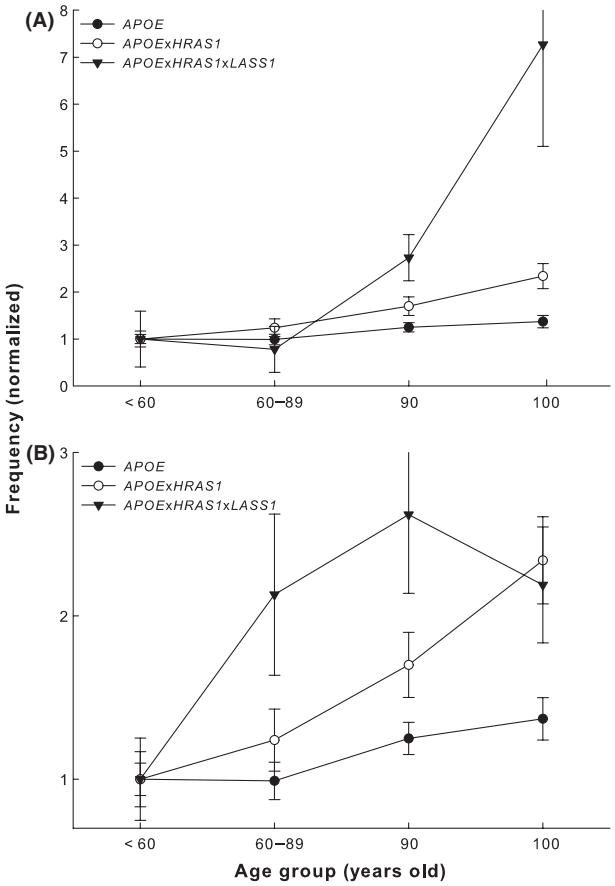

Fig. 2 Haplotype frequencies in different age groups. The frequencies for each haplotype or haplotype combination determined for the European subpopulation (0.9 assignment probability) were normalized to the frequencies in the < 60 year-old-age group from Georgia or Louisiana, respectively, to facilitate comparison. Error bars denote the SD (100 bootstraps). The age groups labeled '90' and '100' are the nonagenarians and centenarians in Georgia and Louisiana, respectively. For clarity, only the haplotypes or haplotype combinations that differ significantly on frequency between age groups are shown: *APOE* (ATTTC), *HRAS1* (CGCGCGT), and *LASS1* (CCT) in A and *APOE* (ATTTC), *HRAS1* (CGCGCGT), and *LASS1* (CGT) in B. Note the difference in scale of the ordinates in A and B. The *APOE* and *APOE·HRAS1* frequencies are identical in the two panels.

(CCT) and (CGT) haplotypes in Fig. 2A,B, respectively. The monotonic increase in the frequencies of the haplotype combinations of *APOE·HRAS1·LASS1* with age indicates a positive association with longevity, although a plateau appears for the combination containing the *LASS1* (CGT) haplotype for the oldest age group. These positive associations with longevity indicate that these are longevity haplotypes.

We next determined the frequencies of the *APOE* (ATTTC) haplotype and its combination with *HRAS1* (CGCGCGT) from the three-gene haplotype combination in the various age groups. Figure 2 shows that in both cases there is a monotonic increase with age. None of the other haplotypes in the individual genes or haplotype combinations changed in frequency with age. The patterns seen indicate that *HRAS1* has a synergistic (epistatic) effect with *APOE,* and this effect is further enhanced by either of the two *LASS1* haplotypes (Fig. 2 and Table 1).

Consistent with the increase in haplotype frequencies with age, the *APOE* haplotype is associated with exceptional survival in the European-origin subpopulations from both Georgia and Louisiana, with OR of 1.53 (CI = 1.08–2.17; Exact $P = 2.12 \times 10^{-2}$; adjusted $P = 2.12 \times 10^{-2}$) and 1.37 (CI = 1.02–1.83; Exact $P = 4.37 \times 10^{-2}$; adjusted $P = 4.37 \times 10^{-2}$) respectively. The *APOE·HRAS1* haplotype combination is also associated with longevity in both of these subpopulations, with OR of 2.83 (CI = 1.88–4.26; Exact $P = 5.68 \times 10^{-7}$; adjusted $P = 1.14 \times 10^{-6}$) and 1.89 (CI = 1.35–2.65; Exact $P = 2.27 \times 10^{-4}$; adjusted $P = 4.54 \times 10^{-4}$) respectively. The greater OR for the two-gene combination supports the epistatic effect of *HRAS1*. None of the other haplotypes in the individual genes or their combinations were associated with longevity (Table S5).

Haplotype combinations in women and men

The plateau in the frequency of the three-gene haplotype combination containing *LASS1* (CGT) in centenarians was puzzling. One possibility is that this reflects the lack of a role for this haplotype in survival at that late age. However, there is an alternate explanation. With advanced age, the ratio of females to males increases. Thus, the plateau we see could be the result of gender bias in the oldest age groups. We therefore examined the haplotype association in the Georgia sample for females and males separately, in the European subpopulation. The results suggest that such a bias indeed exists (Table 1). The association with exceptional survival in female centenarians was found solely for the combined haplotypes of the three genes that included *LASS1* (CCT), showing an OR of 12.52. For male centenarians, it was instead the *LASS1* (CGT) haplotype with an OR of 12.40. The results in the Georgia sample were replicated in the Louisiana population (Table 1). For female nonagenarians, the combined haplotypes that included *LASS1* (CCT) were associated with exceptional longevity with an OR of 3.12. For male nonagenarians this combination included *LASS1* (CGT) and an OR of 4.86.

Gene combination associated with healthy aging

We next examined the question whether the genetic combination that contributes to exceptional longevity is also associated with 'healthy aging.' Healthy aging is a concept that is difficult to define adequately. We elected to quantify healthy aging using the frequency of self-reported deficits (Rockwood & Mitnitski, 2007) in the Louisiana nonagenarians (Table S6). For this purpose, we surveyed subjects for the presence of 42 medical conditions or functional limitations and summed them for each nonagenarian. We then partitioned the subjects into tertiles based on the number of deficits reported; tertile 1 had the fewest deficits, while tertile 3 had the greatest number of deficits. A Kaplan–Meier survival analysis was performed for each of the tertiles (Fig. 3). Examining the entire sample of nonagenarians from Louisiana, the survival

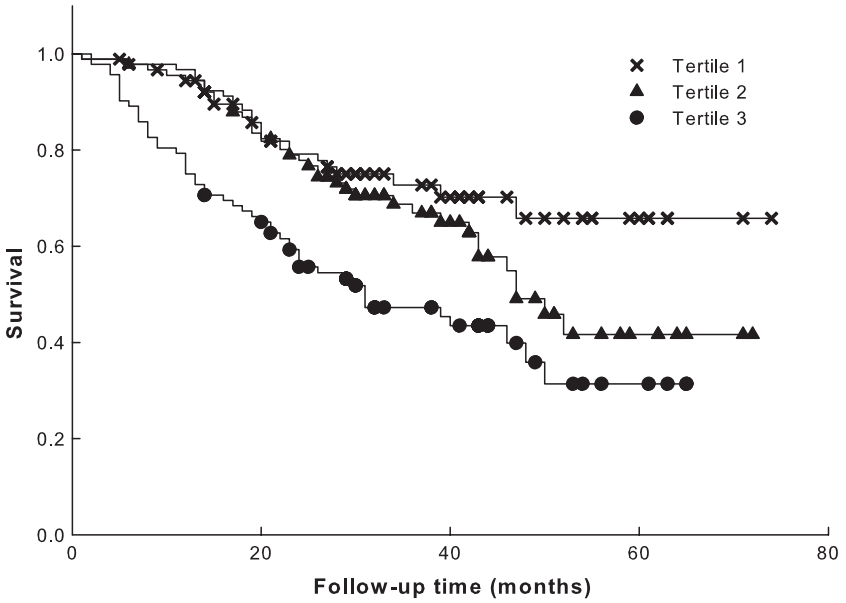

Fig. 3 Survival of nonagenarians as a function of their healthy aging profile. Kaplan–Meier survival of all nonagenarians in the Louisiana sample is plotted after partitioning individuals into tertiles of deficit accumulation. Deficit count increases from tertile 1–3. Mortality was assessed on follow-up after enrollment. Censoring is indicated by the symbols.

curves are significantly different (Wilcoxon $\chi^2_{(2)} = 20.06$, $P < 0.0001$).

We then searched for genetic associations with healthy aging by examining the haplotypes in the three genes in the tertile with the fewest deficits vs. that with the most (Table 1). The results indicate that the combination of the same haplotypes in the three genes is associated with healthy aging but only for the combination including *LASS1* (CGT). The OR for this association was 15.48 for the European subpopulation in Louisiana.

Functional impact of gene variants

The marked difference in the effects of the two *LASS1* haplotypes suggests that they could have functional consequences. Variants in the *APOE* promoter at −491 and −219 are known to affect the expression of this gene at the transcription level (Artiga *et al.*, 1998a,b). The combination we have found in the haplotype associated with exceptional survival elicits a moderate level of expression, approximately one-half of maximal. Because *LASS1* only has an effect when a combination of haplotypes in *APOE* and *HRAS1* is present, and this effect differs depending on the particular *LASS1* haplotype, we examined the variants in the *LASS1* gene for their potential role in transcription of the gene, using a luciferase reporter assay. The results indicate that compared to the AGC haplotype the CCT- and CGT-containing promoters were 48% and 29% more active, respectively (Fig. 4). Thus, the variation observed in our study appears to be functional in determining mRNA expression levels. Perhaps, the more moderate expression of *LASS1* in males promotes healthier aging. It is noteworthy that varying expression of the *LASS1* homolog in yeast, *LAG1*, affects the observed life extension (Jiang *et al.*, 2004).

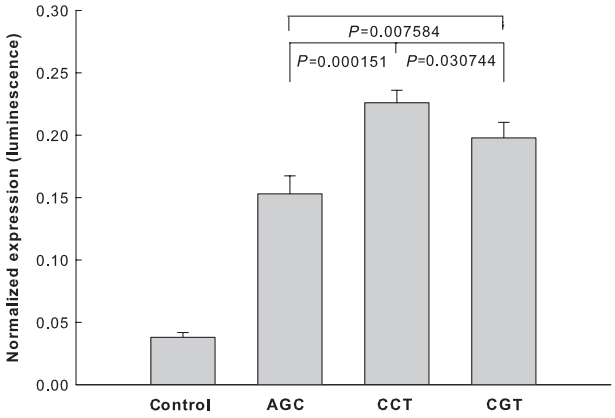

Fig. 4 Impact of polymorphisms in *LASS1* on gene expression. A luciferase reporter assay was used for determining the promoter activity in transfected HeLa cells. Error bars denote SEM for six independent experiments with at least six replicates for each of the four promoter constructs shown. The *LASS1* promoter variants CCT and CGT associated with exceptional longevity were compared to an AGC variant. The control is a reporter lacking inserted promoter sequences.

Discussion

We have found a combination of variants in three genes that is associated not only with exceptional longevity but also with healthy aging. This is a novel finding. An association of *HRAS1* with longevity was found before, and an interaction with the mitochondrial genome was demonstrated (Bonafé *et al.*, 2002). A compensatory effect in late life of variants in one gene for the deleterious effects of variants in a second gene has also been described (Bergman *et al.*, 2007). The combined effect described here involves three genes and extends the association

with exceptional survival to the functional outcome of health in old age. Genetic interactions play an important role in complex traits, but they can be difficult to find unless a hypothesis-driven approach such as we use here is employed that reduces the scale of the search and affects the prior probabilities involved (Thornton-Wells *et al.*, 2004; Flint & Mackay, 2009). Unmasking the interactions of common variants mitigates the necessity of invoking the wide involvement of rare variants in complex traits.

The association with healthy aging and the profile of increasing frequencies of the haplotypes with age indicates that the three genes studied here are not frailty genes or haplotypes but instead genetic determinants of longevity. The foundation for the genetic interaction is laid by the haplotype in *APOE*. *HRAS1* has a synergistic effect, and this combined effect is further enhanced by either of the two *LASS1* haplotypes in a synergistic manner. Our results point to the importance of accounting for population heterogeneity in association studies (Dai *et al.*, 2007). The African-origin subpopulation displays a very low frequency of the crucial *APOE* haplotype, as well as a reduced frequency of the *LASS1* haplotypes. Thus, intrinsic genetic differences between the European and African subpopulations can explain the lack of observation of the three-gene interaction in the latter group. On the other hand, the associations we have discovered in the European-origin sample from Georgia are replicated in the corresponding Louisiana sample, though the haplotype structures in the two samples differ. It will be important to examine samples from additional populations for these associations to further generalize our findings and to explore potential nuances in the haplotypic effects.

There are two different haplotypes in *LASS1* that play a role in the three-gene combination described here. One appears to operate in females and the other in males to determine exceptional longevity. Furthermore, it is only the latter that is associated with healthy aging. Thus, similar but distinct mechanisms of aging are at play. Differences in genetic associations with exceptional longevity between the genders have been noted previously (De Benedictis *et al.*, 2001). Interestingly, the cognition and physical function ability of male centenarians is often greater than for their female counterparts, even though they are outnumbered by them (Terry *et al.*, 2008). Survival to exceptional old age, however, can occur due to delay or escape from morbidity, as well as survival in its presence, regardless of gender (Evert *et al.*, 2003; Terry *et al.*, 2008). The promoter analyses suggest that an enhanced *LASS1* activity is conducive to longevity. They further suggest that healthy aging in nonagenarians depends on a somewhat lower enhancement of this promoter activity. This could, for example, be related to moderation in apoptosis-promoting activity coupled to sufficient enhancement of cholesterol clearance by ceramide. Our results support a model (Fig. 5) postulating that metabolic (lipid) stress induces protective responses. However, at some point, removal of damaged cells is needed. Confirmation of this model will require the longitudinal study of appropriate endophenotypes.

The haplotypes in the three genes are all present at high frequency in the European subpopulation in both Georgia and Louisiana (Table 2). However, the congruence of the respective haplotypes in all three genes is relatively infrequent in the population at large, although it increases in frequency dramatically with age (Table 1 and Fig. 2). Thus, we have uncovered an individual path to exceptional longevity and healthy aging, and others remain to be found.

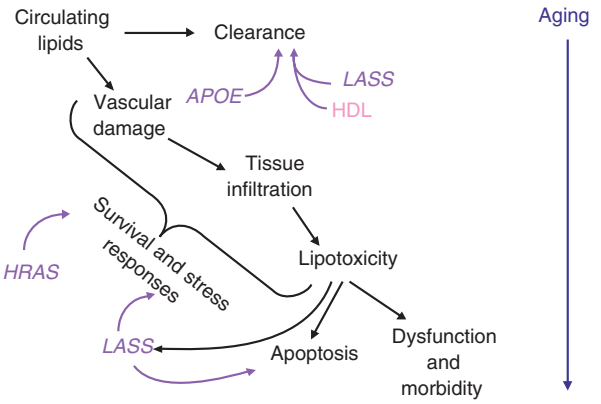

Fig. 5 Hypothetical model of the role of *APOE*, *HRAS1*, and *LASS1* interactions. Circulating lipids are cleared by APOE and by HDL, which is aided by LASS1-generated ceramide. Metabolic (lipid) stress induces protective survival and stress responses, which are mediated/modulated by HRAS1 and LASS1. Lipotoxicity enhances LASS1 production of ceramide, which signals apoptosis removing damaged cells. These mechanisms become overwhelmed with age resulting in dysfunction and morbidity.

Experimental procedures

Study subjects

The Georgia sample (N = 650) was recruited in the Georgia Centenarian Study from 44 counties in Northern Georgia. The sampling frame consisted of voter's registration files and lists prepared from a survey of nursing homes and personal care homes in the catchment area for centenarians and random digit dialing for younger subjects. The Louisiana sample (N = 869) was recruited in the Louisiana Healthy Aging Study from eight parishes within a 40-mile radius of Baton Rouge. The sampling frame consisted of the Center for Medicare and Medicaid Services enrollment database for subjects 65 and older and voter's registration files for younger individuals. Age of subjects was verified using both demographic questionnaires and documentary evidence. Mortality data were collected using Social Security Death Index search. Subjects provided informed consent according to protocols approved by the respective institutional review boards. The demographic characteristics of the samples are tabulated (Table S7).

Genotyping

Small subsets of samples from Georgia and Louisiana were first independently genotyped to identify all detectable variation in the selected genomic regions. Only polymorphisms present at ≥ 0.09 frequency were chosen for subsequent analysis in all

samples. This provided sufficient power (> 0.95) to detect associations with exceptional longevity (Dai *et al.*, 2007). Genomic regions of interest were then amplified by the polymerase chain reaction (PCR). The primers, forward and reverse respectively, used to amplify the DNA regions of interest by the PCR were as follows: (i) the promoter region of the *APOE* gene, 5′-GCGTCTGAGCGTTCACTGT-3′ and 5′-GTCCCAGTCTCGCATTCCT-3′; (ii) exon 4 of *APOE*, 5′-CTTGGGTCTCTCTGGCTCATC-3′ and 5′-GCAGCCTGCACCTTCTCC-3′; (iii) promoter of *LASS1*, 5′-CAGCAAGTGACCCTCAGAGA-3′ and 5′-GACCTGGACCCCGAGAGA-3′; (iv) exon1 of *LASS1*, 5′-GCCTGGGTTTCTCTGCTG-3′ and 5′-GCCCGAGAGACCTTATCCTG-3′; (v) promoter of *HRAS1*, 5′-ATCCCAGCCTTTCCCCAG-3′ and 5′-TTCGCCCCGCGCATGGGCT-3′; (vi) exon 1 of *HRAS1*, 5′-CAGGAGACCCTGTAGGAGGA-3′ and 5′-CCTATCCTGGCTGTGTCCTG-3′.

Amplicons were verified by agarose gel electrophoresis, and cleaned DNA was subjected to cycle-sequencing using the BigDye Terminator Reagent version 3.1 (Applied Biosystems, Foster City, CA, USA) and the same primers, and the 3130xl DNA sequencing system (Applied Biosystems). Identification of alleles was aided by the SNP detection and base-calling software SeqScape v2.5 (Applied Biosystems). To minimize errors, both strands of DNA were sequenced twice independently. The genotyped loci included SNP: rs449647, rs769446, rs405509, rs429358, rs7412, rs8176330, rs8176331, rs8176332, rs8176333, rs8176334, rs8176335, rs12628, rs60774903, rs3746263, and an SNP in exon 1 (+234) in *LASS1* that is not found in dbSNP. The genotyped loci are tabulated (Table S8).

Some of the Louisiana samples were genotyped using the SNPlex technology (Applied Biosystems), which is based on the oligonucleotide ligation assay combined with multiplex PCR amplification. Primers were designed according to the SNPlex Design Pipeline for the following SNP: rs449647, rs769446, rs405509, rs8176330, and rs3746263. Size fractionation of genotype-specific products was performed by capillary electrophoresis using the 3130xl DNA Analyzer, and genotype calls were made using the GeneMapper v4.0 software (Applied Biosystems). The SNPlex genotyping was repeated twice independently.

The two polymorphisms in the coding region (rs429358 and rs7412) account for the occurrence of three alleles, *ε2*, *ε3*, and *ε4*, which result in the three major isoforms, E2, E3, and E4 of APOE, respectively. The E2, E3, and E4 isoforms differ on amino acid sequence at site A (residue number 112) and site B (residue number 158) in exon 4. For *HRAS1* and *LASS1* SNP, their locations are relative to the first nucleotide of the translation start codon, whereas the transcription start site was used for the *APOE* SNP.

Population stratification using Structure

Identification of ethnic origins of individuals using Alu insertion polymorphisms was performed as described (Bamshad *et al.*, 2003; Watkins *et al.*, 2003; Ray *et al.*, 2005). DNA samples were genotyped at 100 separate Alu loci located throughout the genome. The Alu sequences were amplified by PCR using locus-specific primers. The PCR fragments were separated by agarose gel electrophoresis. The primers and methods used have been described (Bamshad *et al.*, 2003; Watkins *et al.*, 2003; Ray *et al.*, 2005). Subjects were classified as one or the other of the possible homozygotes based on the presence of a particular Alu sequence at a given locus or as heterozygotes. The Alu genotypes thus obtained were analyzed to assign ethnic affiliation using Structure (Pritchard *et al.*, 2000). For this purpose, the same Alu genotypes from 715 independent DNA samples from subjects from around the world were used (Bamshad *et al.*, 2003; Watkins *et al.*, 2003; Ray *et al.*, 2005). These samples spanned the geographic regions of Europe, Africa, Asia, and India and included sampling of individuals from many populations in each region (31 total). Subjects were then assigned to populations of origin corresponding to these geographic regions using an assignment probability of 0.9.

Plot of pairwise linkage disequilibrium (LD) comparison

Haplotype blocks for the three genes were examined using Haploview. The standard color scheme is used for D′/LOD values: bright red (D′ = 1, LOD ≥ 2); blue (D′ = 1, LOD < 2); shades of pink/red (D′ < 1, LOD ≥ 2); white (D′ < 1, LOD < 2). The numbers shown inside the boxes are D′ values × 100 (empty boxes represent the D′ value of 1). Haplotype blocks are defined based on confidence bounds on D′. If the one-sided upper 95% confidence bound on D′ lies between 0.7 and 0.98, the SNP pairs involved are considered to be in strong LD (Gabriel *et al.*, 2002). Haploview was also used to examine the Hardy–Weinberg equilibrium. None of the 15 SNP examined in this study showed significance for departure from Hardy–Weinberg proportions in the Georgia and Louisiana samples stratified by Structure at 0.9 assignment probability. Haploview is available at: http://www.broad.mit.edu/mpg/haploview.

Promoter analysis of *LASS1* haplotypes

A 1755-bp DNA fragment containing the putative promoter region and part of the coding region of *LASS1* was PCR-amplified from appropriate genomic DNA samples using *Eco*RV and *Bgl*II-tagged primers and cloned into the Pgl4.10 vector (Promega, Sunnyvale, CA, USA). The cloned sequence is in frame with the firefly luciferase coding sequence in the vector. Then, an additional 1040-bp fragment tagged with *Xho*I and *Eco*RV was added upstream of the original clone. The translational start codon of *LASS1* was changed to TAG by site-directed mutagenesis. The correctness of the final constructs was verified by sequencing. The only sequence differences between the constructs were at the polymorphic sites of interest specified below.

Transfection of HeLa cells was performed using the PolyFect transfection reagent (Qiagen, Valencia, CA, USA) per the protocol provided. Co-transfection was carried out with mixtures

of one of the experimental constructs (0.4 μg) with a control vector, Pgl4.73 (10 ng), which carries a Renilla luciferase coding sequence under the SV40 promoter. The experimental constructs were either Pgl4.10 without *LASS1* promoter sequences or Pgl4.10 with one of three different *LASS1* sequences. These had the following bases at positions −718, +216, and +234, respectively: AGC, CCT, or CGT. Following 24-h incubation, luciferase activities were measured using the Dual-Glo Luciferase Assay (Promega) per the protocol provided, using a Modulus Microplate Luminometer (Turner BioSystems, Sunnyvale, CA, USA). The firefly luminescence readings were normalized to the Renilla luminescence readings. There were at least six replicates of each assay per experiment.

Deficit index

Following previously published reports (Kulminski *et al.*, 2008; Searle *et al.*, 2008), our deficit index was derived as the un-weighted count of the number of deficits divided by the total number of possible deficits for an individual. The self-reported medical history questionnaire was administered to all study participants, and its components were used to construct the deficit index. This was supplemented with items from an activities of daily living (ADL) questionnaire. Responses were grouped to create broader categories where similar ailments were defined more than once. After applying the above criteria, 42 deficits were included in the compilation of the index.

Statistical analysis

There is substantial evidence supporting the hypothesis of association with exceptional longevity of variants in the three candidate genes chosen here for analysis. This information is presented in this paper. *APOE* has been replicated as a longevity-determining gene in numerous studies (Christensen *et al.*, 2006). This gene anchors the interaction described in this report (Fig. 5). The hypothesis we test is related to the role of the apolipoprotein APOE in lipid and lipoprotein metabolism. The BioSystems Database (NCBI) lists 228 genes under the entry 'Metabolism of Lipids and Lipoproteins.' *APOE* and *LASS1* are among them. Thus, we assume the prior probability of an interaction of these genes to be $\sim$ 1.0. It is likely that there are genes involved in lipid and lipoprotein metabolism in addition to the 228, and we conservatively propose there to be 300. We therefore assume the prior probability of identifying another interacting gene, such as *HRAS1*, to be $\sim$ 300/30 000 or $\sim$ 0.01. We set the posterior probability for identification of such a gene at 0.95. Applying Bayes' theorem, we obtain the likelihood ratio *Pr*(*Data*|*Association*)/*Pr*(*Data*|*NoAssociation*) of 1881. These considerations are applicable to the initial test of the association with longevity of the combination of the haplotypes in the three genes in both the Georgia and Louisiana populations. The prior probability increases dramatically in subsequent tests, once the initial association is identified.

APOE allele frequencies and genotype frequencies were compared between centenarians/nonagenarians and young controls with a 2×3 contingency table (for alleles) or with a 2×6 contingency table (for genotypes). The contingency tables were evaluated by Fisher's exact probability test (Sanchez *et al.*, 2006), using SAS v9.13 software (SAS Institute Inc., Cary, NC, USA). A P-value < 0.05 was considered statistically significant. All statistical tests of significance reported in this study are two-sided.

Structure v2.2 (Pritchard *et al.*, 2000) was used to infer population structure for the 650 Georgia subjects and the 869 Louisiana subjects, respectively. Seven hundred and fifteen subjects of known geographic ancestry (European, African, Asian, or Indian) were used as reference. For each individual, Structure 2.2 estimates the proportion of ancestry from each of four populations. We used a burn-in of 15 000 iterations and a run of 20 000 replications. Three replicate runs were performed on each dataset. The largest proportion values in each run were used to calculate the average ancestry proportion for each individual. Individuals were either assigned to a specific geographic origin if the average was larger than a predetermined assignment probability (0.9 in this study) or considered admixed if the average was less. All haplotype analyses were performed on samples stratified for ethnic affiliation, as described here.

Linkage disequilibrium approaches to the analysis of gene–gene interactions have been proposed (Zhao *et al.*, 2006), and the modeling of gene–gene interactions using association analysis of noncontiguous haplotypes has been implemented (Woo *et al.*, 2006). We took a similar approach. The haplotype frequencies for the combination of SNP in *APOE*, *HRAS1*, and *LASS1* were estimated by an Expectation-Maximization (EM) algorithm implemented in Arlequin (Excoffier *et al.*, 2005). The SD was determined by the parametric bootstrap procedure in Arlequin (100 bootstraps). Those haplotype combinations present at a frequency of $\sim$ 0.01 or greater in the 20- to59 -year-old group in Georgia and in Louisiana were analyzed further. This cutoff was taken based on the considerations from comparison of statistical and molecular haplotyping for sample sizes as small as 17 (Tishkoff *et al.*, 2000). The frequency estimates in case and control groups were used to generate 2×2 contingency tables to test significance of associations of the respective haplotype combinations with exceptional longevity. Fisher's exact test was used to assess significance. The sequential Dunn-Sidak procedure was used to adjust the exact P-values for multiple comparisons of haplotype combinations. The adjustment included seven such combinations independently identified in the Georgia and Louisiana samples in the subpopulations of European origin (Table S4). In the African-origin subpopulations, there were seven and three such combinations in these samples, respectively. A stringent criterion of adjusted $P < 0.01$ (two-sided) was used to identify the potentially interacting combination of haplotypes in the three genes.

The two significant haplotype combinations found to be associated with exceptional longevity in the independent analyses in the Georgia and Louisiana samples were then evaluated in men

and women in these two samples, as well as for the tertiles of deficit accumulation in the Louisiana population. The same ~ 0.01 frequency cutoff was applied. This involved adjustment for up to two comparisons, using the sequential Dunn-Sidak procedure. The haplotype combination frequencies differed significantly in the various samples examined. These haplotype combinations are common in the relevant samples characterized by longevity and/or healthy aging. The three-gene haplotype combinations reported here are associated with certain additional healthy-aging phenotypes but not with others (in preparation).

The *APOE* and *APOE·HRAS1* haplotypes derived from the significant haplotype combinations in the three genes were also evaluated independently for association with exceptional longevity, using the above procedures.

Nonagenarians were classified by tertile according to their deficit accumulation, as described above. The Kaplan–Meier survival was plotted for each tertile. The differences between tertiles were evaluated by the Wilcoxon test (a two-sided $P < 0.05$ was considered significant). SAS v9.13 software was used for Kaplan–Meier survival analysis.

The significance of differences in promoter strength determined using the luciferase reporter assay was evaluated for six independent experiments in which the control (no *LASS1* promoter sequences) and the three *LASS1* promoter haplotypes were compared. One-way ANOVA was used to first test for any differences, using a criterion of $P < 0.05$ (two-sided). Then, all pairwise combinations were evaluated in a two-tailed Newman–Keuls test, implemented in StatMost (DataMost Corp., Sandy, UT, USA). A *P*-value of < 0.05 was considered significant.

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Author contributions

S.M.J., L.W.P., M.A.B., D.A.W., L.J.S., D.B.H., E.R., K.E.C., and M.A.W. designed the research; S.K., L.L., X.B., J.C.J., J.A.W., C.M.L., J.V., L.M., L.J.S., D.B.H., M.V.M., and K.E.C. acquired data; J.A. and J.D. contributed new analytic tools; S.M.J., S.K., J.D., X.B., J.A., M.A.B., D.A.W., C.M.L., J.V., L.M., and L.J.S. analyzed and interpreted data; and S.M.J., S.K., and J.D. prepared the manuscript.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Haplotype blocks in *APOE*, *HRAS1*, and *LASS1*. Haplotype blocks for the three genes were examined using Haploview after stratifying the populations using Structure assignment probabilities of 0.8 and 0.9 (in parentheses below) for European origin. A – Georgia (0.9), B – Georgia (0.8), C – Louisiana (0.9), and D – Louisiana (0.8). Among the differences between the two populations, the large haplotype block in *HRAS1* that is evident in the Louisiana population at 0.9 and 0.8 assignment probabilities is only apparent in Georgia at 0.9 assignment probability, and the haplotype block seen in *LASS1* in the Louisiana population is not apparent in the Georgia population. No tag SNP in the haplotype blocks were identified.

Table S1 *APOE* association with exceptional longevity

Table S2 *APOE* association with exceptional longevity in the major population strata

Table S3 Lack of association with exceptional longevity of variants in *HRAS1* and *LASS1* in the major population strata

Table S4 Association of combinations of haplotypes in *APOE*, *HRAS1*, and *LASS1* with exceptional longevity

Table S5 Association of additional haplotypes in individual genes and in combinations of two genes with exceptional longevity

Table S6 List of deficits in deficit accumulation index

Table S7 Age and gender composition of the study samples

Table S8 Genotyped loci

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