Aging Cell (2011) **10,** pp622–628 Doi: 10.1111/j.1474-9726.2011.00698.x

Candidate gene study of *FOXO1*, *FOXO4*, and *FOXO6* reveals no association with human longevity in Germans

Rabea Kleindorp,[1] Friederike Flachsbart,[1] Annibale A. Puca,[2,3] Alberto Malovini,[4] Stefan Schreiber[1,5] and Almut Nebel[1]

[1]Institute of Clinical Molecular Biology, Christian-Albrechts-University of Kiel, Kiel, Germany
[2]IRCCS Multimedica, Milan, Italy
[3]Istituto di Tecnologie Biomediche – Consiglio Nazionale delle Ricerche, Segrate, Italy
[4]Department of Computer Engineering and Systems Science, University of Pavia, Pavia, Italy
[5]Popgen Biobank, Christian-Albrechts-University of Kiel, Kiel, Germany

Summary

In mammals, the forkhead box class O (FOXO) family of transcription factors consists of the four members *FOXO1*, *FOXO3A*, *FOXO4*, and *FOXO6*. The *FOXO* genes are homologues of *daf-16*, a key regulator of the insulin-IGF1 signaling pathway and a modulator of lifespan in *Caenorhabditis elegans*. Recently, variants in *FOXO3A* have consistently been associated with human longevity in various populations worldwide. Given this confirmed finding, it is conceivable that polymorphisms in the other *FOXO* genes might have a similar effect on human longevity. To evaluate whether allelic variation in *FOXO1*, *FOXO4*, and *FOXO6* influences the ability to become long-lived, we performed a comprehensive haplotype-tagging analysis of the three genes in a group of 1447 centenarians/nonagenarians and 1029 younger controls from Germany. This is the first investigation to analyze a possible association of human longevity with *FOXO4* and *FOXO6*, respectively, and the largest and most comprehensive study to date to assess the genetic contribution of *FOXO1* to the phenotype. Our results suggest that in Germans, none of the three genes plays a significant role in the ability to reach old age. With regard to *FOXO1*, this observation is supported by data from an Italian sample and is consistent with several previous reports, but appears to be in contrast to a recent study of Han Chinese. The discrepant association findings in Europeans and Chinese may be explained by their different *FOXO1* linkage disequilibrium structures and could indicate a Chinese- or Asian-specific effect.

Key words: aging; association study; centenarians; *FOXO3A*; long-lived individuals.

Introduction

The forkhead box class O (FOXO) family of transcription factors is evolutionarily conserved and characterized by the so-called forkhead box DNA-binding domain. In mammals, the *FOXO* gene family consists of four members: *FOXO1*, *FOXO3A*, *FOXO4*, and *FOXO6*, which are homologous to the *dauer formation-16* (*daf-16*) gene in the worm *Caenorhabditis elegans* (*C. elegans*). Numerous studies have shown that FOXO proteins play an important role in a wide range of normal biological processes, including cellular proliferation, cell cycle arrest, stress response, and apoptosis (Burgering & Kops, 2002; Stahl *et al.*, 2002; Accili & Arden, 2004), as well as in diseases like cancer or diabetes mellitus (Furukawa-Hibi *et al.*, 2002; Kato *et al.*, 2006). Furthermore, FOXOs interact with several pathways that regulate cellular survival and aging, as it was demonstrated by early studies linking *daf-16* in *C. elegans* as a key regulator of the insulin-IGF1 signaling (IIS) pathway (Kenyon, 2005) to extended lifespan (Tissenbaum & Guarente, 2001). Following on from these findings in model organisms, Willcox *et al.* (2008) have recently identified one of the *daf-16* homologues, *FOXO3A*, as a susceptibility gene for human longevity in ethnic Japanese from Hawaii (Willcox *et al.*, 2008). This association was subsequently replicated in German, Italian, Chinese, US-American, Jewish, and Danish populations (Anselmi *et al.*, 2009; Flachsbart *et al.*, 2009; Li *et al.*, 2009; Pawlikowska *et al.*, 2009; Soerensen *et al.*, 2010).

Human longevity is considered a multi-factorial phenotype with a genetic contribution of about 25% (Christensen *et al.*, 2006). The genetic component has been shown to become stronger with increasing age of the individuals (Hjelmborg *et al.*, 2006; Gögele *et al.*, 2010). Many genetic factors are likely to be involved, each having only a weak to moderate effect (Christensen *et al.*, 2006). Up to date, the ε4 allele of the apolipoprotein E (*APOE*) gene and variation in *FOXO3A* remain the only genetic determinants that have consistently and repeatedly been found to influence survival into old age in numerous populations worldwide (Christensen *et al.*, 2006; Willcox *et al.*, 2008; Anselmi *et al.*, 2009; Flachsbart *et al.*, 2009; Li *et al.*, 2009; Pawlikowska *et al.*, 2009; Soerensen *et al.*, 2010).

Given the confirmed association of *FOXO3A* variation with human longevity, it is conceivable that single-nucleotide

Correspondence
Almut Nebel, Institute of Clinical Molecular Biology, Christian-Albrechts-University, Schittenhelmstrasse 12, 24105 Kiel, Germany. Tel.: +49 431 597 1373; fax: +49 431 597 2196; e-mail: a.nebel@mucosa.de

Accepted for publication *16 February 2011*

polymorphisms (SNPs) in the other *FOXO* members, which are also regulated by the IIS pathway, might have a similar effect on the phenotype. Previously, an analysis of *FOXO1* in the prospective population-based Leiden 85+ study revealed for carriers of a particular 3-SNP haplotype a 1.14-fold increased all-cause mortality risk (*P* value = 0.021, without correction for multiple testing) (Kuningas *et al.*, 2007). More recently, statistically significant frequency differences for two SNPs and two haplotypes in *FOXO1* were reported between long-lived and younger women from China, suggesting a female-specific effect (Li *et al.*, 2009). The results of the single-point analysis were replicated in an additional sample of Chinese. However, this finding is in contrast to other studies showing no evidence for an association of *FOXO1* with human longevity (Bonafe *et al.*, 2003; Kojima *et al.*, 2004; Willcox *et al.*, 2008).

Here, we performed a comprehensive analysis of *FOXO1*, *FOXO4*, and *FOXO6* in a large group of 1447 long-lived individuals (LLI, i.e. centenarians and nonagenarians) and 1029 younger controls from Germany to evaluate whether allelic variation in any of the three genes influences the ability to attain old age.

Results

In this study, 36 haplotype-tagging SNPs (htSNPs) in the gene regions of *FOXO1*, *FOXO4*, and *FOXO6* were analyzed for association with human longevity. The polymorphisms were spaced across the entire genes, covering the majority of the allelic variation. For none of the SNPs, a significant deviation from Hardy–Weinberg equilibrium (HWE) was found in the control population ($P > 0.02$). Single-marker comparisons did not show a significant association in any of the tested SNPs, neither for the complete sample of LLI (Table 1) nor the female (Table 2) or male subgroup (data not shown). A subsequent haplotype-based association analysis using a sliding window with pairs of consecutive SNPs revealed no association in any of the three genes with human longevity (data not shown).

Table 1 Association statistics for 36 haplotype-tagging single-nucleotide polymorphisms (htSNPs) in *FOXO1*, *FOXO4*, and *FOXO6* in German LLI and controls

Gene	Chr.	SNP	bp	minAF LI	minAF controls	*P* allele	*P* geno	OR (95% C.I.)
FOXO1	13	rs2701893	40023636	0.349	0.353	0.772	0.520	0.98 (0.87–1.11)
FOXO1	13	rs2721047	40025682	0.086	0.097	0.177	0.246	0.87 (0.72–1.06)
FOXO1	13	rs17446593	40026085	0.170	0.181	0.338	0.631	0.93 (0.80–1.08)
FOXO1	**13**	**rs2755209**	**40035804**	**0.373**	**0.377**	**0.770**	**0.274**	**0.98 (0.87–1.11)**
FOXO1	13	rs1078892	40036020	0.187	0.193	0.589	0.425	0.96 (0.83–1.11)
FOXO1	13	rs2701859	40039232	0.252	0.272	0.117	0.059	0.90 (0.79–1.03)
FOXO1	13	rs12865518	40041190	0.250	0.237	0.307	0.345	1.07 (0.94–1.23)
FOXO1	13	rs2721069	40041720	0.310	0.325	0.251	0.187	0.93 (0.82–1.05)
FOXO1	**13**	**rs2755213**	**40044301**	**0.090**	**0.094**	**0.626**	**0.876**	**0.95 (0.78–1.16)**
FOXO1	13	rs2701880	40048505	0.060	0.071	0.120	NA	0.83 (0.66–1.05)
FOXO1	13	rs2951787	40059770	0.384	0.376	0.600	0.834	1.03 (0.92–1.16)
FOXO1	13	rs2984121	40059979	0.185	0.178	0.556	0.823	1.05 (0.90–1.21)
FOXO1	13	rs2721044	40060225	0.307	0.330	0.097	0.074	0.90 (0.80–1.02)
FOXO1	13	rs4943794	40071408	0.211	0.223	0.313	0.457	0.93 (0.81–1.07)
FOXO1	13	rs1986649	40076824	0.211	0.225	0.229	0.434	0.92 (0.80–1.06)
FOXO1	13	rs17630266	40092032	0.036	0.033	0.510	NA	1.11 (0.81–1.52)
FOXO1	13	rs12876443	40094877	0.107	0.107	0.989	0.871	1.00 (0.83–1.20)
FOXO1	13	rs7981045	40107236	0.273	0.261	0.349	0.550	1.06 (0.93–1.21)
FOXO1	13	rs9603776	40121886	0.025	0.024	0.778	NA	1.05 (0.73–1.52)
FOXO4	X	rs12013673	70233824	0.437	0.443	0.696	0.548	0.98 (0.86–1.10)
FOXO4	X	rs5981072	70236267	0.299	0.311	0.368	0.976	0.94 (0.83–1.07)
FOXO6	1	rs11585393	41602844	0.301	0.293	0.531	0.466	1.04 (0.92–1.18)
FOXO6	1	rs7539614	41603634	0.444	0.439	0.739	0.904	1.02 (0.91–1.15)
FOXO6	1	rs7547654	41603948	0.458	0.440	0.200	0.356	1.08 (0.96–1.21)
FOXO6	1	rs11581271	41607648	0.401	0.423	0.131	0.097	0.91 (0.81–1.03)
FOXO6	1	rs1317558	41611670	0.119	0.108	0.257	0.195	1.11 (0.93–1.33)
FOXO6	1	rs1317557	41611929	0.028	0.027	0.824	NA	1.04 (0.73–1.48)
FOXO6	1	rs6693260	41612082	0.175	0.178	0.811	0.824	0.98 (0.85–1.14)
FOXO6	1	rs4660532	41612784	0.331	0.348	0.224	0.461	0.93 (0.82–1.05)
FOXO6	1	rs6690527	41614573	0.289	0.287	0.867	0.366	1.01 (0.89–1.15)
FOXO6	1	rs4660192	41614937	0.458	0.464	0.663	0.833	0.97 (0.87–1.09)
FOXO6	1	rs11209971	41616880	0.281	0.293	0.336	0.511	0.94 (0.83–1.07)

Chr., chromosome; bp, base-position of NCBI build 36; minAF, minor allele frequency; LLI, long-lived individuals; *P* allele, *P* value obtained from allele-based case–control comparison using a chi square-test with 1 degree of freedom (df); *P* geno, *P* value obtained from genotype-based case–control comparison using a chi square-test with 2 df; OR, estimated odds ratio for minor allele (based on whole sample); CI, 95% confidence interval of OR; NA, genotype-based case–control comparison was not conducted when there were fewer than five observations. Associated markers from the study of Li *et al.* (2009) are in bold.

Table 2 Association statistics for 36 haplotype-tagging single-nucleotide polymorphisms (htSNPs) in *FOXO1*, *FOXO4*, and *FOXO6* in German female LLI and controls

Gene	Chr.	SNP	bp	minAF LLI	minAF controls	*P* allele	*P* geno	OR (95% C.I.)
FOXO1	13	rs2701893	40 023 636	0.345	0.353	0.617	0.348	0.97 (0.84–1.11)
FOXO1	13	rs2721047	40 025 682	0.082	0.098	0.091	NA	0.82 (0.66–1.03)
FOXO1	13	rs17446593	40 026 085	0.167	0.182	0.239	0.383	0.90 (0.76–1.07)
FOXO1	**13**	**rs2755209**	**40 035 804**	**0.369**	**0.376**	**0.654**	**0.109**	**0.97 (0.85–1.11)**
FOXO1	13	rs1078892	40 036 020	0.183	0.196	0.288	0.054	0.92 (0.78–1.08)
FOXO1	13	rs2701859	40 039 232	0.247	0.269	0.130	0.078	0.89 (0.77–1.03)
FOXO1	13	rs12865518	40 041 190	0.256	0.243	0.383	0.581	1.07 (0.92–1.24)
FOXO1	13	rs2721069	40 041 720	0.309	0.323	0.367	0.303	0.94 (0.82–1.08)
FOXO1	**13**	**rs2755213**	**40 044 301**	**0.087**	**0.093**	**0.548**	**0.808**	**0.93 (0.75–1.17)**
FOXO1	13	rs2701880	40 048 505	0.058	0.070	0.121	NA	0.81 (0.62–1.06)
FOXO1	13	rs2951787	40 059 770	0.382	0.372	0.513	0.806	1.05 (0.92–1.20)
FOXO1	13	rs2984121	40 059 979	0.185	0.181	0.800	0.898	1.02 (0.86–1.21)
FOXO1	13	rs2721044	40 060 225	0.307	0.328	0.170	0.135	0.91 (0.79–1.04)
FOXO1	13	rs4943794	40 071 408	0.212	0.225	0.346	0.402	0.93 (0.79–1.08)
FOXO1	13	rs1986649	40 076 824	0.212	0.227	0.277	0.413	0.92 (0.79–1.07)
FOXO1	13	rs17630266	40 092 032	0.036	0.030	0.315	NA	1.20 (0.84–1.73)
FOXO1	13	rs12876443	40 094 877	0.109	0.108	0.880	0.901	1.02 (0.82–1.25)
FOXO1	13	rs7981045	40 107 236	0.278	0.266	0.435	0.696	1.06 (0.92–1.23)
FOXO1	13	rs9603776	40 121 886	0.027	0.024	0.530	NA	1.14 (0.76–1.72)
FOXO4	X	rs12013673	70 233 824	0.432	0.428	0.800	0.574	1.02 (0.89–1.16)
FOXO4	X	rs5981072	70 236 267	0.298	0.296	0.927	0.982	1.01 (0.87–1.16)
FOXO6	1	rs11585393	41 602 844	0.296	0.288	0.606	0.537	1.04 (0.90–1.20)
FOXO6	1	rs7539614	41 603 634	0.443	0.437	0.707	0.924	1.03 (0.90–1.17)
FOXO6	1	rs7547654	41 603 948	0.460	0.449	0.476	0.763	1.05 (0.92–1.19)
FOXO6	1	rs11581271	41 607 648	0.395	0.408	0.446	0.445	0.95 (0.83–1.09)
FOXO6	1	rs1317558	41 611 670	0.121	0.103	0.084	0.070	1.20 (0.98–1.47)
FOXO6	1	rs1317557	41 611 929	0.028	0.025	0.541	NA	1.14 (0.76–1.71)
FOXO6	1	rs6693260	41 612 082	0.168	0.179	0.388	0.591	0.93 (0.78–1.10)
FOXO6	1	rs4660532	41 612 784	0.342	0.358	0.333	0.598	0.94 (0.82–1.07)
FOXO6	1	rs6690527	41 614 573	0.286	0.283	0.830	0.405	1.02 (0.88–1.17)
FOXO6	1	rs4660192	41 614 937	0.454	0.448	0.725	0.939	1.02 (0.90–1.17)
FOXO6	1	rs11209971	41 616 880	0.281	0.279	0.887	0.990	1.01 (0.87–1.17)

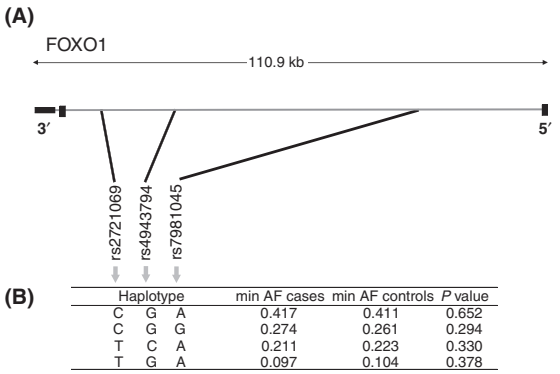
Chr., chromosome; bp, base-position of NCBI build 36; minAF, minor allele frequency; LLI, long-lived individuals; *P* allele, *P* value obtained from allele-based case–control comparison using a chi square-test with 1 degree of freedom (df); *P* geno, *P* value obtained from genotype-based case–control comparison using a chi square-test with 2 df; OR, estimated odds ratio for minor allele (based on whole sample); CI, 95% confidence interval of OR; NA, genotype-based case–controls comparison was not conducted when there were fewer than five observations. Associated markers from the study of Li *et al.* (2009) are in bold.

The markers used for the *FOXO1* haplotype analysis in the Leiden 85+ study (Kuningas *et al.*, 2007) were also subjected to a haplotype analysis in our sample (rs2721069, rs4943794, and rs7981045; Fig. 1A). The frequency of the observed mortality haplotype in the Dutch sample (TCA) was not significantly different in German LLI compared with younger controls (Fig. 1B).

Recently, association signals for the *FOXO1* SNPs rs2755209 and rs2755213 were reported in two samples of female Han Chinese (Li *et al.*, 2009). In Germans, the frequencies of the polymorphisms did not differ significantly between the LLI and the young controls, neither for the complete sample (Table 1) nor the female subgroup (Table 2). When we investigated an additional population of female Italians for the same SNPs, this analysis did neither show evidence of association (Table 3). In view of the very similar *FOXO1* SNP frequencies and linkage disequilibrium (LD) structures in Germans and Italians (Table 5 and Fig. 2), the data from the two populations were pooled. Also the combined sample with an *a priori* power of 84% did not confirm the previous finding of Li *et al.* (Table 3). Furthermore, in the German sample of female individuals (Table 4), we did not detect any association between the two haplotypes (ATG and CCG based on rs2755209, rs2755213, and rs17630266) and the longevity phenotype, as earlier reported in Chinese (Li *et al.*, 2009).

Discussion

In the present study, we performed comprehensive haplotype tagging of the *FOXO* gene family members *FOXO1*, *FOXO4*, and *FOXO6* in an extensive collection of LLI and younger controls, comprising altogether 2476 German individuals. This is the first investigation to analyze a possible association between *FOXO4* or *FOXO6*, respectively, and human longevity, and the largest



Haplotype			min AF cases	min AF controls	*P* value
C	G	A	0.417	0.411	0.652
C	G	G	0.274	0.261	0.294
T	C	A	0.211	0.223	0.330
T	G	A	0.097	0.104	0.378

Fig. 1 (A) Gene structure of *FOXO1*. (B) Results of the haplotype analysis in German long-lived individuals and controls. Single-nucleotide polymorphisms are based on the study of Kuningas *et al.* (2007). minAF, minor allele frequency.

and most comprehensive one to date to assess the genetic contribution of *FOXO1* to the phenotype (1886 German and 382 Italian females). Our overall result suggests that polymorphisms in none of the genes have a significant influence on a person's ability to reach old age.

For *FOXO1*, several longevity association studies have previously been performed, albeit with contradictory findings (Bonafe *et al.*, 2003; Kojima *et al.*, 2004; Li *et al.*, 2009). Bonafe *et al.* (2003) described a negative result in Italians that is – though only a single SNP was tested – consistent with ours. Recently, a study of Han Chinese found two SNPs as well as two haplotypes in this gene to be associated with longevity in women (Li *et al.*, 2009). The results of the single-point analysis were also replicated in an additional sample of Chinese (Li *et al.*, 2009). However, two previous investigations of Asian populations had revealed no association (Kojima *et al.*, 2004; Willcox *et al.*, 2008). One of the studies that examined only male individuals of Japanese descent (Willcox *et al.*, 2008) is not informative with regard to a potential association in females. Another report analyzing Japanese of both genders tested three SNPs (Kojima *et al.*, 2004), which were not haplotype-tagging and thus did not cover the allelic variation in *FOXO1* comprehensively. Of note, one of the SNPs, rs2297627, is in strong LD with one of the significantly associated markers described in Han Chinese (rs2755209, Table 5). A comparison of the two SNPs reveals a comparable increase in their minor allele frequencies in controls relative to LLI (2.9% in Japanese (Kojima *et al.*, 2004) and 4.4% in Chinese (Li *et al.*, 2009). In Japanese, this difference is not significant, which may be because of the relative small sample size used in that study (122 centenarians and 122 controls). Also the other two *FOXO1* SNPs of Li *et al.* (2009) show similar allele frequencies in both Chinese and Japanese (Table 5), which raises the intriguing possibility of a continent-specific effect. This observation is underscored by the notable differences in LD structure and allele/haplotype distribution between European and Asian populations [Tables 4 and 5, Fig. 2 and HapMap data (Consortium 2003)]. Therefore, although our study did not show any evidence for an association of *FOXO1* variants with human longevity in Germans and Italians, this does not exclude an effect that could be restricted to Chinese or Asians. Longevity in different populations is likely to be influenced by varying sets of interacting genetic and environmental factors (e.g. diet) (Caliebe *et al.*, 2010). To confirm the Asian- and female-specific longevity association of *FOXO1*, replications with more extensive DNA collections of, for instance, Japanese origin would be helpful.

Polymorphisms in *FOXO3A* have convincingly and repeatedly been associated in numerous populations with the ability to survive into old age (Willcox *et al.*, 2008; Anselmi *et al.*, 2009; Flachsbart *et al.*, 2009; Li *et al.*, 2009; Pawlikowska *et al.*, 2009; Soerensen *et al.*, 2010). Although here we used a larger sample than the one genotyped for the *FOXO3A* analysis (Flachsbart *et al.*, 2009), our results did not ascertain an association of *FOXO1*, *FOXO4*, or *FOXO6* with longevity in Germans. The members of the *FOXO* gene family share some characteristics (more than 60% sequence identity of the human FOXO domain (Anderson *et al.*, 1998), regulation via the IIS pathway and a seemingly functional redundancy *in vitro* (Coffer & Burgering, 2004)) and yet their physiological roles are thought to be diverse (Wang *et al.*, 2009), a finding that is supported by different expression patterns (Furuyama *et al.*, 2000; Biggs *et al.*, 2001). This functional diversification is also illustrated by the disruption of the *FOXO* genes (Hosaka *et al.*, 2004): while a homozygous knock-out of *FOXO1* is embryonically lethal, *FOXO3A–/–* and *FOXO4–/–* animals are viable and initially develop normal.

Table 3 Association statistics of *FOXO1* single-nucleotide polymorphisms (SNPs) in female Italian individuals and in the pooled sample of Germans and Italians

		minAF LLI	minAF controls	*P* allele	*P* geno	OR (95% C.I.)
SICS						
LLI = 166	rs2755209	0.404	0.391	0.728	0.379	1.05 (0.79–1.41)
Controls = 216	rs2755213	0.124	0.118	0.795	NA	1.06 (0.68–1.64)
Germans + SICS						
LLI = 1258	rs2755209	0.373	0.379	0.702	0.601	0.98 (0.86–1.10)
Controls = 1010	rs2755213	0.092	0.099	0.445	0.271	0.92 (0.76–1.13)

SICS, South Italian Centenarian Study; minAF, minor allele frequency; LLI, long-lived individuals; n, number of individuals; *P* allele, *P* value obtained from allele-based case–control comparison using a chi square-test with 1 degree of freedom (df); *P* geno, *P* value obtained from genotype-based case–control comparison using a chi square-test with 2 df; OR, estimated odds ratio for minor allele (based on whole sample); CI, 95% confidence interval of OR; NA, genotype-based case–control comparison was not conducted when there were fewer than five observations.

Table 4 Comparison of *FOXO1* haplotype association results in German and Chinese females

	Germans			Chinese‡		
Haplotype†	Freq. cases	Freq. controls	*P* value	Freq. cases	Freq. controls	*P* value
ATG§	**0.608**	**0.614**	**0.768**	**0.230**	**0.292**	**0.003**
CTG	0.292	0.287	0.811	0.116	0.127	0.550
CCG	**0.060**	**0.061**	**0.901**	**0.248**	**0.192**	**0.006**
CCT	0.031	0.027	0.581	0.392	0.380	0.604
CTT	0.005	0.007	0.478	0.008	0.004	0.380

Associated haplotypes from Li *et al.* (2009) are in bold.
freq., frequency.
†Haplotype defined by the SNPs rs2755209, rs2755213, and rs17630266.
‡Data from Li *et al.* (2009).
§Haplotype ATG was designated TTG in the study of Li *et al.* (2009).

Table 5 Comparison of minor allele frequencies of *FOXO1* single-nucleotide polymorphisms (SNPs) in German, Italian, Chinese, and Japanese controls

	minAF controls Germans	minAF controls Italians	minAF controls Chinese†	minAF controls Japanese‡
rs2755209	0.37	0.40	0.29	0.33§
rs2755213	0.09	0.11	0.42	NA
rs17630266	0.03	0.02¶	0.37	0.39††

Associated SNPs from Li *et al.* (2009) are in bold.
minAF, minor allele frequency; NA, no information available.
†Data from Li *et al.* (2009).
‡Data from Kojima *et al.* (2004).
§Based on SNP rs2297627 from Kojima *et al.* (2004) that is in strong linkage disequilibrium (LD) with rs2755209 (r^2 = 0.90, using JPT HapMap data).
¶Based on imputed data.
††Based on SNP rs2297626 from Kojima *et al.* (2004) that is in strong LD with rs17630266 (r^2 = 0.95, using JPT HapMap data).

Further studies are needed to clarify in more detail the *in vivo* roles of the *FOXO* genes, and in particular of *FOXO3A*, in human longevity.

Experimental procedures

Study participants

The study sample comprised 1447 LLI with an age range of 95–110 years (mean age: 98.8 years) and 1029 younger controls (60–75 years; mean age: 66.8 years). About 75% of the LLI were women. The controls were similar to the LLI in terms of gender (75% women), ancestry, and geographic origin. There are no mortality data available for the controls. However, based on current predictions, only 1.5% of all 60-year-old and 1.8% of all 75-year-old German women will become 100 years. The study participants provided, as part of a health and family history questionnaire, personal and medical information. A detailed description of the study population and recruitment procedures is provided elsewhere (Nebel *et al.*, 2005). All subjects gave written, informed consent prior to enrolment. The study was approved by the Ethics Committee of the University Hospital Schleswig-Holstein in Kiel.

The Italian women sample consisted of 166 LLI (90–109 years, mean age: 98.39 years) and 216 younger controls (18–48 years; mean age: 31.06 years) obtained from the Southern Italian Centenarian Study (SICS). SICS LLI were thoroughly investigated for demographic and clinical characteristics and they were enrolled by Associazione Longevità (Anselmi *et al.*, 2009). All subjects gave written informed consent to the study. The study was approved by the Institutional Review Board of the 'Istituto di Ricovero e Cura a Carattere Scientifico', Multimedica, Milano, Italy.

Genotyping

Genotyping of the German samples was performed using the SNPlex™ Genotyping System and TaqMan® SNP Genotyping Assays (both Applied Biosystems, Foster City, CA, USA) on an automated platform (Hampe *et al.*, 2001). For quality control purposes, positive controls on each genotyping plate were checked for consistency. All SNPs were tested for deviation from the HWE. The call rate for each marker exceeded 95.8%.

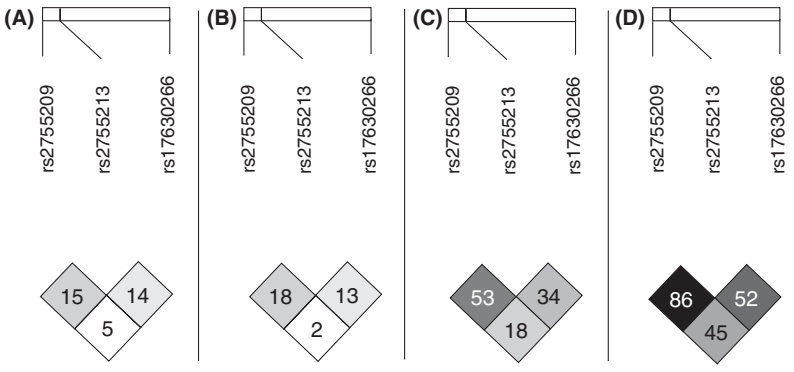


Fig. 2 LD structure of *FOXO1* in Germans (A), Italians (B), Chinese (C), and Japanese (D). The depicted single-nucleotide polymorphisms were used in the study of Chinese (Li *et al.*, 2009). The linkage disequilibrium plots are based on the measure r^2 and were generated with Haploview using HapMap data of Han Chinese (CHB) and Japanese (JPT) as well as data of German and Italian control individuals from this study.

Genotyping of SICS individuals was carried out with the Infinium II Assay-HumanHap 317K duo BeadChip system (Illumina, San Diego, CA, USA) using standard protocols. Individual samples and markers showing a genotyping rate lower than 93% and 95%, respectively, were excluded. Imputation of unobserved genotypes was performed using Impute, version 0.5 (Marchini *et al.*, 2007) with the HapMap CEU release 24 reference panel (http://www.hapmap.org/).

Statistical analysis

Haplotype-tagging SNPs (htSNPs) of *FOXO1*, *FOXO4*, and *FOXO6* were selected based on the HapMap genotypes of Utah residents with ancestry from northern and western Europe (CEU; http://www.hapmap.org/) and the pairwise tagging option implemented in the Haploview v3.32 program (Barrett *et al.*, 2005) (http://www.broad.mit.edu/mpg/haploview/) (minor allele frequency: > 0.05, pairwise $r^2 \geq 0.8$, P_{HWE}: > 0.01, for htSNPs see Table 1). The exact genomic regions for the selection of the htSNPs were as follows: for *FOXO1* chromosome 13: 40 022 274–40 144 279; for *FOXO4* X chromosome: 70 227 715–70 245 118 and for *FOXO6* chromosome 1: 41 596 756–41 624 373 (all NCBI36). The Haploview program was also applied for the haplotype analysis of *FOXO1*. An additional haplotype analysis using a two-marker sliding window was performed for all three genes with the sliding window option implemented in the programme COCAPHASE, version 2.403 that is part of PHASE, version 2.1 (http://www.stat.washington.edu/stephens/; Dudbridge, 2003). Single-point analysis was performed using PLINK v1.07 (Purcell *et al.*, 2007). $P < 0.05$ was considered statistically significant. The *a priori* power calculation for rs2755209 in the German and Italian women sample was carried out with the PS Power and Sample Size Program (PS version 3.0.14; http://medipe.psu.ac.th/episoft/pssamplesize), assuming an OR of 0.77 as observed in the Chinese study (Li *et al.*, 2009) and the minor allele frequency (40%) characteristic of the CEU HapMap sample.

Acknowledgments

We thank all study participants for their cooperation and the laboratory personnel at the Institute of Clinical Molecular Biology and the Popgen biobank for excellent technical assistance.

Funding

This study was supported by the DFG Excellence Cluster 'Inflammation at Interfaces'.

Author contributions

R.K., A.N., S.S. designed research; R.K. performed research; R.K., F.F., A.A.P, A.M. analyzed data; R.K, A.N. wrote the paper.

References

Accili D, Arden KC (2004) FoxOs at the crossroads of cellular metabolism, differentiation, and transformation. *Cell* **117**, 421–426.

Anderson MJ, Viars CS, Czekay S, Cavenee WK, Arden KC (1998) Cloning and characterization of three human forkhead genes that comprise an FKHR-like gene subfamily. *Genomics* **47**, 187–199.

Anselmi CV, Malovini A, Roncarati R, Novelli V, Villa F, Condorelli G, Bellazzi R, Puca AA (2009) Association of the FOXO3A locus with extreme longevity in a southern Italian centenarian study. *Rejuvenation Res.* **12**, 95–104.

Barrett JC, Fry B, Maller J, Daly MJ (2005) Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics* **21**, 263–265.

Biggs 3rd WH, Cavenee WK, Arden KC (2001) Identification and characterization of members of the FKHR (FOX O) subclass of winged-helix transcription factors in the mouse. *Mamm. Genome* **12**, 416–425.

Bonafe M, Barbieri M, Marchegiani F, Olivieri F, Ragno E, Giampieri C, Mugianesi E, Centurelli M, Franceschi C, Paolisso G (2003) Polymorphic variants of insulin-like growth factor I (IGF-I) receptor and phosphoinositide 3-kinase genes affect IGF-I plasma levels and human longevity: cues for an evolutionarily conserved mechanism of life span control. *J. Clin. Endocrinol. Metab.* **88**, 3299–3304.

Burgering BM, Kops GJ (2002) Cell cycle and death control: long live forkheads. *Trends Biochem. Sci.* **27**, 352–360.

Caliebe A, Kleindorp R, Blanche H, Christiansen L, Puca AA, Rea IM, Slagboom E, Flachsbart F, Christensen K, Rimbach G, Schreiber S, Nebel A (2010) No or only population-specific effect of PON1 on human longevity: a comprehensive meta-analysis. *Ageing Res. Rev.* **9**, 238–244.

Christensen K, Johnson TE, Vaupel JW (2006) The quest for genetic determinants of human longevity: challenges and insights. *Nat. Rev. Genet.* **7**, 436–448.

Coffer PJ, Burgering BM (2004) Forkhead-box transcription factors and their role in the immune system. *Nat. Rev. Immunol.* **4**, 889–899.

Consortium TIH (2003) The International HapMap Project. *Nature* **426**, 789–796.

Dudbridge F (2003) Pedigree disequilibrium tests for multilocus haplotypes. *Genet. Epidemiol.* **25**, 115–121.

Flachsbart F, Caliebe A, Kleindorp R, Blanche H, von Eller-Eberstein H, Nikolaus S, Schreiber S, Nebel A (2009) Association of FOXO3A variation with human longevity confirmed in German centenarians. *Proc. Natl Acad. Sci. USA* **106**, 2700–2705.

Furukawa-Hibi Y, Yoshida-Araki K, Ohta T, Ikeda K, Motoyama N (2002) FOXO forkhead transcription factors induce G(2)-M checkpoint in response to oxidative stress. *J. Biol. Chem.* **277**, 26729–26732.

Furuyama T, Nakazawa T, Nakano I, Mori N (2000) Identification of the differential distribution patterns of mRNAs and consensus binding sequences for mouse DAF-16 homologues. *Biochem. J.* **349**, 629–634.

Gögele M, Pattaro C, Fuchsberger C, Minelli C, Pramstaller PP, Wjst M (2010) Heritability analysis of life span in a semi-isolated population followed across four centuries reveals the presence of pleiotropy between life span and reproduction. *J. Gerontol. A Biol. Sci. Med. Sci.* **66**, 26–37.

Hampe J, Wollstein A, Lu T, Frevel HJ, Will M, Manaster C, Schreiber S (2001) An integrated system for high throughput TaqMan based SNP genotyping. *Bioinformatics* **17**, 654–655.

Hjelmborg JvB, Iachine I, Skytthe A, Vaupel JW, McGue M, Koskenvuo M, Kaprio J, Pedersen NL, Christensen K (2006) Genetic influence on human lifespan and longevity. *Hum. Genet.* **119**, 312–321.

Hosaka T, Biggs 3rd WH, Tieu D, Boyer AD, Varki NM, Cavenee WK, Arden KC (2004) Disruption of forkhead transcription factor (FOXO) family members in mice reveals their functional diversification. *Proc. Natl Acad. Sci. USA* **101**, 2975–2980.

Kato M, Yuan H, Xu ZG, Lanting L, Li SL, Wang M, Hu MC, Reddy MA, Natarajan R (2006) Role of the Akt/FoxO3a pathway in TGF-beta1-mediated mesangial cell dysfunction: a novel mechanism related to diabetic kidney disease. *J. Am. Soc. Nephrol.* **17**, 3325–3335.

Kenyon C (2005) The plasticity of aging: insights from long-lived mutants. *Cell* **120**, 449–460.

Kojima T, Kamei H, Aizu T, Arai Y, Takayama M, Nakazawa S, Ebihara Y, Inagaki H, Masui Y, Gondo Y, Sakaki Y, Hirose N (2004) Association analysis between longevity in the Japanese population and polymorphic variants of genes involved in insulin and insulin-like growth factor 1 signaling pathways. *Exp. Gerontol.* **39**, 1595–1598.

Kuningas M, Magi R, Westendorp RG, Slagboom PE, Remm M, van Heemst D (2007) Haplotypes in the human Foxo1a and Foxo3a genes; impact on disease and mortality at old age. *Eur. J. Hum. Genet.* **15**, 294–301.

Li Y, Wang WJ, Cao H, Lu J, Wu C, Hu FY, Guo J, Zhao L, Yang F, Zhang YX, Li W, Zheng GY, Cui H, Chen X, Zhu Z, He H, Dong B, Mo X, Zeng Y, Tian XL (2009) Genetic association of FOXO1A and FOXO3A with longevity trait in Han Chinese populations. *Hum. Mol. Genet.* **18**, 4897–4904.

Marchini J, Howie B, Myers S, McVean G, Donnelly P (2007) A new multipoint method for genome-wide association studies by imputation of genotypes. *Nat. Genet.* **39**, 906–913.

Nebel A, Croucher PJ, Stiegeler R, Nikolaus S, Krawczak M, Schreiber S (2005) No association between microsomal triglyceride transfer protein (MTP) haplotype and longevity in humans. *Proc. Natl Acad. Sci. USA* **102**, 7906–7909.

Pawlikowska L, Hu D, Huntsman S, Sung A, Chu C, Chen J, Joyner AH, Schork NJ, Hsueh WC, Reiner AP, Psaty BM, Atzmon G, Barzilai N, Cummings SR, Browner WS, Kwok PY, Ziv E (2009) Association of common genetic variation in the insulin/IGF1 signaling pathway with human longevity. *Aging Cell* **8**, 460–472.

Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MA, Bender D, Maller J, Sklar P, de Bakker PI, Daly MJ, Sham PC (2007) PLINK: a tool set for whole-genome association and population-based linkage analyses. *Am. J. Hum. Genet.* **81**, 559–575.

Soerensen M, Dato S, Christensen K, McGue M, Stevnsner T, Bohr VA, Christiansen L (2010) Replication of an association of variation in the FOXO3A gene with human longevity using both case–control and longitudinal data. *Aging Cell* **9**, 1010–1017.

Stahl M, Dijkers PF, Kops GJ, Lens SM, Coffer PJ, Burgering BM, Medema RH (2002) The forkhead transcription factor FoxO regulates transcription of p27Kip1 and Bim in response to IL-2. *J. Immunol.* **168**, 5024–5031.

Tissenbaum HA, Guarente L (2001) Increased dosage of a sir-2 gene extends lifespan in *Caenorhabditis elegans*. *Nature* **410**, 227–230.

Wang M, Zhang X, Zhao H, Wang Q, Pan Y (2009) FoxO gene family evolution in vertebrates. *BMC Evol. Biol.* **9**, 222.

Willcox BJ, Donlon TA, He Q, Chen R, Grove JS, Yano K, Masaki KH, Willcox DC, Rodriguez B, Curb JD (2008) FOXO3A genotype is strongly associated with human longevity. *Proc. Natl Acad. Sci. USA* **105**, 13987–13992.