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FOXO3 Gene Variants and Human Aging: Coding Variants May Not Be Key Players

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FOXO3 is generally recognized as a "master" gene in aging since its association with longevity has been replicated in multiple organisms and human populations. A group of single nucleotide polymorphisms in linkage disequilibrium with a coding region has been associated with human longevity, but the actual functional variant is unidentified. Therefore, we sequenced the coding region in our long-lived Japanese American population in order to enhance resources for fine mapping this region. We demonstrate that of 38 published variants, 6 are misalignments with homologous nonallelic sequences from *FOXO3B (ZNF286B)*, a pseudogene on a different chromosome; 2 are attributable to *ZNF286B* only, and the remaining 30 were unconfirmed, indicating that they are very rare and not likely involved in longevity. Furthermore, we identified a novel, unique, nonsynonymous coding variant in exon 3 (*Gly566Ala*; rs138174682) that is prevalent in multiple ethnic groups but appeared too rare for major longevity effects in our study populations.

Key Words: Aging—FOXO3—Genetic—Human longevity.

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THE world's population is rapidly aging and this has enormous implications for society. Therefore, it is vitally important to understand how and why we age in order to enhance our odds of aging with better health and less disability (1). Genetics may account for 10%–50% of the variability in human life span, depending upon the population, but little is known about the effector genes (2–6). Studies of model organisms have identified several evolutionarily conserved biological pathways that have major influence on aging and age-related traits (6–8). *Daf-16*, an evolutionarily conserved gene in nematodes was identified among early candidates as a potential modulator of human aging (8).

On this basis, we tested the hypothesis that the *daf-16* human homologue, *FOXO3*, a gene on chromosome 6 that codes for a forkhead Box O transcription factor, might be important in human aging and longevity (9; see Figure 1). We first reported on associations of three single nucleotide polymorphisms (SNPs) of FOXO3 with healthy aging and longevity in male Americans of Japanese ancestry (rs2764264 [p value = .0002], rs13217795 [p = .0006], and rs2802292 [p < .0001]), associations that were initially confirmed (with effects pointing in the same direction) in German and French populations (10) and later in Italian, American (northern and western European ancestry), Chinese, and several other populations (11–15). Since the association of *FOXO3* with longevity has been observed in genetically diverse populations of European and Asian descent, these results point to *FOXO3* playing an important role in human aging. However, the "functional" SNP has yet to be identified.

SNPs are gene variants that occur on average once every 300 bp. They frequently can be used to discover nearby functional variants that are associated with a disease or other phenotype (16). Once an association between a trait and a gene variant has been discovered, it can be difficult to find which variant is actually responsible for the biologic change that produced the phenotype because several closely linked variants may be in strong linkage disequilibrium (correlation) with the original gene variant. There are numerous SNPs within the *FOXO3* locus that are associated with longevity, but they are all located in a haplotype block on the order of 100 kb in size on chromosome 6. Because there is a high degree of linkage disequilibrium, a "longevity-associated functional" variant could be anywhere in this 100+ kb region, which is centered on the second of four introns (known as "intron 2"; see Figure 2). This putative functional variant might act through any number of mechanisms. For example, it might, when translated, change an amino acid sequence. Or it could be a splice-site variant, a transcription enhancer element, a copy-number variant, or affect some other genetic structure. The quest to identify a functional variant (typically a coding variant) in *FOXO3* will undoubtedly help us better understand the role that this gene plays in

Table 1. Primers Used for DNA Amplification and Sequencing

Primer	Sequence	Tm (°C)	Length (bp)
FOXO3AF:	ATCATCTGGGTGCTCGGTTT	62	1,503
FOXO3AR:	GACCTGCTTTGCCCACTTC	61	
FOXO3A-1F:	CCCAACCAGCTCCTTTAACA	60	
FOXO3A-1R:	TGCATAGACTGGCTGACAGG	60	
FOXO3A-3F:	ACCAATTCTAACGCCAGCAC	60	
FOXO3A-3R:	GAGTCCGAAGTGAGCAGGTC	60	
FOXO3A-7F:	GAATGAGGGAACTGGCAAGA	60	
FOXO3A-7R:	CAGGTCGTCCATGAGGTTTT	60	
FOXO3A-8F:	GACCTGCTCACTTCGGACTC	60	
FOXO3A-8R:	AAATCCAACCCATCAGCATC	60	

Note: DNA sequencing primers used in the current study, which amplifies a 1,503 bp genomic chromosome 6–specific fragment of FOXO3 just outside the region of homology with FOXO3B.

human longevity and healthy aging, but initial sequencing work is required before functional studies are undertaken.

Therefore, we conducted an initial long-range sequencing effort with two purposes: (i) validate known coding variants and (ii) identify novel variants that might be responsible for the association with the longevity phenotype.

Materials and Methods

Study Populations

Ninety-five individuals of various ethnicities (Caucasians, Filipinos, Japanese, Chinese, and multiracial) living in Hawaii contributed DNA for this sequencing study, which yielded 85 intact, complete, high quality sequences for further study; an additional 282 persons with Japanese surnames living in Hawaii were used for allele frequency determination within the Japanese American population, and 675 study participants (Japanese American men) from the Kuakini Honolulu Heart Program were used for a longevity case–control study. Procedures were performed according to institutional guidelines and approved by the Institutional Review Board of Kuakini Medical Center.

Sequencing Strategy

Chromosome 6–specific primers (FOXO3AF and FOXO3AR) were designed using the Primer3 (17) component of Geneious and were used to amplify a 1.5 kb chromosome 6–specific genomic fragment containing exon 3, the location of the majority of the previously reported coding variants in *FOXO3*. Sequences were generated and screened for nucleotide substitutions using an ABI3100 Genetic Analyzer and Geneious software, using eight primers (shown in Table 1 and the triangles in Figure 1).

FOXO3/FOXO3B Sequence Alignments

FOXO3 is known to have a pseudogene on chromosome 17, *FOXO3B* (shown in Figure 1 and 2). *FOXO3* exon 3 was used in a BLAST search (18) to examine this homology in more detail. AF032887 was chosen to represent the homology of *FOXO3B* with FOXO3 (NM_201559), with 1425/1436 identity (99%).

Mapping of FOXO3 Coding Variants

Coding variants for *FOXO3*, published in dbSNP, were used in BLAST queries in an attempt to explore the origins of the sequence variations. For this, a portion of the DNA adjacent to the SNP was used in the NCBI website (megablast) as well as local alignments using the Geneious software (Biomatters Ltd, Auckland, New Zealand).

Results

FOXO3/FOXO3B Sequence Alignment

FOXO3 is 125 kb in size, located on chromosome 6q21, and is composed of four exons (variant 2), of which exons 2 and 3 code for the translated protein. A variant of *FOXO3* (variant 1) is missing the first exon, but this does not result in an amino acid change during translation. *FOXO3* (also referred to as *FOXO3A*) is known to have a pseudogene on chromosome 17, referred to as *FOXO3B*. *FOXO3* exons 2–4 appear to represent the entire *FOXO3B* pseudogene and support the finding that the latter is an integrated processed messenger RNA from *FOXO3*. Figure 2 demonstrates this homology. We observed that a portion of the *FOXO3B* pseudogene is annotated as being contained in exon 4 of the *ZNF286B* gene (Gene ID: 729288 [not shown]).

No Validated Coding SNPs in FOXO3 in the Most Recent dbSNP Build

A recent version of the Genome Reference Consortium (GRCh37, NCBI dbSNP Build 134), released on August 8, 2011 lists *FOXO3* as having 39 polymorphic variants in the coding region (later revised down to 38 polymorphic variants in the November 8 build). This included 21 synonymous, 16 nonsynonymous (including our newly found variant), and 2 frameshift variants. These variants are of great potential interest as one of them might theoretically be the actual causal variant for the longevity and healthy aging phenotypes. We will refer to "transcript variant 2" (NM_201559.2) which describes all four exons for *FOXO3*, but transcript variant 1 (NM_001455.3) describes two exons. Both transcript variants code for the same protein, as exons 1 and 4 are noncoding but may play roles in gene regulation.

We suspected that some of these variants might not be true variants but rather result from misalignment between the coding region of the *FOXO3* gene with its highly homologous pseudogene, *FOXO3B* (Gene ID = 2310). In an attempt to validate these SNPs, we designed long-range primers that were outside the region of *FOXO3/FOXO3B* homology that would amplify a 1,503 bp chromosome 6–specific fragment, including all of exon 3 that would serve as a template for DNA sequencing. We found a paucity of variation in this

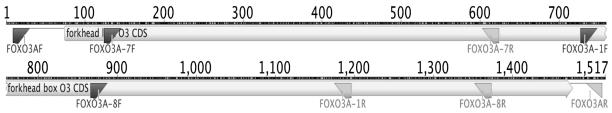

Figure 1. Sequencing strategy. Chromosome 6–specific primers were used to amplify a 1.5-Kb genomic fragment of exon 3. Sequences were generated from 95 individuals of various ethnicities living in Hawaii and screened for nucleotide substitutions. Forward primers are denoted as dark green, whereas reverse primers are light green. The yellow box denotes the protein-coding exon 3, whereas the solid line denotes the noncoding genomic sequence.

region after sequencing DNA from 95 persons in a random sample of persons living in Hawaii. These persons included primarily Caucasians, Filipinos, Japanese, and Chinese, but some were multiracial.

After completely sequencing the region of interest, we were not able to confirm the existence of any (see later) of the 38 sequence variants that were reported in the most recent version (version 134, GRCh37.p2 of November 8, 2011) of the SNP public database accessible through the NCBI website: http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?geneId=2309 (see Table 2.). One variant that was previously reported in dbSNP (August update) is no longer listed. This variant is a T to C transition at position 108985893 (rs113367269), a serine at codon 619 previously described by Watkins and colleagues (19), which does not result in an amino acid change (ie, a synonymous variant). In an attempt to determine the reasons for this failure in confirming the remaining 38 variants, we performed BLAST searches of sequences adjacent to these published *FOXO3* SNPs and found that many of them mapped to chromosome 17 with 100% homologies or were the result of misalignment with the location of the highly homologous *FOXO3* pseudogene (20), known as *FOXO3B* (Figure 2). *FOXO3B* is homologous to exons 2, 3, and 4 of *FOXO3*, and there are a number of nonallelic nucleotides that align with published SNPs. None of the exon 3 SNPs was verified by our long-range chromosome 6–specific sequencing effort. In sum, none of the coding SNPs listed in dbSNP could be verified. Eight of the 38 previously published coding SNPs were found to result from misalignment of sequences between chromosomes 6 and 17, the remaining 30 could not be verified (not found in our multiethnic populations; Table 2). *FOXO3B* itself lies within another gene referred to as "ZNF286B," a putative zinc finger protein 286B (Gene ID: 729288). Collectively, these results indicate that these SNPs are either extremely rare in our multiethnic populations, are the result of misalignment with other highly homologous sequences, or the result of sequencing and/or assembly errors. They are clearly not responsible for the longevity association in our prior work (9) and are unlikely to be important to human longevity.

Newly Identified Coding SNP

One novel variant that we discovered in the current sequencing effort is shown in Table 3; it was a nonsynonymous SNP located at position 108985733 on chromosome 6 which results in a G to C transversion and an alanine being substituted for a glycine at amino acid 566 (SNP ID: rs138174682).

Allele Frequencies of G1697C (Gly566Ala)

A collection of 85 random multiethnic (Caucasian, Filipino, Japanese, Chinese, and mixed ethnicity) genetic samples was chosen for allele frequency determination from the original 95 sequenced samples on the basis of intact, complete, high quality sequences. The ages of these participants ranged from 10 to 78 years. We found that of 85 individuals, 10 were heterozygous and 2 were homozygous for the *G1697C* variant (rs138174682; see Table 4). To determine if this variant might be useful to study longevity in our Kuakini Honolulu Heart Program cohort (American men of Japanese ancestry), we selected 282 persons with Japanese surnames for a further analysis. Of these 282, only 8 were heterozygous and none was homozygous for this variant. Because the allele frequency was not extremely rare, we decided to assess this SNP with our original Hawaii Lifespan Study case–control study sample (9) to assess whether it might be an important contributor to the "longevity phenotype." The cases had a longevity phenotype defined as survival to at least 95 years of age

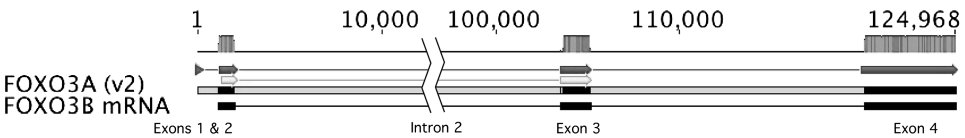

Figure 2. FOXO3 Genomic and FOXO3B mRNA Alignment. Shown is an alignment of the FOXO3 gene (GenBank NM_201559.2), also known as FOXO3A, with the homologous genomic sequences on chromosome 17 (FOXO3B [NR_026718, complement]). Homologies are noted in the top row with green bars. The red arrows refer to the FOXO3 transcript, version 2. The yellow arrows denote the protein-coding exons 2 and 3, and the black boxes denote the noncoding FOXO3B transcript. It is apparent from this alignment that the FOXO3B pseudogene is derived from the FOXO3 processed transcript variant 2, including exons 2–4. The present study assessed single nucleotide polymorphisms from exons 2–3, shown by the yellow arrows. The large intron 2 has been truncated to reduce the size of the figure.

Table 2. Evaluation of Variants in FOXO3 From the dbSNP Public Database

Chromosome Position	mRNA Position	dbSNP rs# Cluster ID	MAF	Function	dbSNP Allele	Protein Residue	Codon Position	Amino Acid Position	Present Results
108882570	502	rs11757217	0.1283	Synonymous	T	Ala [A]	3	53	ZNF286B
				Contig reference	C	Ala [A]	3	53	
108882706	638	rs79884776		Missense	T	Trp [W]	1	99	Not conf.
				Contig reference	C	Arg [R]	1	99	
108882830	762	rs111556510	0.0345	Missense	T	Val [V]	2	140	Not conf.
				Contig reference	C	Ala [A]	2	140	
108882855	787	rs13204476		Missense	T	Ser [S]	3	148	Not conf.
				Contig reference	G	Arg [R]	3	148	
108882873	805	rs146186567		Synonymous	A	Arg [R]	3	154	Not conf.
				Contig reference	G	Arg [R]	3	154	
108882888	820	rs61758963		Synonymous	T	Asn [N]	3	159	Not conf.
				Contig reference	C	Asn [N]	3	159	
108882891	823	rs139172563		Synonymous	A	Leu [L]	3	160	Not conf.
				Contig reference	G	Leu [L]	3	160	
108882906	838	rs149906214		Synonymous	A	Leu [L]	3	165	Not conf.
				Contig reference	G	Leu [L]	3	165	
108882915	847	rs150320900		Synonymous	T	Arg [R]	3	168	Not conf.
				Contig reference	C	Arg [R]	3	168	
108882933	865	rs149189425		Synonymous	A	Pro [P]	3	174	Not conf.
				Contig reference	G	Pro [P]	3	174	
108882987	919	rs142429317		Synonymous	G	Pro [P]	3	192	Not conf.
				Contig reference	C	Pro [P]	3	192	
108984783	1090	rs61756661		Synonymous	A	Arg [R]	3	249	Not conf.
				Contig reference	G	Arg [R]	3	249	
108984823	1130	rs141893794		Missense	G	Gly [G]	1	263	Not conf.
				Contig reference	A	Ser [S]	1	263	
108984834	1141	rs147028825		Synonymous	T	Arg [R]	3	266	Not conf.
				Contig reference	C	Arg [R]	3	266	
108984867	1174	rs112124249		Synonymous	T	Ala [A]	3	277	Not conf.
				Contig reference	C	Ala [A]	3	277	
108984882	1189	rs138297794		Synonymous	T	Asp [D]	3	282	Not conf.
				Contig reference	C	Asp [D]	3	282	
108984883	1190	rs140968061		Missense	A	Asn [N]	1	283	Not conf.
				Contig reference	G	Asp [D]	1	283	
108984951	1258	rs150216371		Synonymous	T	Ala [A]	3	305	Not conf.
				Contig reference	G	Ala [A]	3	305	
108985003	1310	rs145756480		Missense	T	Cys [C]	1	323	Not conf.
				Contig reference	C	Arg [R]	1	323	
108985057	1364	rs145259784		Missense	A	Thr [T]	1	341	Not conf.
				Contig reference	G	Ala [A]	1	341	
108985080	1387	rs149503832		Synonymous	T	Tyr [Y]	3	348	Not conf.
				Contig reference	C	Tyr [Y]	3	348	
108985092	1399	rs149158541		Synonymous	G	Ala [A]	3	352	Misalign
				Contig reference	C	Ala [A]	3	352	
108985094	1401	rs11551770		Missense	T	Ile [I]	2	353	Not conf.
				Contig reference	G	Ser [S]	2	353	
108985119	1426	rs146009555		Synonymous	C	Pro [P]	3	361	Not conf.
				Contig reference	G	Pro [P]	3	361	
108985136	1443	rs34223850		Missense	T	Leu [L]	2	367	Misalign
				Contig reference	C	Pro [P]	2	367	
108985149	1456	rs34754045		Synonymous	C	Asp [D]	3	371	Misalign
				Contig reference	T	Asp [D]	3	371	
108985176	1483	rs34133353		Frameshift	G	Glu [E]	3	380	Misalign
				Contig reference		Asp [D]	3	380	
108985269	1576	rs34079373	0.0147	Synonymous	T	Ser [S]	3	411	Misalign
				Contig reference	C	Ser [S]	3	411	
108985274	1581	rs138742093		Missense	C	Thr [T]	2	413	Not conf.
				Contig reference	G	Ser [S]	2	413	
108985277	1584	rs148296241		Missense	A	Tyr [Y]	2	414	Not conf.
				Contig reference	T	Phe [F]	2	414	
108985280	1587	rs141876866		Missense	T	Leu [L]	2	415	Not conf.
				Contig reference	C	Pro [P]	2	415	
108985306	1613	rs34488332	0.0090	Missense	A	Ser [S]	1	424	Misalign

Table 2. (*Continued*)

Table 2. (*Continued*)

Chromosome Position	mRNA Position	dbSNP rs# Cluster ID	MAF	Function	dbSNP Allele	Protein Residue	Codon Position	Amino Acid Position	Present Results
				Contig reference	G	Gly [G]	1	424	
108985326	1633	rs146169955		Synonymous	C	Phe [F]	3	430	Not conf.
				Contig reference	T	Phe [F]	3	430	
108985552	1859	rs148405845		Missense	T	Cys [C]	1	506	Not conf.
				Contig reference	C	Arg [R]	1	506	
108985557	1864	rs142533192		Synonymous	C	Arg [R]	3	507	Not conf.
				Contig reference	G	Arg [R]	3	507	
108985581	1888	rs150535671		Synonymous	A	Pro [P]	3	515	Not conf.
				Contig reference	G	Pro [P]	3	515	
108985618	1925	rs147010831		Missense	G	Val [V]	1	528	ZNF286B
				Contig reference	T	Leu [L]	1	528	
108985733	2040	rs138174682		Missense	C	Ala [A]	2	566	This study
				Contig reference	G	Gly [G]	2	566	
108985856	2163	rs34600091		Frameshift	C	Ser [S]	2	607	Not conf.
				Contig reference		Phe [F]	2	607	

Note: Chromosome position is in reference to the Genome Reference Consortium (GRCh37.2, NCBI dbSNP Build 132 [NT_025741]) released November 2010. The mRNA position is in reference to the "A" in the AUG start codon, and codon refers to the position in the amino acid codon at which the variant occurs. "ZNF286B" indicates that the SNP has been assigned to the ZNF286B gene on chromosome 17; "not conf." indicates that there is no evidence for this SNP in the present study; "misalign" indicates that the SNP appears to result from misalignment of FOXO3 and FOXO3B sequences. Variant "rs138174682" was identified in the present study and is now listed in dbSNP. MAF = minor allele frequency, mRNA = messenger RNA, and SNP = single nucleotide polymorphism.

(mean attained age 97.9 years), and the controls had an "average-lived phenotype" defined as death by age 81 years (mean attained age 78.5 years). There were 675 study participants. For these men, the allele frequency was 0.01, with only 14 heterozygotes. The small number of carriers precluded any meaningful statistical power for association studies.

Regarding the *C1857T* variant (chromosome position 108985893), at codon 619, only 2 of 85 individuals in the random Hawaii sample were heterozygous—for an allele frequency of 0.012, also precluding any meaningful power for association studies.

Discussion

FOXO3 is generally recognized as a "master" gene for human aging because it represents one of only two genes with strong associations with aging-related phenotypes and longevity that have had replications in multiple human populations (9–15). The other—*ApoE*—is a lipid transport gene, which, while also pleiotropic, appears to act principally via cardiovascular mechanisms on several age-related diseases (15). The *E4* allele is the principal risk allele (15). Conversely, the functional SNP in *FOXO3* is not known—nor is the mechanism(s) of influence over human aging.

The functions of *FOXO3* are highly pleiotropic, ranging from tumor suppression to energy metabolism and thus could affect specific age-related diseases, such as cancer and cardiovascular diseases and/or influence organismal processes, such as oxidative stress, that have wider implications for aging and its related phenotypes (6,21). Important downstream targets of *FOXO3* include autophagy, apoptosis, glycolysis and gluconeogenesis, cell proliferation/differentiation, and stress resistance. Therefore, finding the functional variant and its mechanism would be an important step in understanding human aging.

Although our newly discovered *Gly566Ala* variant (rs138174682) does not appear to be involved in any known modification sites at amino acid 566, such as acetylation, phosphorylation, or ubiquitination, it is three bases away from a cyclic-AMP responsive element binding (CREB-binding protein) site at K569 (22,23). While this is a conservative amino acid substitution, it might change the conformation of the *FOXO3* protein so could theoretically have functional implications. This variant and others identified from further

Table 3. Validated Variants in *FOXO3* exon 3

Chromosome Position	mRNA Position	SNP ID	MAF	Type	Allele	Amino Acid	Codon	Amino Acid
108985733	1697	n/a	0.082	Missense	G	Gly [G]	2	566
					C	Ala [A]	2	566
108985893	1857	n/a	0.012	Synonymous	C	Ser [S]	3	619
					T	Ser [S]	3	619

Note: Chromosome position is in reference to the Genome Reference Consortium (GRCh37, NCBI dbSNP Build 132 [NT_025741]) released October 9, 2010. The mRNA position is in reference to the "A" in the AUG start codon, SNP IDs are those designated in dbSNP, and codon refers to the position in the amino acid codon at which the variant occurs. A multiethnic group of 85 participants were sequenced for a total of 170 chromosomes. MAF = minor allele frequency, mRNA = messenger RNA, and SNP = single nucleotide polymorphism.

Table 4. Allele Frequencies of G1697C in Different Populations

Random Multiethnic in Hawaii (n = 85)	Japanese Surnames in Hawaii (n = 282)	KHHP (n = 675)
14/170 alleles (0.082)	8/564 (0.014)	14/1,350 (0.0104)

Note: Allele frequencies for the G566A variant at chromosome position 108985733 were determined in (1) a random sample of 85 participants living in Honolulu, HI, including Filipinos, Japanese, Chinese, and Caucasian (Random Hawaii); (2) 282 random samples from participants with Japanese surnames living in Honolulu, HI; and (3) 675 participants from the Kuakini Honolulu Heart Program (KHHP).

sequencing efforts will require validation in other populations. Unfortunately, the variant was too infrequent in our study population of Japanese Americans to account for our previous study findings (9), but it might theoretically contribute to longevity in other populations.

While this variant might appear promising, there is a plethora of human studies that have found one or more genetic variants associated with longevity, but the vast majority has not been replicated. This has historically been the rule rather than the exception. For example, in 1987, in the first candidate gene studies of human longevity, several *HLA* genes found in Okinawans (24) were not replicated until over a decade later when one of the original findings (*DR alleles*) were replicated in a European population (25). The second major genetic finding, the *APOE* gene (26) was widely replicated over the next several years (for review, see [15]) and inspired a plethora of studies of new candidate longevity genes and genome-wide scans (27). Over the next decade, multiple novel genetic findings were reported with inconsistent or no replications until the *FOXO3* gene finding (9). Thereafter, multiple replications occurred in less than 2 years (for review, see [15]).

The fact that only two genes (*APOE* and *FOXO3*) have consistent, and widespread replication is puzzling. What is the reason for the general dearth of replications for most preliminary genetic findings? One potential cause that can lead to apparent lack of replication is population-specific variants. Similar phenotypes may result from completely different variants that are common in one population but simply do not exist in other populations, such as population-specific variation within the *CETP* gene. A relation between variation within this gene and human longevity was first reported by Barzilai and colleagues (28) in Ashkenazi Jews but was never replicated until Koropatnick and colleagues (29) tested a population-specific variant in American men of Japanese ancestry from the Honolulu Heart Program and Hawaii Lifespan Study. The particular SNPs thought to be responsible for the shared phenotypes (elevated high density lipoprotein and exceptional longevity) do not exist in the other population. Had Koropatnick and colleagues (29) focused on the specific SNPs found in the initial study of Ashkenazi Jews (and/or those in LD), this may have led to another uninformative study. Other potentially more common reasons for lack of replication include the existence of rare variants, misalignments with other highly homologous sequences, sequencing/assembly errors, among other causes.

Regarding the current study, rapid progress in genomic methodology has made sequencing a viable and cost-effective approach to uncover variants that can then be assessed for functional significance. SNPs found within a coding sequence have generally been of most interest because they are more likely to alter the biological function of a protein than those in noncoding sequences. Therefore, the current investigation focused on all putative coding sequence variants (n = 38) that were reported in the most recent version of the SNP public database (see earlier). Surprisingly, only one of the SNPs (located in exon 3) was verified by our long-range chromosome 6–specific sequencing (a noncoding SNP). Further investigation revealed that, in fact, 8 of the 38 previously published coding SNPs resulted from misalignment of sequences between chromosomes 6 and 17 (Table 2), the remaining 30 are from exon 3 and could not be verified (not found at all in our populations).

This highlights the aforementioned replication issue and points to an important additional challenge for future studies. That is, a significant proportion of SNPs in public databases is, in fact, not well curated and is composed of paralogous sequence variants. Paralogous sequence variants can arise by segmental duplications of the human genome sequence as well as by homologies with pseudogenes (30). Musumeci and colleagues (31) provide evidence that as many as 8.32% of the biallelic coding SNPs listed in the NCBI's dbSNP database are not reliable and appear to be artifacts due to the presence of highly similar genes in the human genome. This problem arises when there is extensive DNA sequence similarity between two or more genomic regions and their sequence differences masquerade as normal SNPs. Care must be taken to reduce this problem in DNA sequencing studies so that primer selection includes unique-copy regions. In the present study, we report that all 38 of the previously reported coding SNPs in *FOXO3* are either invalid or too rare to be useful for phenotype–genotype association and other genetic mapping studies.

The plethora of large-scale genotyping efforts, such as genome-wide association studies that utilize nonvalidated variants from short "shotgun" sequencing efforts, will no doubt add many invalid variants to public available data sets that may lead to wasted efforts and unrealistic expectations when mapping complex genetic traits (32). Through the use of longer range DNA sequencing and careful annotation of the genome, primarily through efforts like the ENCODE Project (33), there should be an improvement in the resources that can help elucidate functional variants that are associated with complex phenotypes.

With regard to discovering the longevity-associated functional variant for *FOXO3*, this is as yet unresolved. It appears from the current study that it is unlikely (although not impossible) that a coding SNP will be identified as the functional variant responsible for human longevity. Interestingly,

Banasik and colleagues (34) did find that one variant in intron 2, rs2802292, a noncoding SNP, demonstrates a small but significant difference in messenger RNA levels in functional studies. This same variant had the strongest association in our previous case–control study of healthy aging and longevity (9). Whether this SNP is the functional variant remains to be proven and more functional studies are required. For example, cell physiology experiments measuring messenger RNA levels need to be performed, keeping all genetic information the same, except for this variant, as well as aforementioned model organism and human studies.

In conclusion, all published nonsynonymous (coding and result in amino acid change) SNPs identified in the *FOXO3* gene, to date, are either invalid or too rare to be of consequence to human longevity. The novel variant that we identified through detailed sequencing appears to be too low in frequency (0.082) to be responsible for our previously reported association of *FOXO3* variants with human longevity (9), although it is conceivable that this novel SNP is associated with a disease phenotype at a younger age or is important in other populations. This suggests that the functional SNP is most likely a noncoding SNP from intron 2, where all previously reported associations have occurred. Future research should include complete sequencing of intron 2 and annotation of regulatory elements, in order to identify candidates for functional study of the *FOXO3* gene and human aging.

In the future, when more evidence accumulates as to a functional SNP(s), rigorous assessment with model organisms and human studies will be needed. Work with model organisms has already provided some clues that might be helpful for mechanistic studies of the effects of *FOXO3* variation in humans. For example, oxidative stress, still a leading theory of how we age (35,36), is one potential mechanism. Indeed, the PI3K/Akt signaling cascade, a downstream target of insulin signaling, is an important inhibitor of *FOXO* function. Important antioxidant enzyme expression, including manganese superoxide dismutase (MnSOD) and catalase, depend on PI3K/Akt/FOXO3 activity (37,38), and oxidative stress appears linked to human longevity (39). Yet, work to date shows that mice deficient in the *FOXO* target MnSOD do not exhibit changes in life span (40). Nor do mice overexpressing the *FOXO* target MnSOD exhibit changes in life span (41). Interestingly, Ferber and colleagues (42) recently found that *FOXO3* activation results in the repression of nuclear-encoded genes with mitochondrial function, this mediated by inhibition of c-Myc (independent of SOD2). *FOXO3* activation also reduced mitochondrial DNA copy number, mitochondrial protein expression, respiratory complexes, and mitochondrial respiratory complexes and activity. More work is needed to assess potential relations between *FOXO3*, oxidative stress, and longevity, such as whether expression of common antioxidant enzymes correlates with genetic variation in *FOXO3* or with other factors that influence *FOXO3* expression.

Some of the most basic groundwork in humans could begin with assessment of the potential relation between mortality from the common diseases of aging, including coronary heart disease, stroke, and cancer, which account for the majority of human deaths, and variation in *FOXO3*. Already, there is growing evidence for *FOXO3* as a tumor suppressor. For example, downregulation of *FOXO3* activity is seen in various cancers (43,44). Indeed, the Hawaii Lifespan Study men who had the longevity phenotype (and were more likely to have a protective *FOXO3 genotype*) had fewer cancers in their medical history (9). Ongoing studies with prospectively collected data can address whether and how cause of death is modified by variation in the *FOXO3* gene. This will provide further clues as to mechanisms for *FOXO3*'s effects on human aging and longevity. Such information may enhance translational efforts that link model organism and human research and could have important implications for human health span (45).

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