Research Paper

FOXO3 longevity genotype mitigates the increased mortality risk in men with a cardiometabolic disease

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ABSTRACT

***FOXO3* is a prominent longevity gene. To date, no-one has examined whether longevity-associated *FOXO3* genetic variants protect against mortality in all individuals, or only in those with aging-related diseases. We therefore tested longevity-associated *FOXO3* single nucleotide polymorphisms in a haplotype block for association with mortality in 3,584 elderly American men of Japanese ancestry, 2,512 with and 1,072 without a cardiometabolic disease (CMD). At baseline (1991–1993), 1,010 CMD subjects had diabetes, 1,919 had hypertension, and 738 had coronary heart disease (CHD). Follow-up until Dec 31, 2019 found that in CMD-affected individuals, longevity-associated alleles of *FOXO3* were associated with significantly longer lifespan: haplotype hazard ratio 0.81 (95% CI 0.72-0.91; diabetes 0.77, hypertension 0.82, CHD 0.83). Overall, men with a CMD had higher mortality than men without a CMD (P=6x10^{-7}). However, those men with a CMD who had the *FOXO3* longevity genotype had similar survival as men without a CMD. In men without a CMD there was no association of longevity-associated alleles of *FOXO3* with lifespan. Our study provides novel insights into the basis for the long-established role of *FOXO3* as a longevity gene. We suggest that the *FOXO3* longevity genotype increases lifespan only in at-risk individuals by protection against cardiometabolic stress.**

INTRODUCTION

Longevity can be accompanied by either healthy aging (i.e., an absence of life-threatening diseases and chronic conditions), or unhealthy aging, in which elderly individuals live long lives with chronic conditions. Genetic factors make a 15–40% contribution to lifespan [1], and may be as high as 48% in the oldest-old [2]. At very old age (> 90 years) specific longevity genes may dominate over environmental influences in determination of eventual lifespan, with evidence that there is a stronger effect in males [3].

Our study focused on *FOXO3*, the gene encoding the forkhead/winged helix box-O3 (FoxO3) transcription factor. Minor alleles of single nucleotide polymorphisms (SNPs) located in *FOXO3* have been found to be strongly associated with human longevity in multiple population subgroups worldwide [4–6]. This includes genome-wide association study data [7]. We

found previously that a haplotype comprised of 14 *FOXO3* SNPs is associated with increased likelihood of living close to 100 years in the Kuakini Honolulu Heart Program/Kuakini and Honolulu-Asia Aging Study (KHHP/KHAAS) cohort [8]. FoxO3 can differentially induce gene expression programs depending on chromatin architecture by binding to specific enhancers, activating these by causing changes in histone acetylation, and subsequent recruitment of RNA polymerase II [9]. In response to cellular stress, *FOXO3* may also function at the genomic level by facilitating long-range gene-gene interactions, changes in chromatin conformation, and interaction of topologically associated domains to regulate multiple neighboring genes involved in various processes that contribute to cell resilience, namely autophagy, stress response, energy/nutrient sensing, cell proliferation, apoptosis, and stem cell maintenance [8, 10]. The expression of at least some of these neighboring genes is increased following cellular stress (see supplement to ref [8]). Thus, *FOXO3* is positioned at the center of an "aging hub." The longevity alleles of *FOXO3* enable enhanced *FOXO3* expression, thereby activating other genes in its functional neighborhood. Physiologically, we now know that the major reason for the increase in lifespan conferred by protective alleles of *FOXO3* is protection against death from coronary heart disease (CHD) [11], although in other cohorts such protection may extend to cancer and stroke [12, 13].

The present study addresses the hypothesis that the *FOXO3* longevity-associated alleles increase cell and organism resilience by protecting against cellular stress caused by chronic conditions of aging. To test this hypothesis, we compared the effect of longevity-associated alleles with lifespan in subjects with and without type 2 diabetes, CHD and hypertension (collectively referred to as cardiometabolic diseases, CMDs).

RESULTS

Characteristics of subjects

Supplementary Table 1 shows age-adjusted baseline (1991–1993) characteristics of men in the study, adjusting for age, genotypes of the strongest SNP (*rs2802292*), and prevalence of medical conditions. By 31 December 2019, 3,548 out of 3,584 of the subjects had died during the 29 years of follow-up from exam 4. At exam 4 (baseline) among the 3,584 participants, there were 21% who had been diagnosed with CHD, 29% with diabetes, 54% with hypertension, and 14% with cancer. Seventy percent had at least one CMD, and 5.2% had all 3 components of CMD. Mean age at death was 88.6 ± 6.1 years for those with at least one of these CMDs, and 89.5 ± 6.0 years for those who did not have any CMD ($p < 0.0001$).

FOXO3 genotype and survival in CMD and non-CMD subjects.

The 14 *FOXO3* SNPs (shown in Supplementary Figures 1, 2) likely function as a *cis*-regulatory unit. Survival curves for men without a CMD and those with a CMD showed that the former lived longer (Kaplan-Meier Log-rank $\chi^2 = 24.8$, $p = 6.4 \times 10^{-7}$). Figure 1 shows survival curves for CMD subjects and subjects without a CMD according to whether they were carriers of the longevity-associated (*G*) allele of the strongest *FOXO3* SNP, *rs2802292*, or were homozygous for the major (*T*) allele. These curves were determined using a Cox proportional hazard model adding an interaction term of *FOXO3* with CMD. Only in CMD subjects was the *G* allele associated with greater lifespan than *TT* ($p = 0.0002$). Subjects without a CMD had the longest lifespans. Moreover, in contrast to CMD subjects, *FOXO3* genotype was not associated with lifespan in subjects without a CMD. Figure 2 shows mortality risk as hazard ratio for CMD subjects and subjects without a CMD according to whether they were carriers of the longevity-associated (*G*) allele of the strongest *FOXO3* SNP, *rs2802292*, or were homozygous for the major (*T*) allele. In men with CMD and the *FOXO3* longevity genotype, mortality was reduced to that of men without CMD.

Table 1 shows age-adjusted baseline characteristics of men with CMD and men without a CMD. Analyses found no evidence of population stratification in the dataset (data not shown). In subjects with *either* diabetes, CHD, or hypertension (CMD) *at baseline*, *G* allele carriers had significantly lower CHD prevalence, but higher diabetes and hypertension (Table 1). In CMD subjects, after adjusting for co-variates, minor allele carriers of each of the 8 *FOXO3* SNPs tested exhibited an association with longer lifespan (Table 2). In contrast, in individuals without any CMD component, there was no difference in lifespan associated with *FOXO3* genotype. Table 3 shows the association with just CHD, just diabetes, and just hypertension for each of the 8 *FOXO3* SNPs and the haplotype of each. Possession of the longevity-associated minor allele was associated with lower hazard ratio for mortality compared with major allele homozygotes in the CMD group when adjusted for age, and, in the full model, after adjustment for additional covariates that represent mortality risk factors.

DISCUSSION

Here we show for the first time that longevity-associated genetic variants of *FOXO3* are associated

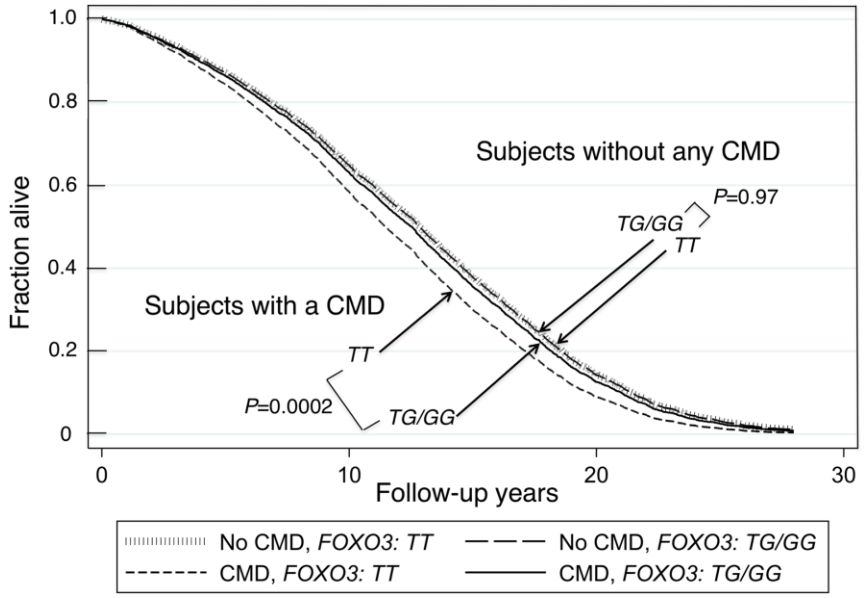

Figure 1. Survival curves spanning the period from baseline (1991–1993) to Dec 31, 2019 for subjects with and without a CMD according to whether they were carriers of the longevity-associated *G* allele of SNP *rs2802292*. The survival probabilities were estimated from the Cox proportional hazard model (see Methods) h(t) = h(t0) * exp(β1*Age + β2*BMI + β3*Glucose + β4*CMD + β5**FOXO3_G* + β6* (CMD**FOXO3_G*)) by fixing age at 75 years, BMI at the mean, 23.5 kg/m^2, and glucose at the mean, 113 mg/dL (where β6 is the effect of the interaction of CMD with *FOXO3* genotype (*G* carriers vs. *TT* genotype) on mortality, giving *P*(β6) = 0.04). The *P* values for comparison of survival curves for the group without any CMD for *FOXO3-G* carriers vs. *FOXO3-TT*, and comparison of survival curves for the group with a CMD for *FOXO3-G* carriers vs. *FOXO3-TT*, were *P*=0.97 and *P*=0.0002, respectively. The *P*-values for comparison of survival curves for *FOXO3-TT* carriers or for *FOXO3-G* carriers for those with a CMD versus those without any CMD, were *P*=0.000039 and *P*=0.28 respectively.

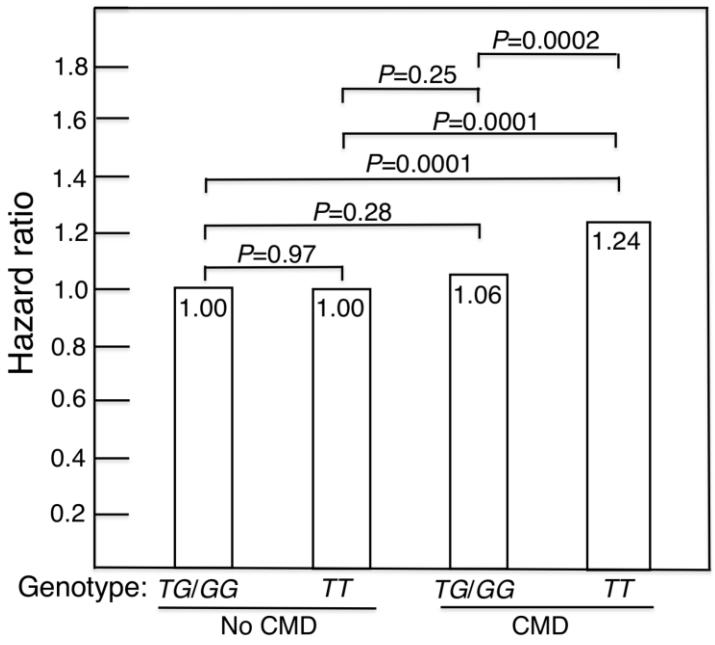

Figure 2. Mortality risk expressed as hazard ratio, adjusted for age, BMI and glucose, for CMD subjects and subjects without a CMD according to whether they were carriers of the longevity-associated (*G*) allele of the *FOXO3* SNP *rs2802292* (*TG*/*GG*) or were homozygous for the major (*T*) allele (i.e., were *TT*). In men with CMD and the *FOXO3* longevity genotype, mortality risk was reduced to normal (i.e., did not differ significantly from that of men without CMD).

Table 1. Age-adjusted baseline characteristics by CMD* and *FOXO rs2802292* genotype.

Variable	With a CMD			Without a CMD		
	TT	*GG/GT*	*P*	*TT*	*GG/GT*	*P*
n	1331	1181		570	502	
Age (years)	77.6 ± 4.5	78 ± 4.6	0.03	77.3 ± 4.4	78.1 ± 4.8	0.053
BMI (kg/m²)	23.7 ± 3.1	23.7 ± 3.0	0.62	22.8 ± 3	23 ± 3.0	0.37
Fasting plasma glucose (mg/dL)	116.8 ± 31.9	119.3 ± 35.2	0.060	101.3 ± 8.5	101.3 ± 8.6	1.00
Smoking (pack-years)	25.4 ± 33.4	28.5 ± 36.1	0.036	24.8 ± 33.4	23.9 ± 32.6	0.67
Alcohol intake (oz/mo)	19.6 ± 42.5	20.2 ± 44.3	0.76	16.2 ± 32.4	16.6 ± 33.4	0.85
Physical activity index	30.8 ± 4.8	30.8 ± 4.3	0.81	30.9 ± 4.3	31.2 ± 4.9	0.23
Depressive symptoms (%)	9.8	11.0	0.36	10.4	11.2	0.67
Stroke (%)	5.2	4.9	0.71	2.5	2.3	0.86
Cancer (%)	14.1	12.5	0.24	14.6	12.8	0.39
CHD (%)	31.7	26.7	0.01	–	–	–
Diabetes (%)	38.5	43.2	0.02	–	–	–
Hypertension (%)	75.9	77.0	0.50	–	–	–

Values shown are age-adjusted mean ± SD for indirect measures and proportion for direct measurements, except age.
*CMD: CHD or diabetes or hypertension

with much lower mortality in elderly individuals who have one or more of the common aging-related conditions consisting of diabetes, CHD and hypertension. In contrast, no reduction in mortality was observed in men with a CMD who did not have the longevity genotype. In addition, surprisingly, in men with CMD and the *FOXO3* longevity genotype, mortality was reduced to normal (i.e., to that of men without a CMD). We propose that longevity-associated *FOXO3* variants confer resilience against the adverse medical conditions that comprise CMD.

FoxO3 serves as a core regulator of cellular homeostasis, stress response, and longevity through its ability to modulate a variety of stress responses during nutrient shortage, oxidative stress, hypoxia, heat shock, and DNA damage [14–17]. By reducing oxidative damage responsible for aging, FoxO3-mediated responses to stress are pivotal to health-span and lifespan [18]. Depending on the stress stimulus and subcellular context, once activated, FoxO3 can induce specific sets of nuclear genes, including cell cycle inhibitors, pro-apoptotic genes, scavengers of reactive oxygen species, autophagy effectors, gluconeogenic enzymes, and others [17]. On the other hand, under glucose restriction, FoxO3 translocates to mitochondria to stimulate transcription of oxidative phosphorylation genes, thus restoring cellular ATP levels [17]. FoxO3 target genes and the pathways that their gene products serve are diverse and sometimes antagonistic, meaning FoxO3 is an adaptable player in the dynamic homeostasis of normal and stressed cells [17].

In unstimulated lymphoblast cell lines, *FOXO3* mRNA expression is significantly higher in *rs2802292 GT* vs. *TT* cells, and in response to H_2O_2-mediated stress, expression increases for each genotype ($P < 0.0001$), reaching a 3-fold higher level in *GT* than *TT* cells [8]. In this study fluorescence in situ hybridization showed that the nuclear position of *FOXO3* changed with regards to its neighbours over a >7 Mb region following stress induction, bringing distant genes into proximity with *FOXO3*. These data indicated that the longevity haplotype is better "primed" for stress response than the common haplotype [8]. If there is no cellular stress, then *FOXO3* appears to play only a small part in cell resilience.

The coronary artery and aortic arch are prone to atherosclerosis and thus CHD. FoxO3 is a central protective factor in safeguarding primate vascular homeostasis – it serves as a master regulator and key driver of aortic and coronary vascular endothelial aging [19]. FoxO3 is the top regulatory factor affecting primate vascular aging, having the highest number of target genes [19]. In various tissues, dysregulation of the developmental program is increasingly regarded as pivotal to cellular aging [20, 21]. A key player in this process is the developmental regulator FOXA2, which becomes upregulated in aging vessels. Crucially, in human aortic endothelial cells, FOXA2 binding to the *FOXO3* promoter suppresses *FOXO3* expression and compromises cell proliferation, the effect being reversed by siRNA-mediated knockdown of *FOXA2* expression [19]. Interestingly the most 5' SNP in our

Table 2. Hazard ratios (HR) of *FOXO3* minor allele carriers vs. major allele homozygotes with total mortality by cardiometabolic disease (CMD)* status.

Model	*FOXO3* SNP	With a CMD (n=2512)		Without a CMD (n=1072)	
		HR (95% CI)	*P*	HR (95% CI)	*P*
Age adjusted	*rs2802292*	0.88 (0.81-0.95)	0.0011	0.99 (0.88-1.12)	0.93
Age adjusted	*rs1935952*	0.90 (0.83-0.97)	0.0070	0.98 (0.87-1.12)	0.81
Age adjusted	*rs2253310*	0.88 (0.81-0.95)	0.0013	0.99 (0.88-1.12)	0.92
Age adjusted	*rs2764264*	0.90 (0.83-0.98)	0.012	0.97 (0.86-1.09)	0.61
Age adjusted	*rs2802288*	0.88 (0.81-0.95)	0.0012	0.99 (0.87-1.12)	0.85
Age adjusted	*rs3800230*	0.92 (0.84-1.00)	0.053	0.97 (0.84-1.11)	0.61
Age adjusted	*rs9398171*	0.90 (0.83-0.98)	0.012	0.98 (0.87-1.11)	0.77
Age adjusted	*rs12212067*	0.90 (0.81-1.00)	0.040	0.98 (0.84-1.14)	0.76
Age adjusted	*Haplotype*	0.86 (0.77-0.96)	0.0069	0.99 (0.84-1.17)	0.92
Covariate adjusted†	*rs2802292*	0.82 (0.75-0.89)	8.1E–06	1.03 (0.90-1.18)	0.63
Covariate adjusted	*rs1935952*	0.83 (0.76-0.91)	4.2E–05	1.06 (0.92-1.21)	0.44
Covariate adjusted	*rs2253310*	0.82 (0.75-0.90)	1.0E–05	1.04 (0.90-1.19)	0.61
Covariate adjusted	*rs2764264*	0.85 (0.78-0.93)	0.0002	1.03 (0.90-1.18)	0.65
Covariate adjusted	*rs2802288*	0.82 (0.75-0.89)	8.1E–06	1.03 (0.90-1.18)	0.70
Covariate adjusted	*rs3800230*	0.88 (0.80-0.97)	0.010	1.03 (0.89-1.20)	0.68
Covariate adjusted	*rs9398171*	0.84 (0.77-0.92)	0.0002	1.05 (0.92-1.21)	0.46
Covariate adjusted	*rs12212067*	0.88 (0.79-0.98)	0.023	1.05 (0.88-1.25)	0.59
Covariate adjusted	*Haplotype*¥	0.81 (0.72-0.91)	0.00031	1.07 (0.89-1.28)	0.49

*CMD: At least one of CHD, diabetes and hypertension.

†Covariates in Cox model: age, BMI, glucose, smoking pack-year, alcohol consumption (ounces per month), physical activity index (PAI), depressive symptoms, cancer and stroke.

¥Haplotype was obtained for 1,740 subjects with a CMD, and 742 subjects without a CMD.

Haplotype comprised minor allele frequencies of SNPs *rs2253310* (allele *C*), *rs2802288* (allele *A*), *rs2802292* (allele *G*), *rs2764264* (allele *C*), *rs9398171* (allele *C*), *rs12212067* (*G*), and *rs3800230* (allele *G*).

Table 3. Hazard ratios (HR) of *FOXO3* minor allele carriers vs. major allele homozygotes with total mortality by diabetes, CHD, and hypertension.

			With a CMD		Without a CMD	
SNP	Cardiometabolic disease	Model*	HR (95% CI) **	*P*	HR (95% CI)	*P*
rs2802292	Diabetes	1	0.84 (0.74-0.95)	0.0064	0.94 (0.87-1.01)	0.11
	(1010, 2525) ***	2	0.78 (0.68-0.89)	0.0003	0.93 (0.85-1.01)	0.08
	CHD	1	0.84 (0.73-0.97)	0.021	0.94 (0.88-1.02)	0.13
	(738, 2846)	2	0.79 (0.67-0.93)	0.0055	0.92 (0.85-1.00)	0.04
	Hypertension	1	0.89 (0.81-0.97)	0.0085	0.94 (0.85-1.04)	0.24
	(1919, 1665)	2	0.83 (0.75-0.92)	0.0003	0.94 (0.84-1.04)	0.22
rs1935952	Diabetes	1	0.84 (0.74-0.96)	0.0075	0.95 (0.88-1.03)	0.25
	(1010, 2521)	2	0.78 (0.68-0.90)	0.0005	0.95 (0.87-1.03)	0.22
	CHD	1	0.85 (0.73-0.98)	0.030	0.95 (0.88-1.03)	0.20
	(738, 2842)	2	0.81 (0.68-0.96)	0.015	0.93 (0.85-1.01)	0.08
	Hypertension	1	0.89 (0.82-0.98)	0.017	0.95 (0.86-1.05)	0.34
	(1917, 1663)	2	0.83 (0.75-0.92)	0.0002	0.97 (0.87-1.08)	0.60
rs2253310	Diabetes	1	0.84 (0.74-0.96)	0.0076	0.94 (0.87-1.01)	0.11
	(1010, 2523)	2	0.78 (0.69-0.90)	0.0004	0.93 (0.85-1.01)	0.08
	CHD	1	0.84 (0.73-0.97)	0.021	0.95 (0.88-1.02)	0.14

	(738, 2844)	2	0.79 (0.67-0.93)	0.0055	0.92 (0.85-1.00)	0.04
	Hypertension	1	0.89 (0.81-0.97)	0.0088	0.94 (0.86-1.04)	0.25
	(1918, 1664)	2	0.83 (0.75-0.92)	0.0003	0.94 (0.84-1.04)	0.24
	Diabetes	1	0.85 (0.75-0.96)	0.0097	0.95 (0.88-1.03)	0.20
	(1009, 2521)	2	0.80 (0.70-0.92)	0.0014	0.95 (0.87-1.04)	0.25
rs2764264	CHD	1	0.85 (0.73-0.99)	0.034	0.95 (0.89-1.03)	0.21
	(736, 2843)	2	0.81 (0.69-0.96)	0.015	0.94 (0.86-1.02)	0.13
	Hypertension	1	0.91 (0.83-1.00)	0.041	0.94 (0.85-1.03)	0.20
	(1915, 1664)	2	0.86 (0.78-0.95)	0.0028	0.95 (0.85-1.06)	0.38
	Diabetes	1	0.84 (0.74-0.95)	0.0062	0.93 (0.86-1.01)	0.10
	(1009, 2516)	2	0.78 (0.68-0.89)	0.0002	0.92 (0.85-1.01)	0.07
rs2802288	CHD	1	0.84 (0.72-0.97)	0.0182	0.94 (0.88-1.02)	0.13
	(736, 2838)	2	0.79 (0.67-0.93)	0.0047	0.92 (0.84-0.99)	0.03
	Hypertension	1	0.89 (0.81-0.97)	0.0084	0.94 (0.85-1.04)	0.22
	(1915, 1659)	2	0.83 (0.75-0.92)	0.0003	0.93 (0.84-1.04)	0.19
	Diabetes	1	0.89 (0.77-1.02)	0.1038	0.96 (0.88-1.05)	0.38
	(998, 2501)	2	0.88 (0.76-1.02)	0.095	0.93 (0.85-1.03)	0.17
rs3800230	CHD	1	0.96 (0.81-1.14)	0.68	0.94 (0.86-1.02)	0.12
	(731, 2817)	2	0.92 (0.76-1.11)	0.3832	0.93 (0.85-1.01)	0.10
	Hypertension	1	0.92 (0.83-1.02)	0.099	0.94 (0.85-1.05)	0.30
	(1901, 1647)	2	0.89 (0.79-0.99)	0.033	0.95 (0.84-1.07)	0.38
	Diabetes	1	0.86 (0.76-0.97)	0.015	0.95 (0.88-1.03)	0.21
	(1002, 2508)	2	0.81 (0.70-0.92)	0.0017	0.95 (0.87-1.04)	0.24
rs9398171	CHD	1	0.85 (0.73-0.99)	0.032	0.96 (0.89-1.03)	0.26
	(732, 2827)	2	0.81 (0.68-0.96)	0.013	0.94 (0.87-1.02)	0.14
	Hypertension	1	0.91 (0.83-0.99)	0.033	0.95 (0.86-1.05)	0.30
	(1905, 1654)	2	0.85 (0.77-0.94)	0.0019	0.96 (0.86-1.07)	0.50
	Diabetes	1	0.92 (0.78-1.08)	0.31	0.95 (0.86-1.05)	0.28
	(1005, 2502)	2	0.88 (0.74-1.05)	0.15	0.94 (0.84-1.05)	0.27
rs12212067	CHD	1	0.90 (0.74-1.10)	0.30	0.94 (0.86-1.03)	0.19
	(732, 2824)	2	0.90 (0.73-1.12)	0.36	0.93 (0.84-1.03)	0.17
	Hypertension	1	0.90 (0.80-1.00)	0.056	0.96 (0.85-1.09)	0.51
	(1903, 1653)	2	0.88 (0.78-0.99)	0.037	0.96 (0.84-1.11)	0.60
	Diabetes	1	0.83 (0.70-0.99)	0.034	0.94 (0.85-1.05)	0.26
	(661, 1793)	2	0.77 (0.64-0.93)	0.0071	0.93 (0.82-1.04)	0.19
Haplotype****	CHD	1	0.85 (0.69-1.05)	0.12	0.93 (0.84-1.03)	0.15
	(526, 1956)	2	0.83 (0.66-1.04)	0.10	0.90 (0.81-1.01)	0.07
	Hypertension	1	0.87 (0.77-0.98)	0.022	0.94 (0.81-1.09)	0.43
	(1331, 1151)	2	0.82 (0.72-0.93)	0.0003	0.93 (0.84-1.04)	0.23

* 1: Age-adjusted; 2: Covariate-adjusted, Covariates in Cox model: age, BMI, glucose, smoking (pack-year), alcohol intake (oz/mo), physical activity index, depression, cancer, and stroke.

** HR (95% CI) of *FOXO3* minor allele carrier vs. major allele homozygotes on mortality estimated from the Cox regression model.

*** Number of subjects with a CMD, without a CMD.

**** Haplotype comprised minor allele frequencies of SNPs *rs2253310* (*C*), *rs2802288* (*A*), *rs2802292* (*G*), *rs2764264* (*C*), *rs9398171* (*C*), *rs12212067* (*G*), and *rs3800230* (*G*).

FOXO3 longevity haplotype was *rs768023*, located in the promoter region, and which we noted previously is the binding site for both FOXA2 and HDAC2 [8]. HDAC2 also deacetylates FoxO3 thus activating it [22]. For the *rs768023* minor allele, binding of FOXA2 and HDAC is abolished, which would increase *FOXO3* expression. The SNP immediately 3' of *rs768023* in the 14-SNP haplotype block, but not included in the present study, is *rs1536057*, the minor (*A*) allele of which abolishes NRF1 and E2F sites and creates a POU5F1 site. The inhibition of *FOXO3* expression by E2F [23] would thereby be lost, so causing an elevation in *FOXO3* expression. NRF1 and POU5FI are involved in cell proliferation and stem cell maintenance, while E2F is involved in DNA damage response.

The full complement of transcription factors that exhibit allele-dependent binding to SNPs in the 14-SNP *FOXO3* haplotype block are shown in Supplementary Table 2. Of these, HDAC1, NRF1, POU5F1, TFCP2L1, MYOG, NKX3-1, MITF, MEF2A, MZF1, NR2F1, PRDM1, FOXP1, MZF1, and ONECUT1 are involved in cell proliferation/stem cell maintenance, whereas HNF3, FOXA2, HNF4A, and ONECUT1 (HNF6) are involved in diabetes/energy response, and E2F1 is involved in DNA damage repair.

We have shown previously that the *G* allele of *FOXO3* SNP *rs2802292* protects against risk of death from CHD [11]. *G* allele carriers had lower plasma TNF-α than non-carriers, suggesting an ability to reduce inflammation as a potential mediating factor for reduction of CHD mortality risk [24]. At the cell and molecular level, cardiovascular benefits of FoxO3 involve stimulation of stress resistance by up-regulation of mitochondrial superoxide dismutase, peroxisomal catalase, and peroxiredoxin expression [25], as well as the promotion of multiple beneficial vascular functions, and reversal of cellular aging through pro-proliferative effects that involve downregulation of CSRP1, which recruits sirtuin-1 to create a suppressive chromatin environment [26]. Longevity-associated genetic variants of *FOXO3* are also associated with lower blood pressure and reduced hypertension prevalence [27]. The association of *FOXO3* SNPs with *self-rated* health in individuals aged 75–87 is influenced solely by cardiovascular disease [28].

Various types of cellular stress induce the recruitment of the evolutionarily conserved transcription factor HSF1 to binding sites involved in controlling gene expression [29]. The fact that only in *G* allele carriers of *FOXO3* SNP *rs2802292* possess the HSF1 binding site [30] explains, at least in part, the association we found between this allele and resistance to mortality in CMD subjects, presumably mediated by HSF1-induced expression of *FOXO3*. Binding of HSF1 to its target site in *rs2802292* leads to formation of a complex resulting in a promoter-enhancer interaction involving the upstream promoter region and the *rs2802292* region of intron 2 [30]. Stress resistance and elevated *FOXO3* transcription were seen following application of the potent oxidative stress inducer H_2O_2 in human dermal fibroblasts having the *GG* genotype, but not in those with the *TT* genotype, thus confirming the mechanism [30]. Unlike cells with the *TT* genotype, *GG* cells also exhibited higher expression of FoxO3 target genes *SOD2*, *CAT*, *GADD45A*, *CCND1*, *RBL2*, *BCL2L11* and *BCL6* in a HSF1-dependent manner [30]. Furthermore, *GG* cells displayed a significantly better DNA repair response. The *FOXO3* SNP *rs2802292* region also contains transcription factor response elements for SP1, GATA1 and ESR1 [30].

Our finding of 13% higher prevalence of diabetes at baseline in *FOXO3 rs2800292 G*-allele carriers is likely due to attrition of non-protective *TT* genotypes at earlier ages owing to mortality from cardiovascular disease (e.g., CHD, stroke) and other complications of diabetes, thereby enriching *G*-allele frequency in the cohort. In a twin study, the *FOXO3 rs2800292 G* allele was associated with more favourable insulin sensitivity and increased *FOXO3* expression [31]. The protection against mortality afforded by this mechanism would explain our finding that diabetes alone was associated with longer lifespan in *G*-allele carriers compared with *TT* subjects. As we have reported previously, the *FOXO3* longevity genotype involves a haplotype that includes at least 14 SNPs that work in concert [8].

We reported previously that the *FOXO3 rs2800292 G* allele was associated with reduced risk of hypertension in women, but not men, from a cohort of Japanese individuals living in Japan [32]. Our finding in the present study that *G*-allele carriers have lower mortality could, as shown here for individuals with diabetes, be due to a higher mortality rate for those with the *TT* genotype, in this instance from hypertension-related cardiovascular events, similar to what has been demonstrated by one of us for *ACE* [33].

In conclusion, we have found for the first time that remaining lifespan of elderly men with longevity-associated alleles of *FOXO3* who have one or more chronic conditions of aging, specifically diabetes, and/or hypertension, and/or CHD (collectively, CMD), live as long as elderly men who lack any of these life-threatening conditions. Thus, favorable *FOXO3* genotype can abrogate the increased risk for mortality in men with prevalent CMD. Since *FOXO3* genotype has no effect on lifespan of men without a *CMD*, the well-established

understanding of *FOXO3* as being a longevity gene is because of an effect on elderly individuals who are at higher risk because they have a CMD.

MATERIALS AND METHODS

Study cohort

Participants were American men of Japanese ancestry living on the island of Oahu, Hawaii. They were recruited in 1965–1968 from World War II Selective Service records for the Kuakini Honolulu Heart Program (KHHP) [34], which continued from 1991 onwards as the Kuakini and Honolulu-Asia Aging Study (KHAAS) [4, 34–36]. The analysis was conducted as part of the Kuakini Hawaii Lifespan Study and the Kuakini Hawaii Healthspan Study, an embedded cohort study of healthy aging drawn from the original KHHP-KHAAS population. Subjects had parents who were both from a limited geographic area of Japan, mostly the western, central and southern regions [34, 37]. Subjects were recruited at the same time and place (Oahu), meaning there was no apparent reason why genetic background should be substantially different. The KHHP cohort is quite robust for phenotype-genotype associations, since the data collection was exceptionally accurate and involved cross validation utilizing an expert Morbidity and Mortality Committee. The Hawaii Japanese population is from Japan, with little outbreeding and, based on the authors' unpublished data, exhibits a smaller degree of genetic diversity than the overall population of Japan.

All participants in the current study were interviewed at Examination 4 of the KHHP (1991–1993). Archived phenotypic data and blood samples from Examination 4 of the KHHP (1991–1993), which coincided with the commencement of the KHAAS, were used as the baseline examination for our study. The KHAAS was begun as an expansion of the KHHP for the study of neurodegenerative diseases, cognitive function, and other aging phenotypes in elderly persons. From 1991–1993, all of the survivors of the KHHP cohort, ranging in age from 71–93 years (mean age: 77.9 ± 4.7 years), were invited to the 4th examination. Response rate was 80% of survivors (including clinic, home, and nursing home visits; n = 3,741).

The study involved 3,584 of 3,741 men aged 71 to 93 (mean age 77.9 ± 4.7 SD years) for whom we had banked DNA so making them eligible for inclusion. Of the 3,584 men, 3,548 had died (mean age at death 89.0±6.2 SD years; range 72–108 years) and 36 were still alive (mean age 101.6 ± 1.9 SD years; range 100–108 years) at the end of the follow-up period, 31 December 2019.

The KHHP was a longitudinal observation study. Subjects from the KHHP/KHAAS population [4] had been followed with regular examinations and blood work until 2015, or death up to the end of 2019.

Information on the prevalence of CHD, stroke, and cancer was identified by the KHHP surveillance system (review of hospital records by an expert panel or matching to Tumor Registry for Cancer). Hypertension was defined as systolic/diastolic blood pressure ≥160/95 mmHg or on anti-hypertensive medication at baseline. Diabetes was defined by fasting serum glucose ≥126 mg/dL or 2-hour post-load glucose ≥200 mg/dL or taking insulin and/or oral hypoglycemic medications at baseline.

Procedures performed were in accord with institutional guidelines and were approved by the Institutional Review Board of Kuakini Medical Center. Written informed consent was obtained from all study participants or from family representatives, if participants could not provide consent.

Genotyping

Leucocyte DNA obtained from participants was used for genotyping. Recruitment, demographic characteristics, and DNA extraction were as described previously [4, 38]. In the HHP cohort, the *FOXO3* longevity haplotype was defined by 14 SNPs (in order of location 5' to 3' with minor [longevity-associated] allele shown in brackets): *rs768023* (*G* allele), *rs1536057* (*T*), *rs2253310* (*C*), *rs2802288* (*A*), *rs2802292 (G)*, *rs2764264* (*C*), *rs12202234* (*G*), *rs17069665* (*G*), *rs12213895 (A)*, *rs12212067* (*G*), *rs9398171* (*C*), *rs73763159* (*T*), *rs3800230* (*G*), and *rs1935952* (*G*)) [8]. Longitudinal genotype data were obtained for 8 of these SNPs. All 14 affect transcription factor binding sites. Specifically, the minor allele of *rs2802292* creates a HSF1 binding site in intron 2 of *FOXO3* [30], *rs1935952* disrupts an MZF1 binding site, *rs2253310* minor allele creates a TFCP2L1 binding site, *rs2764264* disrupts a NKX3 binding site, *rs2802288* creates a MYF binding site, *rs3800230* disrupts a FoxP1 binding site, *rs9398171 creates* NR2F1 and HNF4 site and abolishes a HNF6 site, and *rs12212067* creates a MZF1 site [8]. The SNP *rs768023* (not included in the present longitudinal study) abolishes both a FOXA2 and a HDAC2 recognition site [8].

Genotyping was performed by allelic discrimination assays using TaqMan® (Applied Biosystems, Inc.) and a Life Technologies QuantStudio 12K Flex OpenArray system. Although 88% of participants were born in Hawaii, there is a theoretical possibility of confounding of case vs. control status for allele

frequencies due to geographic origin. Therefore, for certain analyses, cases and controls were stratified by parental prefecture of origin using conditional logistic regression models.

Statistical analyses

General linear models were used to compare age-adjusted indirect measurements between groups, and logistic models were used to compare the age-adjusted direct measurements. Cox proportional models were used to assess the association of *FOXO3* minor-allele carriers with mortality stratified by disease status, such as by CHD, by diabetes, by hypertension, and by chronic conditions defined by presence of any of CHD, diabetes, or hypertension. The Cox proportional hazard assumption was tested for each Cox model. The effect of interaction of disease with *FOXO* genotype on mortality was tested in the Cox model. All statistical analyses were performed using the Statistical Analysis System version 9.4 [39]. Figures were generated using the STATA 12 Graphics [40].

AUTHOR CONTRIBUTIONS

R.C., B.J.M., T.A.D., K.H.M., D.C.W., R.C.A., B.J.W. contributed to the study concept and design; R.C. carried out the statistical analyses; K.H.M. supervised recruitment and data collection; T.A.D. supervised the genotyping; B.J.M. drafted the manuscript; R.C., B.J.M., T.A.D., K.H.M., P.M.C.D., R.C.A., D.C.W. and B.J.W. provided critical input into data interpretation and manuscript preparation.

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CONFLICTS OF INTEREST

These authors declare no conflicts of interest.

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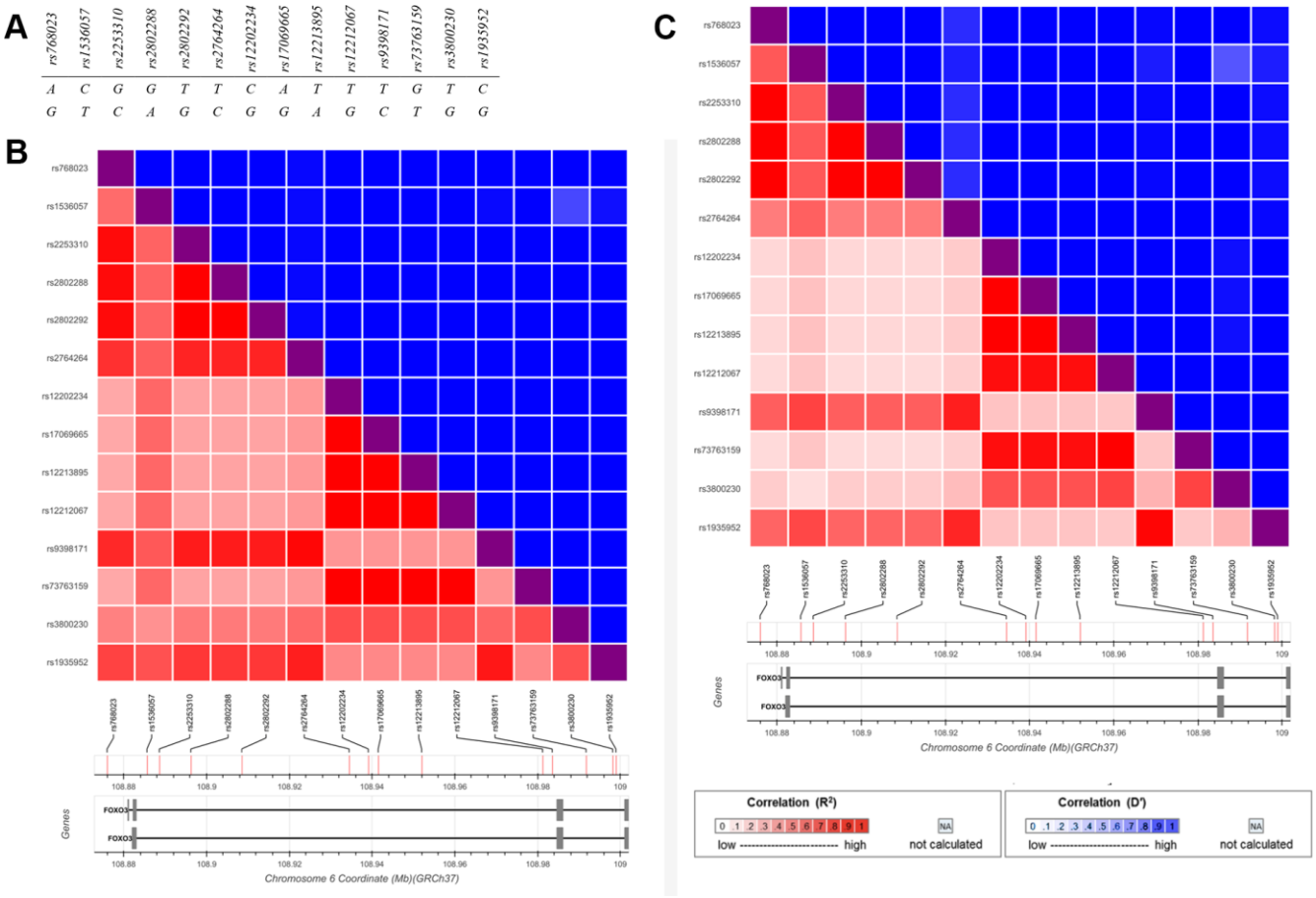
SUPPLEMENTARY MATERIALS

Supplementary Figures



Supplementary Figure 1. Linkage disequilibrium and haplotypes of 14 *FOXO3* longevity-associated SNPs. (**A**) The 14 *FOXO3* longevity-associated SNPs (and alleles) that comprise the full haplotype. (**B**) Linkage disequilibrium in *FOXO3* and SNP locations – Japanese (JPN). (**C**) Linkage disequilibrium in *FOXO3* and SNP locations – Caucasian (CEU). LD matrix plot showing the 14 *FOXO3* SNPs using the program LDLink (https://ldlink.nci.nih.gov). In the Japanese population, shown in part (**B**) of Supplementary Figure 1, the data are from "JPN" population data from Phase 3 (Version 5) of the 1000 Genomes Project (https://www.internationalgenome.org). In the Caucasian population the values are from the "CEU" data. Note that the JPN data have a higher overall level of linkage disequilibrium (i.e., red intensities). Red squares denote blocks that have a Hedrick's multiallelic $R^2 = 1$, whereas blue squares denote blocks that have a R^2 value < 1 [Hedrick PW. Gametic disequilibrium measures: Proceed with caution. *Genetics.* 1987; 117:331-342]. Blue blocks denote *D'* values.

A

RS Number	Position (GRCh37)	Allele Frequencies	Haplotypes					
rs768023	chr6:108876002	A=0.755, G=0.245	A	G	G	G	G	G
rs1536057	chr6:108885623	C=0.841, T=0.159	C	T	T	C	C	C
rs2253310	chr6:108888593	G=0.764, C=0.236	G	C	C	C	C	C
rs2802288	chr6:108896215	G=0.764, A=0.236	G	A	A	A	A	A
rs2802292	chr6:108908518	T=0.764, G=0.236	T	G	G	G	G	G
rs2764264	chr6:108934461	T=0.784, C=0.216	T	C	C	C	T	C
rs12202234	chr6:108939083	C=0.899, G=0.101	C	G	C	C	C	C
rs17069665	chr6:108941468	A=0.899, G=0.101	A	G	A	A	A	A
rs12213895	chr6:108951968	T=0.899, A=0.101	T	A	T	T	T	T
rs12212067	chr6:108981196	T=0.899, G=0.101	T	G	T	T	T	T
rs9398171	chr6:108983527	T=0.788, C=0.211	T	C	C	C	T	C
rs73763159	chr6:108991686	G=0.899, T=0.101	G	T	G	G	G	G
rs3800230	chr6:108998128	T=0.861, G=0.139	T	G	T	G	T	T
rs1935952	chr6:108998905	C=0.808, G=0.192	C	G	G	G	C	C
		Haplotype Count	156	21	9	7	4	3
		Haplotype Frequency	0.75	0.101	0.0433	0.0337	0.0192	0.0144

B

RS Number	Position (GRCh37)	Allele Frequencies	Haplotypes						
rs768023	chr6:108876002	A=0.646, G=0.353	A	G	G	G	A	G	G
rs1536057	chr6:108885623	C=0.737, T=0.263	C	T	T	C	C	C	T
rs2253310	chr6:108888593	G=0.646, C=0.353	G	C	C	C	G	C	C
rs2802288	chr6:108896215	G=0.646, A=0.353	G	A	A	A	G	A	A
rs2802292	chr6:108908518	T=0.646, G=0.353	T	G	G	G	T	G	G
rs2764264	chr6:108934461	T=0.702, C=0.298	T	C	C	T	C	C	T
rs12202234	chr6:108939083	C=0.919, G=0.081	C	C	G	C	C	C	C
rs17069665	chr6:108941468	A=0.919, G=0.081	A	A	G	A	A	A	A
rs12213895	chr6:108951968	T=0.919, A=0.081	T	T	A	T	T	T	T
rs12212067	chr6:108981196	T=0.924, G=0.076	T	T	G	T	T	T	T
rs9398171	chr6:108983527	T=0.732, C=0.268	T	C	C	T	T	C	T
rs73763159	chr6:108991686	G=0.924, T=0.076	G	G	T	G	G	G	G
rs3800230	chr6:108998128	T=0.899, G=0.101	T	T	G	T	T	G	T
rs1935952	chr6:108998905	C=0.727, G=0.273	C	G	G	C	C	G	C
		Haplotype Count	120	31	15	13	6	5	5
		Haplotype Frequency	0.6061	0.1566	0.0758	0.0657	0.0303	0.0253	0.0253

Supplementary Figure 2. *FOXO3* longevity haplotypes. (**A**) Japanese Haplotype – allele frequency of haplotype = 0.10. (**B**) Caucasian haplotype – allele frequency of haplotype = 0.76. Tables of observed haplotypes were generated using LDHap (https://ldlink.nci.nih.gov/?tab=ldhap). Haplotypes with frequencies greater than 1% are displayed vertically and ordered by observed frequency in the selected query sub-population. Variant bi-allelic genotypes and frequencies are reported in rows and are sorted by genomic position. Links are available to dbSNP (https://www.ncbi.nlm.nih.gov/snp/) RS numbers and coordinates in the UCSC Genome Browser (http://genome.ucsc.edu/cgi-bin/hgGateway; version GRCh37 is shown). Reference populations were "JPN" Japanese, shown in "A", and "CEU" Caucasian, shown in "B", using data from Phase 3 (Version 5) of the 1000 Genomes Project (https://www.internationalgenome.org). Rectangles highlight the longevity haplotype (minor alleles noted in Supplementary Figure 1) that has a frequency of 0.101 in the Japanese population and 0.758 in Caucasians.

Supplementary Tables

Supplementary Table 1. Age-adjusted characteristics of subjects at baseline for *FOXO rs2802292 TT* genotype and *G*-allele carriers.

Variable	*TT*	*GT/GG*	p
n	1901	1683	
Age (years)	77.0 ± 4.5	78.0 ± 4.7	0.0008
BMI (kg/m²)	23.5 ± 3.2	23.5 ± 3.1	0.81
Fasting plasma glucose (mg/dl)	112.3 ± 28.0	113.9 ± 31.0	0.090
Smoking (pack-years)	25.4 ± 33.5	27.0 ± 35.3	0.20
Alcohol intake (oz/mo)	18.7 ± 39.8	19.0 ± 41.4	0.83
Physical activity index	30.9 ± 4.7	30.9 ± 4.5	0.94
Depressive symptoms (%)	10.0	11.2	0.29
Stroke (%)	4.5	4.4	0.85
Cancer (%)	14.3	12.8	0.22
CHD (%)	22.3	18.7	0.009
Diabetes (%)	27.0	30.4	0.027
Hypertension (%)	53.1	54.1	0.55
CHD or diabetes or hypertension (%)	70.0	70.2	0.92

Values shown are age-adjusted mean ± SD for indirect measures and proportion for direct measurements.

Supplementary Table 2. Effects of minor alleles on transcription factor (TF) binding.

SNP	Result	TF	Biological Pathways	Tissue*
rs768023	abolish	FOXA2	stem cell maintenance, vascular epithelia, diabetes, energy response	Liver, Lung, Pancreas, Stomach
	abolish	HDAC	stem cell maintenance	Lymphoblasts, Colon, Esophagus, Ovary, Small Intesting, Spleen, Thyroid
rs1536057	abolish	NRF1	stem cell maintenance	Brain
	abolish	E2F	DNA damage response	Lymphoblasts
	create	POU5F1	stem cell maintenance	-
rs2253310	create	TFCP2L1	stem cell maintenance	Kidney, Salivary Gland, Skin, Thyroid
rs2802288	create	MYF	stem cell maintenance, muscle atrophy	Muscle
rs2764264	abolish	NKX3	stem cell maintenance	Salivary Gland, Prostate
rs2802292	create	HSF1	stress response	most tissues, not Brain
rs12202234	abolish	FOXA	stem cell maintenance, vascular epithelia, diabetes, energy response	Bladder, Breast, Prostate
rs17069665	abolish	MITF	stem cell maintenance	Cervix, Uterus
rs12213895	create	MEF2	stem cell maintenance	Artery, Lung, Skin
rs12212067	create	MZF1	stem cell maintenance	Brain, Lung, Ovary, Pituitary, Prostate, Thyroid, Uterus
rs9398171	create	NR2F1	stem cell maintenance	Bladder, Brain, Cervix, Fallopian Tube, Lung, Nerve, Ovary, Prostate, Uterus
	create	HNF4	diabetes, energy response,	Colon, Liver, Small Intestine
	abolish	ONECUT1	stem cell maintenance, diabetes, energy response	Liver, Pancreas
rs73763159	abolish	PRDM1	stem cell maintenance	Esophagus, Vagina
rs3800230	abolish	FOXP1	stem cell maintenance	-
rs1935952	abolish	MZF1	stem cell maintenance	Brain, Lung, Ovary, Pituitary, Prostate, Thyroid, Uterus

The 14 SNPs modify 19 TF binding sites shown below, along with their functions. SNP ID# is variant name from the database dbSNP, create/abolish refers to whether the minor allele creates or abolishes a TF binding site.
Significant variants were identified previously [Donlon, T.A., et al. FOXO3 longevity interactome on chromosome 6. Aging Cell. 2017; 16:1016-1025] using HaploReg (https://pubs.broadinstitute.org/mammals/haploreg/haploreg.php), which is a tool for exploring annotations of the noncoding genome at variants on haplotype blocks, such as candidate regulatory SNPs at disease-associated loci [Ward, L.D. & Kellis, M. HaploReg: a resource for exploring chromatin states, conservation, and regulatory motif alterations within sets of genetically linked variants. Nucleic Acids Res. 2012; 40 (Database issue):D930-934]. Expression data are from GTEX; * >100 TPM.