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Gene expression analysis of mTOR pathway: association with human longevity

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Summary

mTOR signalling is implicated in the development of disease and in lifespan extension in model organisms. This pathway has been associated with human diseases such as diabetes and cancer, but has not been investigated for its impact on longevity *per se*. Here, we investigated whether transcriptional variation within the mTOR pathway is associated with human longevity using whole-blood samples from the Leiden Longevity Study. This is a unique cohort of Dutch families with extended survival across generations, decreased morbidity and beneficial metabolic profiles in middle-age. By comparing mRNA levels of nonagenarians and middle-aged controls, the mTOR signalling gene set was found to associate with old age ($P = 4.6 \times 10^{-7}$). Single gene analysis showed that seven of 40 mTOR pathway genes had a significant differential expression of at least 5%. Of these, the *RPTOR (Raptor)* gene was found to be differentially expressed also when the offspring of nonagenarians was compared with their spouses, indicating association with familial longevity in middle-age. This association was not explained by variation between the groups in the prevalence of type 2 diabetes and cancer or glucose levels. Thus, the mTOR pathway not only plays a role in the regulation of disease and aging in animal models, but also in human health and longevity.

Key words: aging; longevity; mTOR; human; gene expression.

Introduction

Mammalian target of rapamycin (mTOR) is an evolutionarily conserved nutrient-sensing protein kinase that belongs to the phosphoinositide 3-kinase (PI3K)-related protein kinase family and acts as a central regulator of growth and cell division (Stanfel *et al.*, 2009). The core kinase mTOR is present in two distinct multiprotein complexes called mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), each with different functions (Polak & Hall, 2009), although their exact respective roles remain to be elucidated. The main known functions of mTORC1 include translation initiation, protein synthesis and autophagy, while those of mTORC2 include cytoskeletal organization (Laplante & Sabatini, 2009). In addition, the two complexes are connected in that mTORC2 regulates Akt phosphorylation, part of an upstream pathway that controls activation of mTORC1 (Stanfel *et al.*, 2009). Inhibition of the mTOR signalling pathway in yeast (Kaeberlein *et al.*, 2005), worms (Vellai *et al.*, 2003), flies (Kapahi *et al.*, 2004) and mice (Harrison *et al.*, 2009) results in lifespan extension. In mouse models, inhibition of the mTOR signalling pathway provides cardiovascular benefits and improved metabolic function (Stanfel *et al.*, 2009). Additionally, a primary hallmark of calorie restriction (CR) in rodents, which leads to inhibition of the mTORC1 pathway, is a dramatic reduction in age-associated cancer incidence and growth rate (Spindler, 2005), implying that enhanced mTOR signalling plays a central role in cancer progression. In rats, the mTOR pathway has also been implicated in both Type 1 and Type 2 diabetes, with chronic activity of mTORC1 contributing to obesity and insulin insensitivity (Newgard *et al.*, 2009). Because reduced mTOR signalling in model organisms extends lifespan and is associated with better metabolic health, we postulated that a similar reduction in this signalling pathway may also contribute to longevity and healthy aging in humans.

Thus far, mTOR signalling has been investigated in humans mainly by studying disease. Its activity has been found to be elevated in different cancers including lymphomas, melanomas, breast and prostate (Guertin & Sabatini, 2005; Stanfel *et al.*, 2009). Elevated mTOR signalling in human tumours is correlated with poor tumour prognosis, and inhibitors of this pathway are showing promising results in clinical trials (Le *et al.*, 2008; Strimpakos *et al.*, 2008; Yap *et al.*, 2008). Additionally, intermediate phenotype insulin resistance in human diabetes can be prevented by mTOR inhibitors (Krebs *et al.*, 2007). Metformin, a commonly prescribed anti-diabetic drug which inhibits mTORC1 downstream pathways through activation of AMP kinase, has also been shown to reduce tumour growth (Dowling *et al.*, 2007). Human CR studies used as model for down-regulation of mTOR are much more short term in comparison with animal studies, but nevertheless, CR may reduce the risk of age-related diseases even in non-obese humans (Fontana *et al.*, 2004; Holloszy & Fontana, 2007). Thus, accumulating evidence is showing that mTOR signalling can play an important role in human disease. In contrast, it has not been investigated whether natural variation in mTOR signalling could also contribute to disease risk.

Recently, we have identified 360 genes, the expression of which was associated with human longevity (Passtoors *et al.*, 2012). *MTOR* (also known as *FRAP1*) was among these, suggesting a role for the mTOR signalling pathway in human longevity. In the current study, we investigated whether common natural genetic and transcriptional variation among the 40 genes of the mTOR pathway (Fig. 1) was associated with human longevity. To do this, we took advantage of the Leiden Longevity Study (LLS), a Dutch cohort in which families are selected on the basis of nonagenarian sibling pairs (Schoenmaker *et al.*, 2006). The middle-aged offspring of these nonagenarians have a

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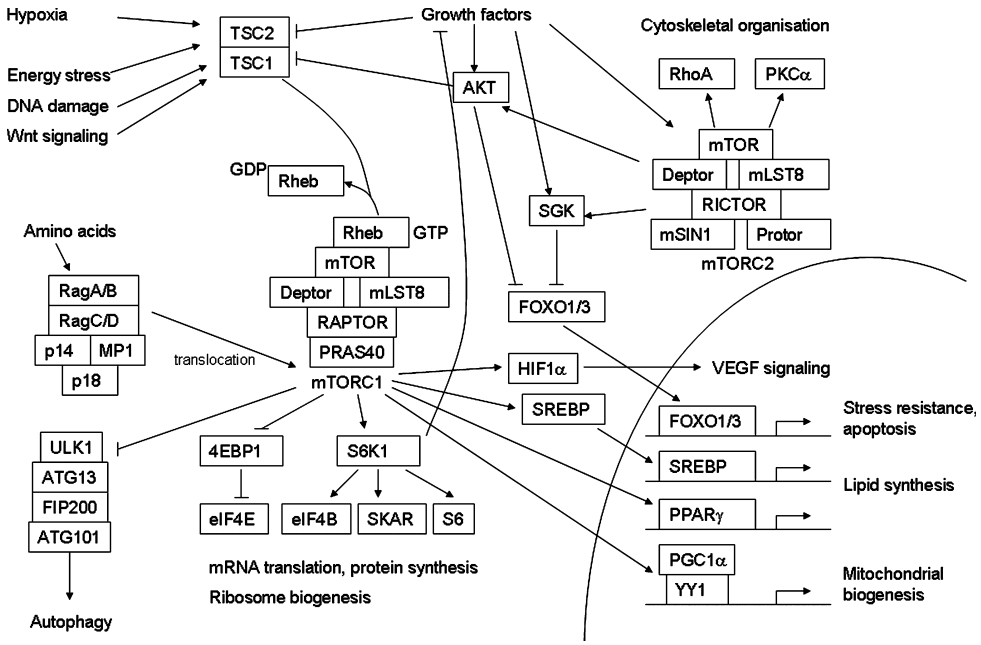

Fig. 1 mTOR signalling pathway of which genetic variation and expression of 40 genes are investigated for their association with human aging and longevity. mTORC1 receives signals from growth factors, nutrients, energy status and a range of stressors and positively regulates cell growth and proliferation by promoting many anabolic processes, including biosynthesis of proteins, lipids and organelles, and by limiting catabolic processes such as autophagy (Sengupta *et al.*, 2010). mTORC2 phosphorylates and activates Akt, serum- and glucocorticoid-regulated kinase (sGK), RhoA and protein kinase C (PKC), which regulate cell survival, cell cycle progression and cytoskeleton organization (Sarbassov *et al.*, 2005; Garcia-Martinez & Alessi, 2008; Ikenoue *et al.*, 2008).

decreased prevalence of myocardial infarction and type 2 diabetes (Westendorp *et al.*, 2009) and display healthier intrinsic metabolism reflected by lipid profiles (Barzilai *et al.*, 2001; Heijmans *et al.*, 2006), glucose metabolism and preservation of insulin sensitivity (Rozing *et al.*, 2009; Wijsman *et al.*, 2011) than age-matched controls. They also have different immune profiles in that they fail to show a lower number of CD8+ naive T cells and a higher number of CD8+ late-stage differentiated memory cells dependent on cytomegalovirus infection often seen as a hallmark of immune aging (Derhovanessian *et al.*, 2010). To investigate whether expression of mTOR signalling genes is associated with familial longevity, we compared the levels of mRNA for these genes in peripheral blood of nonagenarians from families enriched for longevity and younger controls. In addition, genetic variation in mTOR pathway genes of LLS nonagenarians is compared with that of younger controls. Next, mRNA levels of the top longevity-associated genes were compared between the offspring of the nonagenarians and similarly aged controls to exclude the possibility that differential expression of these genes did not simply mark old age, but are a defining characteristic of the long-lived families in middle-age. Finally, we examined whether the association of the expression of mTOR signalling genes with longevity is independent of metabolic health parameters, namely plasma glucose levels, and the prevalence of type 2 diabetes and cancer, because these factors are known characteristics of the long-lived families and are suggested to be influenced by mTOR signalling.

Results

Selection of mTOR pathway genes

For the mTOR pathway, we selected 40 genes encoding proteins that belong to the well-described core of the mammalian mTOR pathway. First, we selected the two mTOR complexes mTORC1 and mTORC2 including their downstream targets often described in the literature (Laplante & Sabatini, 2009; Polak & Hall, 2009). Next, we included the direct activators and inhibitors of these two complexes. Figure 1 shows the mTOR pathway as we investigated.

Gene expression in whole blood: nonagenarians vs. controls

We measured gene expression levels for each of the 40 mTOR signalling genes using RT-qPCR on whole blood samples from 87 LLS nonagenarians, 337 of their middle-aged offspring and 321 middle-aged LLS controls (Table 1). When comparing long-lived individuals to younger controls differentially expressed genes may associate either with calendar age, familial longevity or both. We tested the total gene set for such association using the globaltest method (Goeman & Oosting, 2011). We observed that the gene expression levels of the mTOR signalling gene set did associate significantly with age and/or familial longevity in these families ($P = 4.6 \times 10^{-7}$).

Second, to investigate which genes are primarily responsible for this association, single gene analysis using linear regression was performed on all 40 mTOR signalling genes (Table 2). After Bonferroni correction for multiple testing, seven genes showed significant differential expression with at least a 5% expression difference: *FOXO1* and *RPTOR*

Table 1 Analysed samples: gene expression

Samples	Number	Mean age	Age range	Men/women
Long-lived individuals	87	94.3	89–102	40/47
Offspring	337	61.3	34–78	194/143
Controls	321	61.2	32–81	146/175

Table 2 Linear regression results of gene expression of long-lived individuals compared with controls

Protein	Genename	*N* cases	*N* controls	Mean	SD	FC	*P*
4EBP1	***EIF4EBP1***	**76**	**303**	**2.37**	**0.048**	**1.19**	**2.1×10^{-8}**
AKT	*AKT1*	76	303	2.27	0.103	1.18	0.030
ATG101	*C12orf44*	71	296	1.02	0.020	1.00	0.644
ATG13	*ATG13*	71	296	1.10	0.019	0.99	0.095
Deptor	*DEPTOR*	70	296	0.05	0.001	1.00	0.001
eIF4B	*EIF4B*	70	295	0.47	0.018	1.00	0.572
eIF4E	*EIF4E*	71	296	1.10	0.045	0.94	0.012
FIP200	*RB1CC1*	71	295	2.41	0.153	1.29	0.005
FOXO1	***FOXO1***	**71**	**296**	**1.50**	**0.034**	**0.90**	**1.0×10^{-11}**
FOXO3	*FOXO3*	71	296	1.73	0.057	1.05	0.166
HIF1a	*HIF1A*	71	296	4.05	0.322	1.90	0.005
mLST8	*MLST8*	71	296	0.42	0.010	1.00	0.446
MP1	*MAPKSP1*	71	296	1.64	0.039	0.97	0.112
mSIN1	*MAPKAP1*	70	294	1.42	0.018	0.98	0.002
mTOR	*MTOR*	68	294	0.71	0.013	0.97	6.0E-06
p14	***LAMTOR2***	**71**	**296**	**1.50**	**0.049**	**1.08**	**9.1×10^{-5}**
p18	*CDKN2C*	70	295	0.71	0.020	1.00	0.630
PGC1a	*PPARGC1A*	66	287	0.02	0.003	1.00	0.762
PKCa	*PRKCA*	71	296	0.57	0.016	0.95	5.4×10^{-9}
PPARg	*PPARG*	70	294	0.03	0.002	1.01	8.4×10^{-5}
PRAS40	***AKT1S1***	**70**	**296**	**0.87**	**0.049**	**1.14**	**1.9 × 10−4**
Protor1	*PRR5*	71	296	4.47	0.197	0.82	0.035
Protor2	***PRR5L***	**71**	**296**	**7.03**	**0.211**	**1.81**	**4.9×10^{-5}**
RagA	*RRAGA*	71	295	1.09	0.038	0.95	0.009
RagB	*RRAGB*	71	296	0.67	0.019	0.99	0.028
RagC	*RRAGC*	71	296	1.69	0.041	1.02	0.231
RagD	*RRAGD*	70	296	0.66	0.015	1.02	0.035
Raptor	***RPTOR***	**79**	**312**	**0.70**	**0.037**	**0.92**	**0.001**
Rheb	*RHEB*	71	296	0.73	0.028	0.97	0.060
RhoA	***RHOA***	**71**	**296**	**2.15**	**0.037**	**1.08**	**5.6×10^{-5}**
Rictor	*RICTOR*	71	296	6.24	0.182	0.99	0.874
S6	*RPS6*	71	295	1.92	0.073	0.93	0.055
S6K1	*RPS6KB1*	76	303	3.05	0.110	0.94	0.648
SGK	*SGK1*	71	296	0.79	0.019	1.01	0.247
SKAR	*POLDIP3*	67	260	1.00	0.020	1.05	1.3×10^{-4}
SREBP	*SREBF1*	71	296	0.72	0.031	1.00	0.758
TSC1	*TSC1*	71	296	1.02	0.020	0.98	0.019
TSC2	*TSC2*	71	296	0.78	0.021	1.04	3.8×10^{-4}
ULK1	*ULK1*	76	303	2.47	0.081	1.19	0.002
YY1	*YY1*	71	296	1.73	0.037	1.01	0.740

Significance level for *P*-value after Bonferroni correction for multiple testing is 0.00125. Genes significantly differentially expressed with at least 5% are depicted in bold.

Mean, mean relative expression level; SD, standard deviation of relative expression level; FC, fold change, above one indicated higher expression in long-lived individuals. *P*, raw *P*-value from linear regression model.

were expressed at a lower level, and *EIF4EBP1*, *LAMTOR2*, *AKT1S1*, *PRR5L* and *RHOA* at a higher level in nonagenarians.

Genetic analysis: nonagenarians vs. controls

Since we had GWAS data available in the LLS study, we analysed the set of GWAS-based SNPs within a 10-kb window around the 40 mTOR signalling genes for differences in variation between 417 unrelated nonagenarian participants and 476 younger controls from the LLS (Supplementary Table S1) (Deelen *et al.*, 2011a). Using the PLINK set-based test, we investigated 1,018 SNPs in the 40 mTOR signalling genes simultaneously in one test (*P*-value < 0.05 for significance) (Supplementary Table S2). We observed a significant association of genetic variation in the mTOR pathway as a whole with familial longevity ($P = 0.009$). However, the single gene analysis (of 40 SNP sets), for which the significance level for *P*-values after Bonferroni correction for multiple testing is 0.00125, did not result in significant associations. (Supplementary Table S3), suggesting that effects of single common variation in mTOR signalling genes on familial longevity is very small.

Gene expression in whole blood: offspring of nonagenarians vs. controls

To investigate whether the differences in expression of the seven genes differentially expressed between nonagenarians and controls are a characteristic of the long-lived families and not just a marker of old age, we compared their expression in the middle-aged offspring of nonagenarians to the controls of similar age (Supplementary Table S5). While the association for six genes was not significant after correction for multiple testing, as compared to the controls, the offspring expressed significantly less *RPTOR* mRNA (Table 3). Thus, in two generations, the *RPTOR* gene expression was decreased as compared to controls. As association of *PRR5L* was borderline significant and displayed the largest effect size (FC = 0.74), we also included *PRR5L* for further follow-up in this article Gene expression levels of *PRR5L* were increased in the nonagenarians, but decreased in their offspring. The gene expression differences between offspring and control groups may be due to variation in gene expression level indeed or could (partially) be explained by differences in between the groups in blood cell subsets or in disease prevalence.

Relation of gene expression in whole blood with cell subtypes

Whole blood consists of several cell subtypes and subsets of differentiated immune cells that may have different mRNA expression profiles. To investigate whether the proportions of different blood cell types could explain differential expression levels of *RPTOR* and *PRRL5*, the comparisons between offspring and controls were corrected for the relative counts of leucocytes, thrombocytes, neutrophils, lymphocytes, monocytes, basophils and eosinophils present in the whole blood samples (see Experimental procedures). We observed that variation between cell counts in offspring and controls did not influence the associations of the gene expression with familial longevity.

Recently, an increasingly important role for mTOR in directing T-cell activation and differentiation has become apparent (Derhovanessian *et al.*, 2010). We therefore examined whether the difference in expression levels of *RPTOR* and *PRR5L* between offspring and partners is influenced by the level of T-cell differentiation reflected in the distribution of different T-cell phenotypes. To this end, the frequency of naïve (CD45RA+ CCR7+ CD27+ CD28+), central memory (CD45RA−CCR7+ CD27+ CD28+), effector memory (CD45RA−CCR7−CD27−CD28−) and late-stage differentiated (CD45RA+CCR7−CD27−CD28−) T cells was assessed in 71 offspring and 73 controls. The largest effect was seen when correcting for the relative amount of effector memory T cells present in this sample; it reduced the expression difference between offspring and controls for *PRR5L* by about 60% (the beta changed from −0.44 to −0.17, Supplementary Table S4). This indicated that differences in the proportion of effector memory T cells in these groups explained about half of the differential gene expression effect for *PRR5L*. No

Table 3 Linear regression results of gene expression of offspring compared with controls

Protein	Genename	*N* cases	*N* controls	Mean	SD	FC	*P*
4EBP1	*EIF4EBP1*	320	303	2.03	0.295	1.03	0.311
FOXO1	*FOXO1*	311	296	2.18	0.238	1.00	0.878
p14	*LAMTOR2*	311	296	1.93	0.397	0.99	0.852
PRAS40	*AKT1S1*	311	296	1.67	0.278	0.96	0.282
Protor2	*PRR5L*	311	296	5.59	1.143	0.74	0.041
Raptor	***RPTOR***	**332**	**312**	**0.99**	**0.184**	**0.92**	**0.001**
RhoA	*RHOA*	311	296	2.15	0.265	1.00	0.927

Significance level for *P*-value after Bonferroni correction for multiple testing is 0.00714. Genes significantly differentially expressed with at least 5% are depicted in bold.

Mean, mean relative expression level; SD, standard deviation of relative expression level; FC, fold change, above one indicated higher expression in offspring of nonagenarians; *P*, raw *P*-value from linear regression model.

remarkable changes in effect size were found for *RPTOR* when correcting for the above-mentioned T-cell differentiation markers (Supplementary Table S4).

Association of gene expression in whole blood with disease and glucose phenotypes

Since in the literature expression levels of mTOR pathway proteins have been implicated in the pathogenesis of type 2 diabetes (Newgard *et al.*, 2009) and progression of cancer (Guertin & Sabatini, 2005; Stanfel *et al.*, 2009) and since the prevalence of diabetes differed between LLS offspring and controls (Westendorp *et al.*, 2009), we tested whether *RPTOR* and *PRR5L* expression were simply marking differences in disease prevalence. We therefore performed the same analysis between offspring and controls without known diabetics and cancer patients, which yielded similar associations to those described above (Supplementary Table S6).

Because glucose levels differed between offspring and controls (Supplementary Table S5), we investigated whether the mRNA differences of *RPTOR* and *PRR5L* depended on glucose levels in non-diabetic participants. The association of familial longevity with *RPTOR* was not affected by adjustment for glucose levels, and the association with *PRR5L* expression gained significance (Supplementary Table S7). Thus, the expression level of the *RPTOR* gene in blood mark familial longevity independent of the prevalence of type 2 diabetes and cancer and glucose levels. Due to the influence of T-cell differentiation, glucose levels and diabetes prevalence on its gene expression, the association of *PRRL5* with familial longevity is more complex.

Discussion

By comparing mRNA levels in blood of nonagenarians and middle-aged controls from the LLS, we found that the mTOR signalling gene is differentially expressed in old age. Single gene analysis showed that seven of 40 mTOR pathway genes had a significant differential expression of at least 5%, up to 1.81-fold. The expression of the genes *EIF4EBP1*, *LAMTOR2*, *AKT1S1*, *PRR5L* and *RHOA* were higher in nonagenarians, whereas *FOXO1* and *RPTOR* were lower. The directions of these differential gene expressions indicate separate effects on mTORC1 and mTORC2 complexes. Two of the seven differentially expressed genes, *RPTOR* and *PRR5L,* were expressed to a lower level in middle-aged members of the longevity families as compared to similarly aged controls. The significant differential expression of *RPTOR* and suggestive differential *PRR5L* expression indicate that expression of these genes may not just mark old age but familial longevity *per se*.

We also compared nonagenarians and controls in a genetic approach. Variation in the mTOR gene set associated with longevity in the LLS due to the joint contribution of small effects in several genes. This association could not pinpoint the main effector genes or SNPs, so we conclude that this finding must be replicated in much larger studies to test whether genetic variation in the pathway and specific genes truly associate with familial longevity.

Decreased mTORC1 protein activity is associated with lower glucose levels and a lower prevalence of diabetes in humans (Liu *et al.*, 2004; Krebs *et al.*, 2007), while impaired mTORC2 is associated with glucose intolerance and insulin resistance (Lamming *et al.*, 2012). The offspring of the nonagenarian siblings in the LLS have a lower prevalence of diabetes and lower glucose levels than similarly aged controls. Since adjustment for glucose levels and excluding diabetic patients provides similar associations, differences in the prevalence of type 2 diabetes or plasma glucose levels between offspring and controls did not explain the association of *RPTOR* and *PRR5L* mRNA level with familial longevity.

Our results could be explained if *RPTOR* gene expression in blood marks familial longevity and metabolic health in middle-age and would thus be an early marker of familial longevity. The altered gene expression levels in the longevity family members may be the consequence of aging, or merely a trait shared by the long-lived families or may truly contribute to human longevity. In view of the findings in animal models (Vellai *et al.*, 2003; Kapahi *et al.*, 2004; Kaeberlein *et al.*, 2005; Harrison *et al.*, 2009), it is not unlikely that a familial and genetic background contributing to low mTORC1 signalling activity (and preservation of low mTOR throughout life) contributes to metabolic health and an extended life expectancy across species.

In our study, we found *RPTOR*, *AKT1S1* and *EIF4EBP1* to be associated with old age and/or familial longevity, and the directions of association indicate a transcriptional down-regulation of mTORC1. Even in middle-age, a down-regulation of *RPTOR* mRNA was associated with familial longevity, providing another example of the highly conserved role mTOR signalling has in lifespan regulation across different species.

Not only mTORC1, but also mTORC2 activity has been linked to health and lifespan. Rictor, an important component of mTORC2, contributes to glucose homeostasis in murine muscle tissue (Kumar *et al.*, 2008), TORC2 *C. elegans* mutants show modulation of lifespan by affecting feeding and metabolism of different diets (Soukas *et al.*, 2009), TORC2 is a mediator of proliferative and survival signals in cancer cells (Fang *et al.*, 2012) and a study in mouse embryonic fibroblasts showed that mTORC2 regulates the TLR-mediated inflammatory response via FoxO1 (Brown *et al.*, 2011). In addition, *PRR5L*, which is part of mTORC2, has been suggested to play a role in apoptosis in HeLa cells, but whether it is pro-apoptotic or anti-apoptotic remains unclear (Thedieck *et al.*, 2007). Recently, Lamming *et al.* showed that disruption of mTORC2 can result in glucose intolerance and insulin resistance in rodents and may be relevant in the pathogenesis of type 2 diabetes and metabolic syndrome (Lamming *et al.*, 2012). Our results regarding the association of *PRR5L*, *RHOA* and *FOXO1* with old age and/or familial longevity suggest a transcriptional up-regulation of mTORC2, which would be in concordance with these earlier findings in animal models. As we provide the first evidence that mTORC2 is beneficial for healthy aging in humans, further research is required to replicate these findings.

We observed that *PRR5L* expression was higher in nonagenarians, but lower in their offspring as compared to the controls. If expression of a gene is associated with familial longevity in this population, we would expect to find a similar direction in both long-lived nonagenarians as well as their offspring. Late-differentiated memory T-cells accumulate with age and this hallmark feature of immunosenescence is believed to contribute to the weakened immune status in the elderly. It has also been shown that offspring of the long-lived nonagenarians have less accumulation of these effector memory T cells in comparison with controls (Derhovanessian *et al.*, 2010). Therefore, varying proportions of T-cell subsets might influence observed *PRR5L* expression levels in the different groups of the LLS. When testing for association with cell subtype measurements in a subset of middle-aged LLS participants, we found that *PRR5L* expression is positively associated with the amount of CCR7−CD45RA−CD27−CD28− late-differentiated effector memory cells. Data on T-cell subset counts are not available for the LLS nonagenarians, but Koch and colleagues showed the same subset of effector memory cells to be increased in the elderly (Koch *et al.*, 2008). This may explain the higher *PRR5L* expression levels we found in the nonagenarians, while lower amounts of effector memory cells (Derhovanessian *et al.*, 2010) and also lower *PRR5L* expression was found in the middle-aged offspring compared with controls. From this, we conclude that *PRR5L* expression may be a marker for the differentiation state of T cells, indicating a more late-differentiated T-cell compartment in nonagenarians as compared to younger controls, but a less-differentiated phenotype in the offspring in middle-age. The link between the mTOR pathway and T-cell activation and differentiation is perhaps not surprising when it is considered that rapamycin was first identified and exploited as a T-cell-directed immunosuppressive agent in transplantation (Morath *et al.*, 2007). The differential distribution of T-cell phenotypes contributed approximately to 60% of the *PRR5L* effect, suggesting that *PRR5L* might have another role in longevity other than marking/influencing T-cell population subtypes; further research is required to identify such a role.

In conclusion, the expression level in blood of genes belonging to the two mTOR complexes 1 and 2 associated with old age showing down- and up-regulation in the long-lived respectively. *RPTOR* gene expression was further significantly associated with familial longevity in middle-age independent of glucose levels and the prevalence of type 2 diabetes and cancer. At the level of suggestive evidence, this was also the case for *PRRL5*. These results suggest that the mTOR pathway may not only be involved in lifespan regulation in animal models, but also with the metabolic health and extended lifespan of human longevity families.

Experimental procedures

Study population

The individuals investigated in this study are participants of the LLS. The families participating in this study have at least two siblings with a minimum age for men of 89 years and for women of 91 years (Schoenmaker *et al.*, 2006). The offspring of these long-lived individuals, who have an increased potential to become long-lived individuals (30% reduced standardized mortality rate) were also included. In addition, the partners of the offspring were included as population controls of similar age and environmental exposures as the offspring and as a young control group for the nonagenarian siblings. Blood samples were taken from all the participants. The LLS was approved by the Medical Ethical Committee of Leiden University Medical Centre, and all participants gave written informed consent.

Selection of mTOR pathway

For the mTOR pathway, we selected 40 genes encoding proteins that belong to the well-described core of the mammalian mTOR pathway. First, we selected the two mTOR complexes mTORC1 and mTORC2 including their downstream targets often described in the literature (Laplante & Sabatini, 2009; Polak & Hall, 2009). Next, we included the direct activators and inhibitors of these two complexes. Figure 1 shows the mTOR pathway as we investigated.

Sample collection and RNA preparation

Eighty-seven non-related long-lived siblings, 337 offspring and 321 partners belonging to 281 nuclear families were selected for the current study (Table 1 and Supplementary Table S5). These samples were randomly selected, but in such a way that age and gender were balanced between the groups and age range was as large as possible. Additionally, individuals with outlying cell counts (beyond 3 SDs below or above the standard error of the mean) were excluded. This subpopulation is representative for the whole LLS regarding disease prevalence and parameters involved in metabolic syndrome (Supplementary Table S5) (Westendorp *et al.*, 2009; Rozing *et al.*, 2010). From these non-fasted individuals, peripheral blood was harvested using PAXgeneTM tubes (Qiagen, Venlo, the Netherlands). The tubes were frozen and kept at −20 °C for ~3–5 years. After thawing at room temperature for at least 2 h, total RNA was extracted from the approximately 2.5 mL of peripheral blood in each tube following the manufacturer's recommended protocol (PAXgene Blood RNA Kit Handbook, Qiagen, Venlo, the Netherlands). The quality and integrity of the total RNA was evaluated on the 2100 Bioanalyzer (Agilent Technologies, Amstelveen, the Netherlands), and the concentration was measured using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Quality criteria included a 28S/18S ratio as measured by the Bioanalyzer of at least 1.2, and a total RNA yield of at least 3 μg.

RT-qPCR

For all 40 mTOR signalling genes, the suggested Taqman® assay (Applied Biosystems, Bleiswijk, the Netherlands) was selected. Reverse transcription was performed with total RNA from blood of in total 790 samples, which passed QC using the First Strand cDNA Synthesis Kit according to the manufacturer's protocol (Roche Applied Science, Almere, the Netherlands). cDNA was amplified using the DNA Engine Tetrad® 2 Peltier Thermal Cycler (Bio-Rad, Veenendaal, the Netherlands). qPCR was then performed with the Taqman® method using the BiomarkTM 48.48 and 96.96 Dynamic Arrays (Fluidigm, Amsterdam, the Netherlands). Relative gene expression of the BioMarkTM Array data were calculated using the $2^{-\Delta\Delta Ct}$ method, in which C_t indicates cycle threshold, the fractional cycle number where the fluorescent signal reaches the detection threshold (Livak & Schmittgen, 2001). YKT6 was used as internal control and commercially available human total reference RNA (Clontech Laboratories, Mountain View, CA, USA) as reference RNA.

Geneset analysis of gene expression data

The Globaltest methodology was designed to determine whether the common expression pattern of genes within a pre-defined set is significantly related to clinical outcome (Goeman *et al.*, 2004; Goeman *et al.*, 2005). A generalized linear model is used to estimate a

'*Q*-statistic' for each gene set, which describes the correlation between gene expression profiles, X, and clinical outcomes, Y. The *Q*-statistic for a gene set is the average of the *Q*-statistics for each gene in the set. The Globaltest method was used to perform geneset analysis comparing two groups of individuals (either nonagenarians vs. controls or offspring vs. controls) including age (in offspring vs. controls only) and gender and their interaction as covariates. Global test package in R (Goeman & Oosting, 2011) has been used to perform analyses.

Single gene analysis of gene expression data

Differences in expression level between long-lived siblings, their offspring and the partners of their offspring were assessed using linear regression. In these analyses, expression level was the dependent variable and the two groups of individuals (either nonagenarians vs. controls or offspring vs. controls) were included in the model as a categorical variable together with age (in offspring vs. controls only), gender and their interaction as covariates. To take into account dependencies within sibships, robust standard errors were used, that is, the variance was computed from the between family variation. *P*-values were also based on these robust standard errors. Analyses were performed using the software package STATA/SE 11.0 (DPC Software, StataCorp 2009).

Sample and SNP collection for genetic association analysis

The SNP set analysis was performed in the same manner as described in Deelen *et al.* (2011b). We used the genotype data of 417 unrelated long-lived individuals and 476 young controls from the LLS, typed with Illumina HumanOmniExpress BeadChips (Supplementary Table S1). We removed SNPs with a SNP call rate <0.95, MAF < 0.01 or $P_{HWE} < 10^{-4}$ in nonagenarian cases and controls and 1018 analysed SNPs within a 10-kb window around the 40 genes from the MTOR pathway (Fig. 1) using the PLINK set-based test.

PLINK set-based statistical analysis

In the PLINK set-based test [–set-test, http://pngu.mgh.harvard.edu/purcell/plink (Purcell *et al.*, 2007)], a SNP analysis of the pathway or gene SNP set is performed. For the SNP set, a mean SNP statistic is calculated from the single SNP statistics of a maximum amount (–setmax) of independent SNPs below a certain *P*-value threshold (–set-*P*). If SNPs are not independent, that is in case linkage disequilibrium (r^2) is above a certain threshold (–set-r^2), the SNP with the lowest *P*-value in the single SNP analysis is selected. The same analysis is performed with a certain amount (–mperm) of simulated SNP sets in which the phenotype status of the individuals is permuted. An empirical *P*-value for the SNP set is computed by calculating the number of times the test statistic of the simulated SNP sets exceeds that of the original SNP set. For the analysis in this study, the parameters were set to –set*P* 0.05 –set-r^2 0.5, –set-max 99 999 and –mperm 10 000. Bonferroni correction for the pathway analysis is not necessary as it contains just one test. When SNP sets were tested per gene (40 SNP sets), the significance level for *P*-values after Bonferroni correction for multiple testing is 0.00125.

Blood cell subtypes

To further investigate the candidate genes, their expression level was again tested for association with familial longevity, but now adjusted for several blood cell counts. In the whole blood samples of the participants, the following cell subtypes were counted using the automated Siemens ADVIA 1200 system (SMSD, Tarrytown, NY, USA) in the Leiden Medical Diagnostical Center: leucocytes, thrombocytes, neutrophils, lymphocytes, monocytes, basophils and eosinophils. Both longevity-associated genes in middle-age were adjusted for each of these cell counts separately when associating with familial longevity, which analysis is described above.

T-cell differentiation measurements

Data on distribution of different T-cell subsets were generated in 71 offspring and 73 controls, of whom also gene expression data were available, as described previously (Derhovanessian *et al.*, 2010). Briefly, PBMCs viably cryopreserved with DMSO were thawed and treated with human Ig, GAMUNEX (Bayer, Leverkusen, Germany) and ethidium monoazide (EMA) (Invitrogen, Karlsruhe, Germany). Cells were first stained indirectly for KLRG-1 using a primary antibody kindly provided by Prof. Hans-Peter Pircher, Freiburg, Germany and a Pacific-Orange-conjugated secondary antibody (Invitrogen). After blocking with mouse serum (Chemicon/Millipore, Schwalbach, Germany), the following directly conjugated monoclonal antibodies were added: CD3-PE (Calltag; Invitrogen), CD4- PerCP, CD8-allophycocyanin-Cy7, CCR7-PE-Cy7 (BD Biosciences, Heidelberg, Germany), CD27-allophycocyanin, CD45RA-Pacific Blue, CD28- Alexa Fluor 700, (BioLegend, San Diego, CA, USA) and CD57-FITC (Immunotools, Freiburg, Germany). After 20 min incubation on ice, cells were washed and analysed immediately on an LSR II cytometer with FACSDiva software (BD Biosciences). The spectral overlap between all channels was calculated automatically by the BD FACSDiva software, after measuring negative and single-colour controls. Data were analysed using FLOWJO software (Tree Star, Portland, OR, USA). For data analysis, EMA-positive dead cells were excluded. T cells were characterized as naïve (CD45RA+CCR7+ CD27+ CD28+), central memory (CD45RA− CCR7+ CD27+ CD28+), effector memory (CD45RA−CCR7−CD27− CD28−) and 'terminally differentiated' effector memory (TEMRA; CD45RA+ CCR7−CD27−CD28−) T cells according to previously published models (Koch *et al.*, 2008).

Association of gene expression with insulin-related phenotypes

Since the mTOR pathway has been implicated in insulin-related phenotypes, their effect on the expression of the longevity-related genes was further investigated. Blood samples were taken at baseline for the determination of non-fasted serum parameters. For the serum measurements, the Hitachi Modular or the Cobas Integra 800, both from Roche, Almere, the Netherlands were applied. CVs of these measurements were all below 5%. Information on medical history was requested from the participants' general practitioners. As described before, differences in expression level between offspring and controls were assessed using linear regression using the same model, but excluding known Type 2 diabetes patients. The association of glucose and insulin levels with expression levels of the seven longevity-associated genes was also analysed using linear regression in controls only. To investigate the influence of glucose and insulin levels on the association with longevity of these seven genes, linear regression between offspring and controls was performed again, including one of these parameters as covariate.

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Author contributions

WMP, MB and PES designed the experiments, interpreted data and wrote the paper. WMP, JD, RB, BG and ED performed the experiments and analysed data. DH, AJMC and PES generated the study concept and study design. GP contributed to the study concept, interpretation of data and revised the paper.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Table S1 Analysed samples: genetic variation.

Table S2 Measured SNPs in the mTOR pathway.

Table S3 Results of gene set analysis of the mTOR pathway gene SNP sets.

Table S4 Linear regression results of gene expression of offspring compared with controls, including adjustments for T-cell differentiation markers.

Table S5 Descriptives of prevalence of disease and metabolic syndrome related parameters in offspring and controls (n = 658).

Table S6 Linear regression results of gene expression of offspring compared with controls, excluding diabetics and cancer patients.

Table S7 Linear regression results of gene expression of offspring compared with controls (without diabetics), including adjustments for glucose plasma levels.