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Study of transcriptional factors which regulate the enhancer activity of a VNTR located within the *SIRT3* gene

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SOMMARIO

Nell'ultimo decennio nuovi ed importanti risultati sono stati ottenuti nell'ambito della ricerca sull'invecchiamento. Gli studi sperimentali condotti negli organismi modello (lievito, verme, drosofila, topo) hanno permesso di comprendere il ruolo funzionale di una serie di pathways molecolari e di identificare differenti geni coinvolti nella regolazione del processo di invecchiamento e nella lifespan. Gli studi di associazione nell'uomo hanno, inoltre, evidenziato come la variabilità individuale osservata nell'invecchiamento umano e nella lifespan è da porre in relazione a quella genica e che i geni coinvolti in tale fenomeno sono omologhi a quelli identificati negli organismi modello. Questo legame suggerisce che i pathways molecolari relativi al controllo della lifespan sono evolutivamente conservati. In questo contesto, di particolare interesse risulta essere il gene *SIRT3*, uno degli omologhi umani del gene *Sir2* (*Silent information regulator-2*) quest'ultimo responsabile del processo di silenziamento genico, nonché coinvolto nel mantenimento della stabilità cromosomica e nel controllo della lifespan negli organismi modello. *SIRT3* codifica per una deacetilasi NAD^+-dipendente a localizzazione mitocondriale la cui attività è ancora poco conosciuta. Recentemente, studi condotti nel tentativo di comprendere il ruolo del gene *SIRT3* nel processo d'invecchiamento hanno dimostrato l'esistenza di un'associazione tra la longevità nell'uomo e un polimorfismo VNTR (sequenza core 72 pb), localizzato nel quinto introne del gene. Questo VNTR, caratterizzato da sei alleli contenenti da 1 a 6 ripetizioni, è in grado di modulare l'espressione del gene reporter della luciferasi in modo allele-specifico. In particolare, tale espressione è correlata sia al numero di ripetizioni che alla presenza o assenza del sito di regolazione trascrizionale GATA2. La presenza/assenza di tale sito è legata ad una variante (T/C) localizzata nel secondo repeat degli alleli del VNTR. Il sito GATA2 risulta, inoltre, parzialmente sovrapposto ad un sito AP-1, che non viene modificato dalla variante (T/C). Gli studi sperimentali da noi condotti hanno evidenziato che l'allele mancante del sito GATA2 (allele2b) è caratterizzato da una drammatica riduzione dell'attività enhancer se paragonato a quello che contiene tale sito (allele2a). Questo stesso allele sembra avere un effetto sfavorevole sulla longevità, data la sua completa assenza nei centenari maschi.

Alla luce di questi risultati nel presente lavoro di tesi ci siamo proposti di determinare il ruolo del fattore GATA2 sulla regolazione dell'attività di enhancer mostrata dagli alleli 2a

e 2b e sugli alleli del VNTR che contengono un'unica ripetizione (alleli 1a e 1b). Inoltre, mediante successivi esperimenti è stata valutato il ruolo funzionale del sito AP-1 e la probabile interazione tra i fattori GATA2, c-Jun e c-Fos, quest'ultimi generalmente riconosciuti quali fattori leganti in sito AP-1. A tal fine sono stati effettuati esperimenti di co-trasfezione utilizzando i costrutti contenti gli alleli specifici del VNTR e i vettori di espressione che codificano sia per le proteine GATA2 Wild Type e GATA2 Deleto che per i fattori c-Jun e c-Fos. Questi esperimenti hanno permesso di osservare che la variante (T/C) è in grado di modulare l'espressione del gene reporter della luciferasi attraverso il sito GATA2. Questo risultato è stato confermato dagli esperimenti di co-trasfezione dove GATA2 Wild Type è sostituito dalla proteina GATA2 Del. non funzionale dove l'attività della luciferasi ritorna ai valori basali.. D'altra parte, la variante (T/C) sembra modulare l'attività di enhancer del *SIRT3*-VNTR solo quando sono presenti due ripetizioni. Infatti, gli esperimenti di co-trasfezione effettuati utilizzando gli alleli 1a e 1b dimostrano che l'induzione dell'espressione del gene reporter non è da correlare alla variante (T/C).
I risultati ottenuti evidenziano che l'attività di enhancer degli alleli analizzati non è solo specificamente dovuto al fattore GATA2 ma dipende anche dai fattori c-Jun e c-Fos e, quindi, dal sito AP-1.
Mediante successivi esperimenti di co-trasfezione si è anche osservato che entrambi i siti, GATA2 e AP-1, sono in grado di cooperare in modo sinergico al fine di determinare l'incremento dell'attività di enhancer sia dell'allele2a che di quello 2b, anche se tale incremento risulta più evidente nel caso del primo dei due alleli. Gli esperimenti condotti hanno permesso di sottolineare, inoltre, che quando il numero di ripetizioni e, quindi, di siti GATA2, è maggiore di due l'attività di enhancer dipende esclusivamente dal numero di repeats e che quindi gli alleli che contengono da tre a sei ripetizioni non evidenziano differenze significative nei livelli di espressione tra le categorie alleliche a e b.
Lo studio condotto fornisce le prime evidenze sperimentali che i fattori GATA2, c-Jun e c-Fos giocano un ruolo chiave nel modulare l'attività di enhancer del *SIRT3*-VNTR e, quindi, sembrano essere fondamentali per la regolazione dell'espressione del gene *SIRT3*. Probabilmente l'allele 2b è sfavorevole per il raggiungimento della longevità nei maschi perché non può sostenere l'attività di enhancer richiesta per la funzione ottimale del gene *SIRT3*.
In relazione allo studio dei fattori genetici e delle vie molecolari che sono coinvolti nella modulazione della lifespan, nel presente lavoro di tesi abbiamo analizzato anche il ruolo svolto dal promotore nella regolazione dell'espressione del gene *SIRT3*. Abbiamo scoperto

che tale gene presenta una organizzazione strutturale "testa-testa" con il gene *PSMD13*, che codifica per la subunità p40.5, non ATP-asica, del proteasome 26S. Studiando l'attività trascrizionale della regione compresa tra i due gene abbiamo osservato che questa agisce da promotore bidirezionale. Le analisi, mediante trasfezioni transienti, di diversi deleti della regione *SIRT3-PSMD13* mostrano, inoltre, che i fattori di trascrizione Sp-1 sono importanti per la regolazione dell'attività del promotore in orientamento *SIRT3* e che questi stessi fattori devono agire in sinergia con altre proteine di trascrizione per indurre la trascrizione in orientamento *PSMD13*. Mediante analisi di linkage disequilibrium tra i due geni, effettuata utilizzando un ampio campione di popolazione comprendente anche soggetti centenari, abbiamo dimostrato che la regione intergenica *SIRT3-PSMD13* è associata con l'invecchiamento.

Nel complesso, i risultati ottenuti sono in linea con l'osservazione che esiste una relazione tra un fenotipo complesso quale la longevità e diversi geni. Tali risultati possono inoltre contribuire a comprendere il fine meccanismo che regola l'espressione dei geni e quindi chiarire l'intrigante interazione tra i livelli di espressione genica e la sopravvivenza.

SUMMARY

During the last decade, significant new insights have been gained in the research on human aging. Experimental studies on model organisms (yeast, worm, drosophila, mice) have shed light on molecular pathways, and allowed the identification of relevant genes, involved in regulating the rate of aging and lifespan. Moreover, association studies suggested that the individual variability observed in human aging and lifespan is related to the variability of the genes which are homologue to those modulating these phenomena in model organisms. These findings suggest an evolutionary conservation of pathways related to lifespan control. In this context, the *SIRT3* gene, a human homologue of *Silent information regulator-2* (*Sir2)* which is involved in gene silencing, in chromatin folding and in control of lifespan in model organisms, is especially interesting. This gene encodes for a deacetylase located in mitochondria. We found a significant association between longevity and a VNTR polymorphism (72 bp core sequence) located in the fifth intron of *SIRT3*. This VNTR displays six alleles spanning 1-6 repeats that are able to modulate the expression of luciferase reporter gene in an allele-specific way. In particular, the expression of this gene changes according to the number of repeats and the occurrence of a GATA2 site in the second repeat of the length allele2. The presence or absence of this site is correlated to a nucleotide variation (T/C) located in the second repeat. Furthermore, the sequence of GATA2 site is partially overlapping with an AP-1 site, which is not modified by the (T/C) variation. It was observed that the allele 2 lacking of the GATA2 site (allele2b) was characterized by a dramatic reduction of enhancer activity with respect to allele 2 having this site (allele2a). Besides, the allele 2b was completely absent in centenarian males while it was present in the younger group, suggesting an unfavourable effect on human longevity.

Thus, we investigated whether the presence of the GATA2 site can modulate the enhancer activity displayed by alleles 2a and 2b. Moreover, we analyzed VNTR with a single repeat (alleles 1a and 1b) and then we verified the functional role of AP-1 site and its possible interaction with GATA2 site.

Thus, we carried out co-transfection experiments by using reporter constructs containing the specific alleles described above together with expression vectors encoding either wild type or deleted GATA2 protein or expression vectors encoding c-Jun and c-Fos factors,

which generally bind AP-1 site. We showed that the (T/C) polymorphism affects the expression of the reporter gene through the GATA2 site. This effect was confirmed by co-transfection experiments where GATA2 wild type is replaced by deleted GATA2. In fact, in this case we observed that the induction of the luciferase reporter gene equals the basal value. On the other hand, the (T/C) polymorphism seems to modulate the *SIRT3*-VNTR enhancer activity only when two repeats are present. In fact, we observed in co-transfection experiments carried out by using allele1a and allele1b that the induction of luciferase expression was independent of the (T/C) variation. Furthermore, co-transfection experiments carried out using c-Jun and c-Fos factors we observed that the enhancer activity of *SIRT3*-VNTR is not exclusively due to the GATA2 factor. In fact, the AP-1 complex is able to increase the enhancer activity in a allelic-specific way and independently of the T/C variation.

Finally, in co-transfections with GATA2 and the AP-1complex we observed a synergistic effect of these factors on the enhancer activity of both alleles 2a and 2b, although this effect is enhanced when allele 2a is used. In the light of these observation it is interesting to underline that when the number of repeats, and therefore of GATA2 sites, is higher than two the enhancer activity solely depends on the number of repeats. In fact, the alleles containing from three to six repeats do not show significant differences between a and b allelic categories.

The results presented above provide the first experimental evidence that GATA2, c-Jun and c-Fos could be very important for the enhancer activity regulation of the *SIRT3*-VNTR and then *SIRT3* gene expression. So, the allele 2b is likely to be unfavourable for male longevity because it is not able to support the enhancer activity required for the optimal function of the *SIRT3* gene.

In the context of the research for genetic factors and molecular pathways which are involved in lifespan modulation, a part of the present thesis was also aimed to analyze the promoter activity in the regulation of *SIRT3* gene expression. We discovered that the *SIRT3* gene is linked in a head-to-head configuration with *PSMD13* gene, encoding for the p40.5 regulator subunit of 26S proteasome. By studying the transcriptional activity of the region shared between the two genes we observed that this region acts as a bidirectional promoter. Furthermore, transfection analyses of deletion mutants of the intergenic region showed that Sp-1 transcriptional elements play a critical role for the regulation of the promoter activity in *SIRT3* orientation, but they need to cooperate with other transcriptional factors in the *PSMD13* orientation.

In addiction, we demonstrated linkage disequilibrium analysis carried out in a large population sample including centenarians, that the intergenic region *SIRT3-PSMD13* is associated with aging.

On the whole, the results obtained in this work are in line with the observation that a relationship between a complex phenotype such as longevity and different genes exists. The findings of this work may contribute to understanding the fine mechanism that regulates gene expression and then to clarify the intriguing interplay that links expression level of gene and survival at old age.

List of Abbreviations

ADHD	Attention Deficit Hyperactivity Disorder
ATP	Adenosine Tri-phosphate
BRCA1	Breast Cancer 1 gene
CR	Calorie Restriction
D4DR	D4 Dopamine receptor
DAT1	Dopamine transporter gene 1
DBSs	Double Break Strands
DMEM	Dulbecco's Modified Eagle Medium
DNMT 1	DNA Methyl-transferase
ET-1	Endothelin-1
FOXO	Forkhead transcription factors
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
HAT	Histone Acetyltransferase
HDAC	Histone-deacetylase
HM	Heterochromatin Mating
HPG	Human Genome Project
HRAS1	v-Ha-ras Harvey rat sarcoma viral oncogene homolog 1
IDDM	Insulin Dependent Diabetes Mellitus
IGF2	Insulin-like Growth Factor 2
IL-13	Interleukin-13
INS	Insulin
LD	Linkage Disequilibrium
NADH	Nicotinamide Adenine Dinucleotide
NF-kB	Nuclear Factor *kappa* B
PCR	Polymerase Chain Reaction
PNC1	Pyrazinamidase/Nicotinamidase-1
rDNA	ribosomal DNA
RFLPs	Restriction Fragment Length Polymorphisms
ROS	Reactive Oxygen Species
RT-PCR	Real Time-Polymerase Chain Reaction
SEC14L	Secretory14 *S. cerevisiae* Like1 gene

SIR	Silent Information Regulator
SNP	Single Nucleotide Polymorphism
SP1	Specificity Protein 1
SSRs	Simple Sequence Repeats
STR	Short Tandem Repeat
TGIF	5’-TG-3’ Interacting Factor
TH	Tyrosine Hydroxylase
TSA	Tricostatin
UPS	Ubiquitin Proteasome System
VNTR	Variable Number of Tandem Repeat

1. Introduction

The discovery of the *Silent information regulator 2* gene (*Sir2*) in *Saccharomyces cerevisiae* (Ivy et al., 1986; Rine and Herskowitz, 1987; Kaeberlein et al., 1999) has represented one of the major breakthroughs of recent research in molecular biology. The protein encoded by this gene (a NAD^+-dependent deacetylase involved in DNA silencing) has proved to be a key determinant of lifespan regulation in this organism. In fact, Guarente, (2000) and Tissenbaum and Guarente, (2001) showed that lifespan of *S. Cerevisiae* was shortened by null mutations of *Sir2* while it was extended by its over-expression (extra-copies). In subsequent studies, the *Sir2* orthologue of *Caenorhabditis elegans* and *Drosophila melanogaster* were also shown to affect lifespan (Tissenbaum and Guarente, 2001; Rogina and Helfand, 2004; Wood et al., 2004). These observations triggered an enormous interest in *Sir2* and in homologous genes as they suggested the possibility of better understanding the molecular basis of the genetic control of lifespan. Thus, in recent years a large amount of data have accumulated regarding the molecular biology of these genes and on their effects on lifespan in different organisms (Haigis et al., 2006; Bordone, 2006). In humans, as in other mammals, seven different homologues of the *Sir2* gene have been described, and this has triggered many studies on the regulation of these genes and on their ability to contribute to longevity in humans. In this context *SIRT3*, one of the closest human homologues of Sir2, proved to be extremely interesting because of its localization in mitochondria which are well known as one of the most important check-points for lifespan control, and because of its expression in brown adipocytes, known for their correlation with metabolism and lifespan control. In addition, human *SIRT3* is located in the 11p15.5 chromosomal region which has been shown to encompass different genes correlated with longevity. Indeed, a case control study carried out on a Southern Italian population indicated a correlation between *SIRT3* variability and longevity (Rose et al., 2003). However, a silent polymorphism was used in this study furthermore the functional variant causing the observed association remained unknown.

During the three years of my PhD appointment I was involved in the study of the regulation of *SIRT3* gene expression and defining the molecular basis of the previously observed association between the variability of this gene and human longevity. In my work

I took advantage of the most up to date knowledge concerning the regulatory capacity of repetitive DNA sequences, as the main regulator of *SIRT3* proved to be a VNTR.

1.1 Chromatin Silencing and Lifespan

Gene silencing is a global mechanism of transcriptional control. This process assembles specific regions of eukaryotic chromosomes into transcriptionally inactive chromatin structures to prevent the transcription of genes located in these regions. Silencing involves specialized regulatory sites that are known as silencers and a combination of general DNA-binding proteins and other proteins specifically dedicated to silencing (Yang et al., 2000). On the other hand, an important role in the regulation of this process is played by the basic unit of chromatin: the nucleosome. The histones of nucleosomes are modified by methylation, phosphorylation, ubiquitination and acetylation. All these processes are known to have profound influences on the regulation of gene expression and other processes which lead to the structuring of genomic DNA in to chromatin. Histone acetylation occurs at lysine residues and at positions that are conserved in eukaryotes, especially for histones H3 and H4. Histone acetylation is regulated by the opposing activities of histone acetyltransferase (HAT) and histone deacetylase (HDAC) enzymes and is correlated to the regulation of gene activity.

These observations suggest that the reversible acetylation of histones plays a critical role in the mechanisms of silent chromatin formation. Thus, the identification and characterization of numerous transcriptional regulators possessing HAT or HDAC may provide a very important basis for understanding the regulation of transcription, replication and the recombination machinery (Struhl, 1998). Since the HDACs are well conserved, their basic functions have been successfully studied in the less complex and experimentally manageable model organisms, such as yeast (*Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*) and *Caenorhabditis elegans* and then in human cell lines.

In *S. cerevisiae Sir2* gene was characterised (Ivy, 1986; Rine and Herskowitz, 1987; Kaeberlein, 1999). It encodes for a protein that exhibits histone deacetylase activity which is nicotinamide dinucleotide phosphate (NAD^+)-dependent (Frye, 1999; Smith, 2000; Imai, 2000). This deacetylase contains a conserved catalytic core domain conferring the NAD^+-dependent protein deacetylase and ADP-ribosyltransferase activity (Imai, 2000). In fact, mutations that lowered NAD^+ synthesis *in vivo* impaired the activity of Sir2 proteins (Guarente, 2000; Hasty, 2001). Sir2 is involved in gene silencing and in many biological processes, such as chromatin folding, control of the cell cycle, DNA repair, and lifespan control (Smith and Boeke, 1997; Guarente, 1999; Blander and Guarente, 2004). In

particular, the yeast S*ir2* gene has been reported to be a key determinant of lifespan regulation in this organism. In fact, Kaeberlein et al., (1999) and Guarente, (2000) showed that null mutations of *Sir2* shortened lifespan while an over-expression (extra-copies) extended lifespan, likely through chromatin silencing in the ribosomal DNA repeats. In fact, *Sir2* promoted the increase of cell survival in the non mitotic state (replicative longevity) by repressing the recombination of repetitive ribosomal DNA (rDNA) and the subsequent formation of extra-chromosomal rDNA circles (Kaeberlein, 1999; Guarente, 2000). The NAD^+-dependence of deacetylation mediated by Sir2 suggested this protein could represent a biological link between energy metabolism, genome maintenance, and aging. In line with this hypothesis, Calorie Restriction (CR), which extends the lifespan of yeast mother cells was found to up regulate the silencing activity of *Sir2* (Fig.1.1) (Lin, 2002; Bordone and Guarente, 2005).

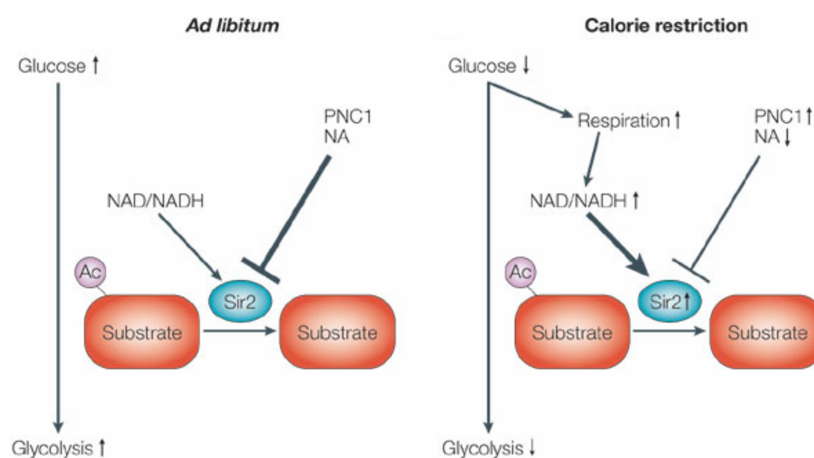


Fig (1.1) *Sir2 might connect cellular energetics to lifespan in yeast (Bordone and Guarente, 2005)*

The link between mitochondrial respiratory changes and Sir2 activity is thought to be due to an alteration in the NAD/NADH ratio or, alternatively, to the increased expression of PNC1 (pyrazin-amidase/nicotinamidase-1), which encodes a nicotinamide that lowers the levels of nicotinamide, another Sir2 inhibitor (Anderson et al., 2002; Lin et al., 2004). Subsequent studies showed that the homologues of *Sir2* were able to mediate longevity also in other organisms. In fact, it was reported that both in the nematode and in drosophila the *Sir2* gene homologues were able to extend the lifespan of these organisms by affecting pathways relative to nutritive state and stress response (Tissenbaum and Guarente, 2001; Rogina et al., 2004; Wood et al., 2004).

The results obtained in these organisms indicate the possibility that *Sir2* homologues may modulate different pathways linked to aging also in higher organisms including mammals and possibly in humans.

1.1.1 Human Histone Deacetylases (HDACs)

The HDACs act in different ways in histone deacetylation and, therefore, in the general process of regulation of gene expression. Eighteen distinct human HDACs have been identified and separated into three classes on the basis of their similarity to yeast histone deacetylases (Grey and Ekstrom, 2001).

CLASS I (RPD3-like Histone Deacetylases): The isolated human histone deacetylase HDAC1,-2,-3,-8 and -11 has 60% identity to the yeast RPD3 at the protein level (Tauton et al., 1996). Members of the Class I HDACs have been demonstrated to be associated with other complexes including 5'-TG-3'-interacting factor (TGIF/Smads), glucocorticoid receptor, DNA methyltransferase 1 (DNMT1), and with other important proteins including Beta-Catenin and Sp1 (Tauton et al., 1996). All four members of this Class of histone deacetylases have been demonstrated to be sensitive to histone-deacetylase-specific inhibitors such as trichostatin A (TSA) and n-butyrate (Dangond and Gullans, 1998). In addition, they were shown to be predominantly nuclear proteins expressed in most tissues and cell lines and have been mapped to different chromosomal locations (Fischle et al., 2001).

CLASS II (HDA1-like Histone Deacetylases): Members of Class II histone deacetylases are homologous to the yeast HDA1 and are subdivided into two sub-Classes, IIa (HDAC4,-5,-7 and -9 and its splice variant MITR) and IIb (HDAC6 and HDAC10), based on sequence homology and domain organization (Verdin et al., 2003). The function of Class II has yet to be fully delineated, but emerging evidence points to a role in cellular proliferation/differentiation. In addition, it has been demonstrated that these deacetylases are associated with the MEF2 family of transcription factors (Lemercier et al., 2000). Furthermore, members of this Class have been shown to be actively maintained in the cytoplasm and are imported to the nucleus when required (Verdel et al., 2000).

Fig.(1.2) represents the phylogenetic relationships of yeast and human Class I and II HDACs.

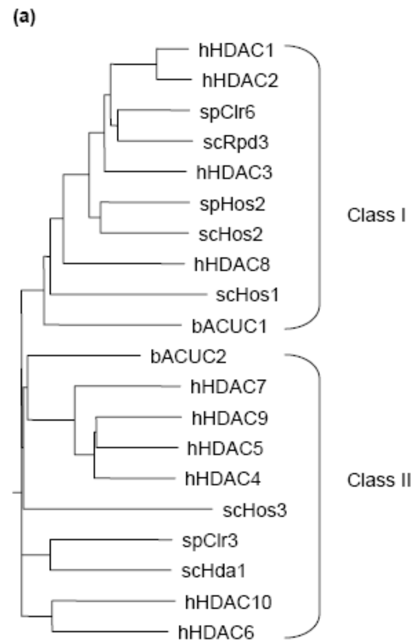


Fig.(1.2) *Dendograms representing the phylogenetic relationships of yeast and human HDACs (a) The Class I and II HDAC subfamilies (Ekwall, 2005)*

CLASS III (SIR2-like Histone Deacetylases): The deacetylase enzymes of Class III, Fig.(1.3), also called sirtuins, are homologues to the Silent information regulator 2 (Sir2) protein (Guarente, 2000; Ekwall, 2005).

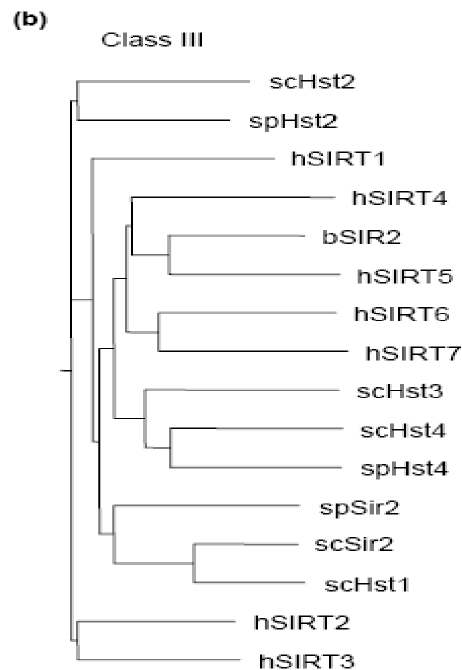


Fig.(1.3) *Dendograms representing the phylogenetic relationships of yeast and human HDACs (b) The Class III HDAC family (Ekwall, 2005)*

Molecular phylogenetic analysis of 60 sequences of the conserved sirtuin core domain from a wide variety of organisms (including archaens, bacteria, yeast, plants, protozoans, and metazoans) shows that there are several short motifs of conserved amino acids. Some short motifs seem to mediate protein-protein or protein-DNA interactions. Other short conserved sequences in this domain are reported to be important for promoting catalytic activity, as mutations of these motifs have been shown to abrogate this activity (Imai et al., 2000). The aforementioned molecular phylogenetic analysis showed that eukaryotic Sir2-like proteins could be divided, according to sequence homology, into four main branches designated as sirtuin-Classes I-IV plus an additional ***U***ndifferentiated class called "sirtuin - Class U" (including the *Sir2* homologues of vertebrate, insect, nematode, protest, and plant lifeforms) (Frye, 2000). Thus, these data suggest that sirtuins from organisms representing diverse phyla can be grouped into distinct sequence-related Classes. In particular, *S. cerevisiae* has five sirtuins from sub-Class I, while *C. elegans* and *D. melanogaster* have sirtuin genes from sub-Classes I, II, and IV. There are no Class I or IV prokaryotic sirtuins whereas most bacterial sirtuins are of the Class III variety (Gray, 2001).

1.2 Human homologues of the Sir2 gene

In the human genome seven different gene homologues to the *Sir2* gene (*SIRT genes)* have been identified. These data were demonstrated to be in line with observations carried out in other mammals, such as the mouse.

The human sirtuins are defined by a conserved core domain. In some instances additional N-terminal or C-terminal sequences are present. On the basis of their sequence, each of the seven identified human sirtuin genes fall into different sirtuin-Classes: *SIRT1*, *SIRT2*, and *SIRT3* fall in Class I, *SIRT4* in Class II, *SIRT5* in Class III, and *SIRT6* and *SIRT7* in Class IV (Table 1.1).

Class I	**Class II**	**Class III**	**Class IV**
SIRT1 (10q21.3) *SIRT2* (19q13) *SIRT3* (11p15.5)	*SIRT4* (12q)	*SIRT5* (6p23)	*SIRT6* (19p13.3) *SIRT7* (17q25)

Table 1.1 *SIR2-like Histone Deacetylases and Chromosomal location*

Sirtuins have been found to have different cellular localization (Michishita et al., 2005; Sinclair and Guarente, 2006). SIRT1 is located in the nucleus, SIRT2 protein is observed in the cytoplasm, while SIRT3, SIRT4 and SIRT5 are found in mitochondria. Finally, SIRT6 and SIRT7 are associated with heterochromatic regions and nucleoli, respectively (Imai, et al., 2000; Marry, et al., 2004; Bordone and Guarente, 2005; Sinclair and Guarente, 2006). Fig. (1.4) summarizes the different localization of human sirtuins.

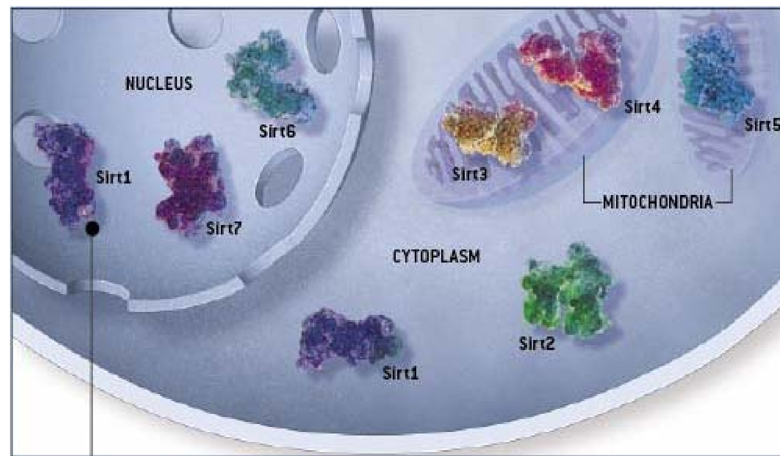


Fig.(1.4) *Cellular localization of the seven human protein homologues to Sir2 (SIRT1-SIRT7) (Sinclair and Guarente, 2006)*

The functional roles played by the different human sirtuins have yet to be established only for some of them. In fact, Vaquero et al., (2004) suggested that SIRT1 could promote the transcriptional control of a specific set of single-copy genes through conserved deacetylase activity, and could regulate the chromatin structure and transcription of ribosomal DNA clusters (Vaquero, et al., 2004; Picard, et al., 2004). In addition, *in vivo* deacetylation assays demonstrated the ability of SIRT1 to deacetylate cellular proteins, such as p53 and FORKHEAD and to modulate cellular stress responses and survival through regulation of NF-kB signaling and FOXO transcription factors (Motta et al., 2004; Yeung et al., 2004). It is important to underline that SIRT1 is expressed in all mammalian somatic and germline tissues and hence it may be a good candidate to regulate the known effects of calorie restriction (CR) in the soma and germline (McBurney et al., 2003). SIRT1 function has also been examined in the context of cellular senescence in cell cultures. Moreover, preliminary studies examining links between SIRT1 function and cancer suggest that forced reduction of SIRT1, by RNA interference (RNAi), led to growth arrest and/or apoptosis in human epithelial cancer lines but not primary epithelial cells (Chua et al.,

2005; Ford et al., 2005; Ota et al., 2006). Consistent with these reported results, this sirtuin is postulated to protect against neurodegeneration and diabetes but to promote tumorogenesis (Ota et al., 2006). However, its role in disease progression and aging in mammals remains to be fully determined. In this context it is worth noting that a Knock In mouse containing an extra copy of *SIRT1* has been developed recently (Bordone et al., 2006). This is likely to lead to the unveiling many mechanisms of SIRT1 function and its effects on life span. The other sirtuin proteins showed different activities. SIRT2, was reported to deacetylate alfa-tubulin, regulating in this way the microtubule and then the cell cycle (North et al., 2003). The functional properties and the roles played by the *SIRT3* gene will described in detail in the following paragraph. SIRT4 and SIRT5 are the most distant sirtuins from Sir2 and SIRT1 from a phylogenetic standpoint. RT-PCR analysis of SIRT4 and SIRT5 expression profiles from adult and fetal tissues demonstrated that these genes are highly expressed in all tissues although their roles have yet to be discovered (Hubbert et al., 2002; Blander and Guarente, 2004; Michishita et al., 2005; Haigis et al., 2006). SIRT6 and SIRT7 are located in the nucleus and are reported to play a role in the dynamic regulation of chromosome structure and function during mitosis (Frye, 2000; North and Verdin, 2004). Despite their nuclear location, which is similar to the cellular location of yeast Sir2, SIRT6 and SIRT7 are unable to deacetylate the histone H4 peptide containing the acetylated lysine 16 residue, a target site of yeast Sir2 deacetylase, whereas SIRT1 showed strong NAD^+-dependent deacetylase activity (Liszt et al., 2005).

1.3 The *SIRT3* gene

The *SIRT3* gene is characterized by 7 short exons and very long introns. It lies at the telomeric terminal on 11p15.5 chromosome and spans 20Kb (www.ncbi.nlm.nih.gov/omim: MIM 604481; contig NT_035113) (Fig. 1.5).

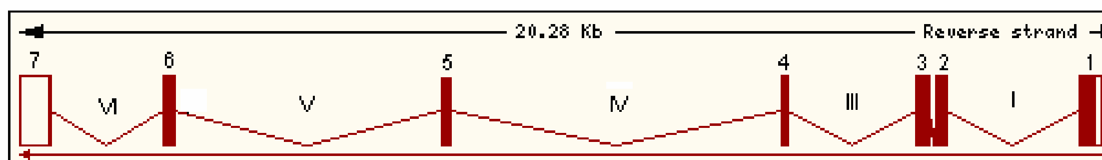


Fig.(1.5) *Organization in exons (1-7) and in introns (I-VI) of SIRT3 gene*

From a phylogenetic point of view it is very close to *Sir2* and *SIRT1*, Fig (1.3) and it is the closest paralog of *SIRT2* (Frye, 2000).
Northern blot analysis demonstrated that the gene is ubiquitously expressed, particularly in metabolically active tissues, such as brain, heart, liver, kidney, testis, and muscle (Yang et al., 2000; Onyango et al., 2002). The *SIRT3* gene encodes for a protein that is located in the mitochondrial matrix (Onyango et al., 2002; Schwer et al., 2002). Mitochondrial import of SIRT3 is dependent on the NH_2-terminal amphipathic alfa-helix which is rich in basic residues. SIRT3 is proteolytically processed in the mitochondrial matrix into a 28 KD product. These observations demonstrate the existence of a latent Class III deacetylase that becomes catalytically activated upon import into the human mitochondria (Onyango et al., 2002; Schwer et al., 2002). SIRT3 has NAD^+-dependent deacetylase activity but the functional properties and the biological targets of this protein are still largely unknown (Blander G. and Guarente, 2004; Onyango et al., 2002; Schwer et al., 2002). A hypothesis regarding the role played by SIRT3 protein postulates that it might deacetylate mitochondrial proteins directly involved in apoptosis and that it could be involved in mitochondrial ADP-ribosylation. In fact, ADP-ribosyl transfer has been reported for some sirtuins (Onyango et al., 2002). In any case, both its localization in mitochondria and NAD^+-dependent activity are of particular interest as they suggest a possible role for SIRT3 as a sensor of metabolic or oxidative states of cells capable of regulating cellular functions (Shi et al., 2005; Hallows et al., 2006; Schwer et al., 2006).
An important aspect of the molecular biology of SIRT3 is that, similarly to yeast Sir2*,* it seems to be able to mediate the effects metabolism and caloric restriction. In fact, SIRT3 is able to activate mitochondrial functions and play an important role in adaptive thermogenesis in brown adipose tissues. Calorie restriction activates SIRT3 expression in both white and brown adipose but SIRT3 expression in the latter is elevated in response to cold while it is reduced in a thermoneutral temperature. Constitutive expression of SIRT3 in brown adipocytes not only increases the expression of genes related to mitochondria function and thermogenesis, such as PGC-1α and UCP1, but it also reduces $\Delta\psi m$ and ROS and increases oxygen consumption. Finally, the down-regulation of SIRT3 and related mitochondrial genes, such as COX II and ATP synthetase, in brown adipocytes correlates with obesity. Thus, the reduction of thermogenesis may contribute to the development of obesity (Shi et al., 2005). Besides, an intriguing recent study has demonstrated that SIRT3 protein could directly control the activity of the mitochondrial Acetyl-CoA synthetases2 (AceCS2). Some data provide direct evidence that mitochondrial AceCS2 is inactivated by

acetylation on Lis-635, and suggest that SIRT3 could function as the regulatory deacetylation in mitochondria. On the whole, these results suggest that SIRT3 may regulate acetyl-CoA levels through modulation of AceCS2 activity in mitochondria. AceCS2 is the first example of a mitochondrial protein to be controlled by reversible lysine acetylation and the first target protein for a mitochondrial sirtuin deacetylase. A schematic overview of the regulation of AceCS2 by reversible lysine acetylation is given in Fig.(1.6) (Hallows et al., 2006; Schwer et al., 2006).

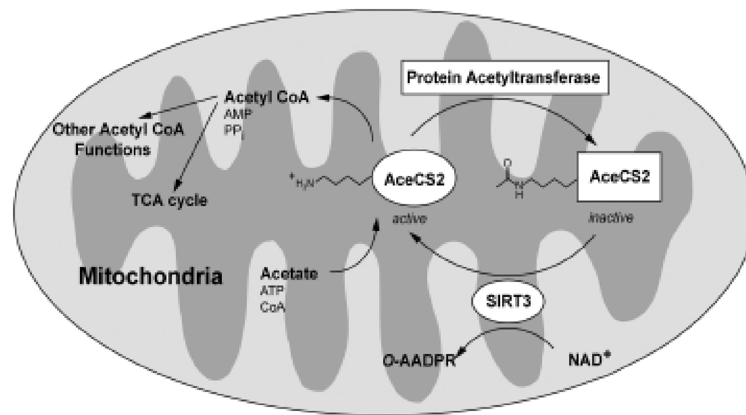


Fig.(1.6). *Proposed model for the regulation of mammalian AceCS2 SIRT3. In the mitochondria, SIRT3 catalyzes the deacetylation of AceCS2 on Lys-635 (murine). Free acetate, generated from endogenous cellular reactions or absorbed from the gut, is converted to acetyl-CoA that can be used in metabolic pathways, such as tricarboxylic acid cycle in mitochondria.*

Furthermore, with regard to studies correlating cancer progression and sirtuin expression, increased levels of *SIRT3* transcription have been associated with node positive breast cancer and it was demonstrated that there is a differential sirtuin expression profile for non-malignant and malignant breast tissue, with consequent diagnostic and therapeutic implications (Ashraf et al., 2006).

On the whole, the *SIRT3* gene can carry out differential effects on apoptosis, stress responses, mitochondrial function and thermogenesis in brown adipocytes, ROS production, oxidative damage, and on the regulation of metabolic pathways. On the basis of these observations the *SIRT3* gene is a good candidate for modulating longevity, linking cellular metabolism, genome maintenance and aging (Guarente, 2000; Hasty, 2001). In addition to the above evidence, it is interesting to report that the *SIRT3* gene lies at the telomeric terminal on the chromosome 11p15.5 region containing other genes whose

polymorphisms were found to be associated with longevity. These genes from telomere to centromere position are: *PSMD13* (Proteasome 26S, non-ATPase 13 gene), *HRAS1* (v-Ha-ras Harvey rat sarcoma viral oncogene homolog 1), *IGF2* (Insulin-like Growth Factor 2), *INS* (Insulin) and *TH* (Tyrosine Hydroxylase) (De Benedictis et al., 1998; Meloni et al., 1998; De Luca et al., 2002; Bonafè et al., 2002; Chondrogianni et al., 2003; Chondrogianni and Gonos, 2004).

On the basis of these observations, an association study was carried out in a large population sample including centenarians in order to verify if a silent marker, G477T, located in the coding region of the *SIRT3* gene was associated with survivorship in the elderly (Rose et al., 2003). Indeed, an association was found between the variability of this marker and survivorship. This finding raised a great interest to identify the functional variant causing such an association.

1.4 Sequence variations: a genome overview

Recent decades technological developments have provided new methods of analysis for molecular genetics which have led to new insights in many aspects of human genetics. In particular, the success of the Human Genome Project (HGP) has given an unprecedented understanding of the structure and organization of our genome. This has allowed a reappraisal of the theories on evolution of the human genome and on the nature and dynamics of its variability.

The HGP generated the draft sequence from a physical map covering more than 96% of the euchromatic part of the human genome. The data obtained, together with additional sequence found in public databases cover about 94% of the genome. The current estimate of the size of the human haploid genome is about 3,200 Mb of DNA and it is estimated to contain no more than 32,000-40,000 genes. These figures are slightly higher than those reported for the nematode (23,000 genes) or the fruit fly (26,000 genes). This has challenged the pre-existing general assumption that higher vertebrates with greater phenotypic complexity have more complex genomes. The modest number of human genes indicates that the complexity of human development and the sophisticated signaling system that maintains homeostasis is mainly due to the regulation of these genes and to their interaction. Human DNA was found to include sequences that appear to be unique or rarely

repeated and sequences that have been found to be repetitive. The latter have been estimated to account for about 15% of the entire genome.

The repetitive DNA can be subdivided into two types, depending on whether the repeat units are dispersed singularly (interspersed repetitive DNA) or clustered together. The latter are subsets of repetitive sequences consisting of tandem repeats, which are consecutive perfect or slightly imperfect copies of DNA motifs of variable lengths (Charlesworth et al., 1994; Bennett et al., 2000). Blocks of tandemly repeated sequences are widespread in the genome, such as centromeres, telomeres, the short arms of acrocentric chromosomes and ribosomal gene clusters (International Human Genome Sequencing Consortium, 2001). In general, based upon both the size of the individual repeating unit and overall repeat length, tandemly repeated sequences have been classified into three major subgroups which have been termed satellites, minisatellites, and microsatellites although some confusion in the precise definition of the different repeated sequences is present in the literature (Li 1997; Naslund et al., 2005). Satellite DNA was the first type of tandem repeated DNA to be discovered. It was named “satellite DNA” because of the appearance of minor or “satellite” bands which seemed to be separated from the “bulk” DNA upon buoyant density gradient centrifugation (Britten and Kohne, 1968). The basic repeat unit of satellite DNA can vary from five to several hundreds base pairs. These sequences are not transcribed, and are usually organized as large clusters in the heterochromatic regions of chromosomes near centrosomes and telomeres. Satellite DNA is also found abundantly on the Y chromosome (Charlesworth et al., 1994; Csink and Henikoff, 1998; Durdica and Miroslav, 2002). Some satellites constitute important functional components of chromosomes; for instance alpha satellite, which is a component of centromeres, has an important role in the formation of heterochromatic compartments essential for the chromosome migration occurring in mitosis and meiosis (Schueler et al., 2001; Durdica and Miroslav, 2002).

Microsatellites, which are widely known as Short Tandem Repeats (STR) or Simple Sequence Repeats (SSRs) consist of specific DNA sequences which are moderately repetitive and contain mono, di, tri, or tetra tandem repeats. In the human genome about 30,000 microsatellite loci have been observed to be uniformly distributed in the euchromatin. These sequences are highly variable for the number of the repeats, and have proved to be inherited according to Mendelian rules. Due to these features and due to the ease of their typing by PCR, STRs are widely used as genetic markers in human genetics.

Finally, minisatellites**,** also called Variable Number of Tandem Repeats (VNTRs), are usually defined as tandem repeats of a short (6-100 bp) motif ranging from 0,5 Kb to several Kbs. The scanning of the human genome sequence has underlined that VNTRs are generally widespread throughout the genome and, in particular, appear to be more abundant in subtelomeric and centromeric regions (Denoeud et al., 2003; Naslund et al., 2005). It is interesting to note that there is an inverse relationship between copy number and unit size. In fact, minisatellites with a high copy number are generally composed of short repetitive units while minisatellites with large repetitive units usually display a few repeats. In addition, it has been observed that minisatellites with a very high number of repeats tend to show less sequence similarity between units (<70%). This seems to be due to the different evolution rate of different types of minisatellites. In fact, minisatellites with large units tend to be more stable than minisatellites with short units and thus less prone to expand and this seems to have led to the inverse relationship between copy number and unit size, and to the low sequence similarity of large VNTR (Naslund et al., 2005). Minisatellites frequently exhibit length polymorphism, which results from the variation in the number of internal copies. In fact, VNTR, have represented valuable genomic markers that for a long time were thought to be neutral. VNTR provided the first highly polymorphic multi-allelic markers for linkage studies, forensic applications and were used in the early stages of human genome mapping (Bell et al., 1982; Nakamura et al., 1987; NIH/CEPH Collaborative Mapping Group, 1992; Nakamura et al., 1988). Denoeud and coll. (2003) have defined sequence-based predictive criteria to identify polymorphic and hypermutable minisatellites in the human genome by scanning chromosomes 21 and 22. Assuming that these chromosomes are representative of all human chromosomes and given that they represent ~2% of the whole genome, the authors demonstrated that the entire human genome contains ~6,000 minisatellites, including 4,800 polymorphic and 2,500 very polymorphic ones. A few tens of these might be expected to qualify as hypermutable minisatellites. Interestingly, these hypermutable VNTR were found preferably to be within coding sequences (Denoeund et al., 2003).

More recent studies have reported that VNTRs may have important functional roles (Nakamura et al., 1998; Naslund et al., 2005). In particular, minisatellites were shown to contribute to genome function in different ways. In fact, VNTRs were found to be involved (*i*) in the binding sites of different transcriptional factors, such as the 5' region of genes where they participate to the transcription regulation, or within introns where they participate to splicing regulation (Kennedy et al., 1995; Turri et al., 1995; Paquette et al.,

1998; Vergnaud and Denoeud, 2003); (*ii*) in open reading frames, which may or may not display polymorphism in human populations (Bois and Jeffreys, 1999; Vergnaud and Denoeud, 2000); (*iii*) in chromosome fragile sites, as they have been found in the vicinity of a number of recurrent translocation breakpoints and in the switch recombination site in immunoglobulin heavy chain genes (Brusco et al., 1999; Vergnaud and Denoeud, 2003). In addition, minisatellites have been proposed as intermediates in chromosome pairing initiation in some eukaryote genomes, which might be related to their proposed recombinogenic properties (Wahls and Moore, 1998; Sybenga, 1999).

1.5 VNTR sequences as functional regulators

The evidence for the role of VNTR as transcriptional or translational regulator as well as post transcriptional function modulators have accumulated in recent years.

A very interesting example of VNTR that may influence transcription, was first found within the insulin gene (*INS*). In fact, Lucassen et al., (1993) observed an association of the length of this VNTR with susceptibility to Insulin-Dependent Diabetes Mellitus (IDDM). Subsequent analysis discovered that the alleles with 140-210 repeats of the *INS*-VNTR lead to an increased expression of insulin transcript in the fetal thymus. This high level of insulin expression in the thymus promotes negative selection of insulin specific T-lymphocytes and plays a critical role in the pathogenesis of IDDM (Pugliese et al., 1997; Vafiadis et al. 1997).

An additional example of VNTRs as transcriptional regulators is represented by the tetranucleotide repeat (TCTAT)n located in the first intron of the Tyrosine Hydroxylase (*TH*) gene (HUMTHO1 microsatellite) which acts as a transcriptional enhancer *in vitro* (Meloni et al., 1998; Albanese et al., 2001). This effect was found to be independent of the sequence orientation and was correlated with the number of repetitions. Another example of VNTRs as transcriptional regulators is represented by a VNTR present in the 3' flanking region of the v-Hs-ras Harvey rat sarcoma viral oncogene (*HRAS*). This VNTR is highly polymorphic and it ranges from 1000 to 2500 bp corresponding to 35-90 repeats. The presence of one or two rare alleles appears to be associated with a higher than normal risk of multiple cancers, such as colon and breast carcinomas (Krontiris et. al., 1993; Nakamura et al., 1998). Moreover, Phelan et al. (1996) suggested that some rare alleles of the *HRAS*-VNTR may influence the penetrance of ovarian cancer in subjects who carry a mutation in

the breast cancer susceptibility gene Breast Cancer 1 gene (*BRCA1*). In fact, the risk of developing ovarian cancer was doubled in women with mutated-*BRCA*1 who also carried one of two specific *HRAS*-VNTR rare alleles (Nakamura et al., 1998). Further, VNTR that acts as transcriptional regulator was identified in the 15th exon of *DAT1*, the gene encoding the human Dopamine Transporter (hDAT). The sequence unit of this VNTR, 40 bp, is repeated 3-13 times (Kang, 1999; Mitchell et al., 2000; VanNess et al., 2005). Different studies demonstrated that *hDAT*-VNTR may influence the regulation of dopaminergic neurotransmission and it is implicated in conferring genetic vulnerability for ADHD (Attention Deficit Hyperactivity Disorder) (VanNess et al., 2005).

It is interesting to note that this polymorphic variation may be evolutionarily recent, as a homologue of the *hDAT*-VNTR has been observed in humans, chimpanzees, and *Cynomologus macaques*, but not in lower mammals including rats and mice (Miller et al., 2001). It is important to underline that the functional activity of VNTR sequences is correlated with the presence of specific sites binding transcriptional factors. These sequences often are typical of promoter or enhancer regions. For example, VNTR sequences present in the 3' flanking region of the *HRAS* gene can bind some members of the rel/NF-kB family of transcriptional regulators and this process has been shown to activate transcription of a reporter gene in some cell lines (Trepicchio and Kronitiris, 1992). Other important members of transcriptional regulators binding VNTR sequences are some members of GATA family. The GATA factors bind the consensus DNA sequence (A/T)GATA(A/G) with one or two distinctive zinc-finger domains. These factors have been shown to play critical roles in development, regulation of differentiation and control of cell proliferation (Patient and McGhee, 2002). In particular, among GATA proteins, GATA2 has emerged as a key transcriptional factor in the control of proliferative self-renewal, hematopoietic differentiation progenitor cells, and it is also an important factor for the regulation of a number of cell-specific enhancers (Yamamoto et al., 1990). An additional essential protein binding motif identified within VNTR sequences is the AP-1 (TCAGTGAG) site. Both members of Jun and Fos families are able to bind to AP-1 site, although Fos members can bind only in association with a Jun member (Lee et al., 1991). The AP-1 sites have been shown to be involved in the control of cellular proliferation, transformation processes, survival and cellular death. Furthermore, these sites are able to modulate the expression of cyclin D1 and proteins such as p53, p21, p19, p16 (Shaulian and Karin, 2000). Several studies have investigated the regulation of the activity of AP-1 complex. This complex is induced by growth factors, cytokines, and some physic-chemical

stress agents. This activity of the AP-1 complex seems to increase with aging (Basheer and Shiromani, 2001).

For many years GATA2 and the AP-1 complex were thought to be the terminal part of independent transduction pathways. By contrast, several studies suggested that GATA, c-Jun and c-Fos factors participate in gene regulation through common sites of regulation thereby increasing transcription through cooperative synergic activity (Masuda A., et al., 2004). In fact, it has been observed that GATA and AP-1 sites are found in close association in the core hypersensitivity sites of the globin locus control region and in a number of erythroid promoters (Ney et al., 1990). Moreover, this interaction between GATA2 and the AP-1 complex has a critical role in the regulation of IL-13 (Interleukin-13) and Th2 cytokine gene transcription in mast cells (Hural et al., 2000). Finally, in the promoter region of the Endothelin-1 (ET-1) gene the c-Jun/c-Fos heterodimer was found to cooperate with GATA2 to activate transcription (Kawana et al., 1995). This interaction between different transcription factors has then provided a functional link between separate signal transduction pathways.

As reported above, VNTRs can also act as translational regulators. An example of VNTRs with translational regulator activity is the VNTR in the 3'UTR region of the *Secretory14 S. cerevisiae Like1* (*SEC14L*) gene. In a study carried out to verify the different efficiencies of the *SEC14L*-VNTR alleles that are most common in the Japanese population, it was found that large VNTR alleles determine a reduction of the translation efficiency by affecting the stability of mRNA (Nakamura et al., 1998).

Finally, VNTRs have been identified within the coding sequences of different genes (Chinen et al., 1996). For instance, a VNTR leading to a tandemly repeated 20-aminoacid sequence has been identified in the human epithelial mucin gene. However, to date, no evidence has been documented on the influence of the variable number of this 20-aminoacid sequence on the physiological functions of the protein.

A further example of a VNTR within the coding sequence showing an allele specific effect on the phenotype, is present in the DNA sequence corresponding to the putative third cytoplasmic loop of the D4-Dopamine Receptor (*D4DR*) gene (Lichter et al., 1993; Nakamura et al., 1998). The *D4DR* VNTR leads to a variable number of a 16 aa repeat in the protein. As this gene is expressed at a high level in limbic areas of the brain, an association of this polymorphism with cognitive and emotional behaviours has been suspected since it was characterised. Indeed, differences in ligand-binding affinity between D4DR molecules containing different numbers of the 16 aa sequence have been observed

(Van Tol et al., 1992; Asghari et al., 1994). On the basis of these observation, Benjamin et al., (1996) examined the relationship between the length of the *D4DR*-VNTR and some personality traits and found that differences in the number of repeated sequences is implicated in "novelty seeking" behaviour. The effect of this polymorphism on the physiological activity of the gene product was proposed to be due to the alteration of the protein structure caused by the different number of repeated sequences.

The information reported here underlines the importance of VNTR sequences as functional elements of the genome. Thus, the identification of additional human genes containing VNTR within exons, introns, or flanking regions will contribute to an increased understanding of the regulation of the transcriptome and, consequently, to elucidate complex human traits.

Human aging and longevity are complex traits with a genetic component exerted by the modulation of the activity of many genes. Thus, it seemed important to study VNTRs with functional activity influencing the regulation of genes that contribute to this multifactorial phenomenon.

2. GATA2 and AP-1 complex transcription factors regulate the enhancer activity of the *SIRT3*-VNTR in an allelic specific way

2.1 Background

In a recent paper (Bellizzi et al., 2005 enclosed in the section of published papers) we reported the discovery of a VNTR polymorphism (72bp repeat core) in the intron 5 of the *SIRT3* gene. According to the number of repeats, we named the VNTR alleles 1-6. Through a preliminary analysis of the VNTR polymorphism in a population sample of 20-80 years old subjects (n=703), we observed that out of the six alleles, four were common (alleles 1-4) while two were rare (alleles 5 and 6). We verified whether nucleotide variability occurred within the VNTR alleles by sequencing the alleles with 1-4 repeats from the homozygous subjects. The second repeat of each allele showed a $(T/C)_{63}$ SNP. The occurrence of T identifies a putative positive transcription modulator (GATA2 site), while the occurrence of C modifies this site into a putative negative transcription modulator (DeltaEF1 site). Depending on the presence of T_{63} or C_{63} in the second repeat, we categorized the VNTR alleles length as a or b alleles, respectively.
By transient transfection experiments, we demonstrated that the different *SIRT3*-VNTR alleles are able to modulate the expression of a reporter gene according to number of repeat in an allele-specific way. Furthermore, transient transfections using constructs including the allele2 harbouring the DeltaEF1 (2b) site showed a dramatic reduction of the enhancer activity with respect to allele2 lacking this site and having the GATA2 site (2a). However, the enhancer activity of alleles 3 and 4 was independent of the presence/absence of the DeltaEF1 site. Furthermore, bioinformatic analysis has shown that the GATA2 site partially overlaps with an AP-1 site. It is worth noting that the two sites have been found to be integral parts of enhancers associated with a number of eukaryotic genes (Wisdom et al., 1999; Bossis et al., 2005).
Given the homology of *SIRT3* with *Sir2* we explored a possible role of the VNTR in human longevity. Genotypic and allelic frequencies of a sample composed of 20-80 year old subjects with those of a sample composed of 90-106 year old subjects were compared. We showed that in males, but not in females, both genotypic and allelic distributions were different in the group selected for longevity compared to the younger group. In particular, we found that the DeltaEF1-allele2 (allele 2b) was completely absent in males older than

90 years while it was present in the younger group, thus indicating that it is detrimental for male survival at oldest ages. These data confirm that longevity factors are sex-specific and that males and females probably follow different strategies to attain longevity (Ivanova et al., 1998; Tan 2001; De Luca et al., 2002).

On the whole, the results presented are in line with the possibility that VNTR acts *in vivo* on *SIRT3* and that the expression of the sirtuin genes may affect lifespan in humans, similarly to model organisms (Campisi, 2002).

On the basis of these data, the aim of this study was to highlight a few point remained not clear about this polymorphic enhancer. In particular, the role of GATA2 and AP-1 sites and a probable their interaction in the *SIRT3*-VNTR transcriptional regulation were verified. On the other hand, we looked for a possible correlation between the expression of the *SIRT3*-VNTR and the functional role of the *SIRT3* gene in the longevity.

2.2 *Materials and Methods*

2.2.1 *Bioinformatic analysis*

Prediction of putative transcriptional factor binding sites was performed using TFSEARCH software (www.cbrc.jp/research/db/TFSEARCH.html).

2.2.2 *Plasmid vectors*

In this study the following plasmids were used:

pGL3-Promoter vector: It contains a modified coding region for firefly (*Photinus pyralis*) luciferase that has been optimized for monitoring transcriptional activity in transfected eukaryotic cells and the SV40 promoter localized upstream of the luciferase gene.

pRL-CMV vector: It is a reporter plasmid used as internal control in co-transfection experiments in eukaryotic cells to normalize the effect of transfection efficiencies. It contains a cDNA (R*luc*) encoding *Renilla* luciferase, the CMV enhancer and early promoter elements to provide high-level expression of *Renilla* luciferase in co-transfected mammalian cells.

pBabeGATA2 Wild Type and pBabeGATA2 Deleted expression vectors: They contain respectively the cDNA encoding human GATA2 wild type and a deleted GATA2 lacking of the DNA binding region cloned into the EcoRI site.
They were supplied from Dr. Qiang Tong of the Children' s Nutrition Research Centre of the Baylor College of Medicine, Houston (Texas).

c-Fos and c-Jun expression vectors: They contain the cDNA coding for the c-Fos and c-Jun factors**,** respectively.
These vectors were supplied from Dr. Marcello Maggiolini of the University of Calabria.

pGL3/allele2a, pGL3/allele2b and pGL3/allele1a: These vectors contain allele2a, allele2b and allele1a sequences cloned into the SmaI site of the pGL3-Promoter vector.
Their construction was described in the paper by Bellizzi et al, (2005).

pGL3/allele1b: This vector contains allele1b sequence cloned into SmaI site of the pGL3-Promoter vector.

2.2.3 pGL3/allele1b construction

A 72bp oligo containing the allele1b sequence was synthesized *in vitro* by the Sigma Company. A PCR amplification using the oligo as template was carried out with the forward primer 5'-CTCCCCGGGTGGGAACTCCCTGGAGGTT-3' and the reverse primer 5'-CTCCCCGGGTGGCACCAGCCCTGGAAG-3'. 100 ng of template were amplified in 25 µl final volume containing each primer at 0.08 µM; 200 µM dNTP; 0,3 U Finnzymes *Taq* DNA polymerase; 1,5 mM MgCl2; and 1X thermophilic buffer. The following thermal cycling conditions were used: 1 cycle at 95°C for 45 s and 25 cycles of denaturation for 1 min at 95°C, annealing for 1 min at 55°C, and elongation for 1 min at 72°C. An extra step was performed at 72°C for 5 min. The PCR product was purified by agarose gel in TAE buffer and digested with SmaI restriction enzyme. After purification by phenol extraction, the fragment was ligated to SmaI-digested and dephosphorylated pGL3-Promoter vector, upstream of the *Luc*-promoter transcriptional unit. After preparing electrocompetent cells the ligation mixture was transformed into Top10 *E. coli* cells by electroporation.

2.2.4 *Preparation of electrocompetent E. coli cells and electroporation*

In order to prepare electrocompetent *E. coli* 300 ml of Luria Bertrani-Broth (LB- Broth) (1% Bacto-Tryptone, 0,5% Bacto-yeast extract, 0,5 % NaCl) containing streptomycin (50 µg/ml) were inoculated with 6 ml of a overnight Top10 *E. coli* cell culture and incubated on a rotary shaker at 37°C. The cell growth was monitored by photometer measuring the optical density at 600 nm (OD_{600}), every 45min-1hr. When the OD_{600} value was equal to about 0.6 (log phase growth), the cells were removed from the shaker and placed on ice. Then the cells were centrifuged twice at 4000 rpm for 15 min at 4°C. and the pellets were gently resuspended in decreasing volumes of ice-cold sterile water (300 ml and 150 ml respectively). Again the cells were centrifuged at 4000 rpm for 15 min at 4°C. The supernatants were removed and the pellets were resuspended in 6 ml of ice-cold 10% sterile glycerol. Then the cells were centrifuged at 4000 rpm for 15 min at 4°C. The pellet was resuspended in 600 µl of ice-cold 10% glycerol. 20 µl aliquots of cells were prepared in pre-chilled 1.5ml eppendorf tubes.

20 µl of the electrocompetent cells and 2 µl of the ligation mixture were transferred to a 0,2 cm cuvette (BIORAD) and immediately electroporated at 250 kV, 25 µF, 200 ohms (Dower et al., 1988). 480 µl of liquid Luria-Bertani medium (LB) (1% Bacto-Tryptone, 0,5 % Bacto-yeast extract, 1 % NaCl,) were added. Then the electropored cells were placed for 45 min on a rotary shaker at 37 °C. 100 µl of electropored cells were plated in a selective solid LB medium (1.6 % Agar) containing ampicillin (50µg/ml), streptomycin (50µg/ml). The following day, single colonies were selected from the solid medium, transferred to a liquid LB medium and incubated at 37°C. Finally, the plasmid containing allele1b was extracted using *QIAprep Spin Miniprep Kit* (QIAGEN). The sequence of the insert and its orientation in the reporter vector was confirmed by automated DNA sequencing.

2.2.5 *Eukaryotic cell cultures*

HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) containing 4.5 g/l glucose and 2mM L-Glutamine supplemented with 5% fetal bovine serum (FBS, Invitrogen) and 1% Penicillin/Streptomycin (Invitrogen). The cells were cultured in a water-humidified incubator at 37° C in 5% CO_2 95% air. 1 x 10^5 HeLa cells

were transferred into 24-well plates with 500 µl of regular growth medium/well the day before co-transfection.

2.2.6 Co-transfection experiments

Co-transfections were performed with the Fugene6 Reagent as recommended by the manufacturer (Roche Molecular Biochemcals) with a mixture containing 1 µg of each reporter plasmid, 0,5 to 10 µg of each expression vector for the Dose-Response Assays and 2 ng of pRL-CMV (Promega). The cells were lysed 24 h after co-transfection by applying 50µl Passive Lysis Buffer from the Dual Luciferase Reporter Assay Kit (Promega) into each well of the 24-well plates. Twenty micro-liters of cell lysate were used for luciferase reporter assay by using the same kit according to the manufacturer's protocol. Light intensity was quantified in a Lumat LB9507 luminometer (EG&G Berthold). The Luciferase activity of the reporter plasmids was normalized to the *Renilla* luciferase activity.

2.2.7 Statistical analysis

Co-transfection experiments were carried out three times in triplicate, and the values of luciferase gene expression were represented as mean of three independent experiments and reported as fold induction respect to pGL3-Promoter Vector. The statistical significance of the differences among the luciferase expression of the different reporter constructs and the expression vectors was tested by ANOVA.

2.3 Results

2.3.1 Computer-assisted analysis of the SIRT3-VNTR

Nuclear transcription factors which interact with *SIRT3-VNTR* were analyzed by using TF-SEARCH software. Fig.(2.1) shows the sequence of *SIRT3*-VNTR, the position of the T/C variation at 63nt on the second repeat and potential GATA2 and AP-1 motifs marked with arrows indicating the functional direction.

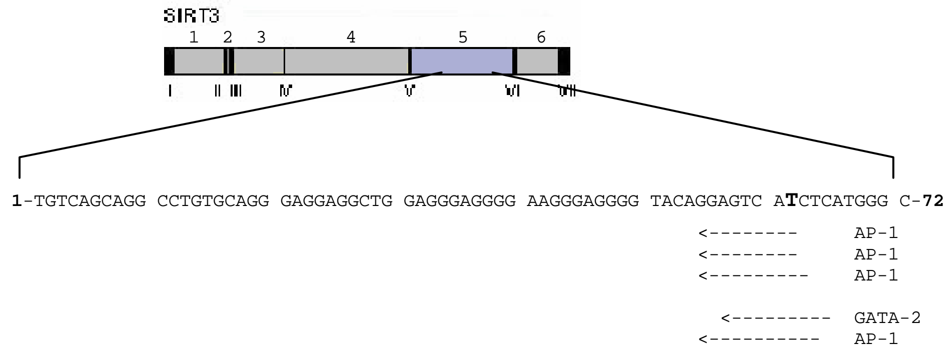


Fig.(2.1) *A schematic representation of the SIRT3 gene and the $VNTR_{72bp}$ sequence core discovered in intron 5 of this gene.*

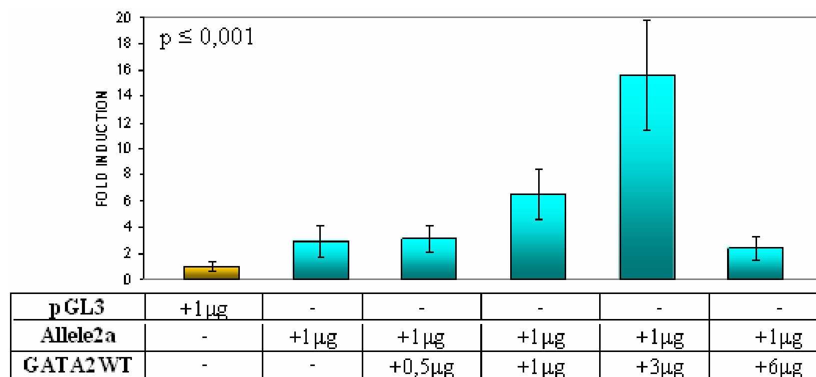
This analysis revealed that the presence of T identifies a putative GATA2 site, while the presence of C modifies the GATA2 site into a putative DeltaEF1 site. Furthermore we observed that the GATA2 site sequence is partially overlapping with an AP-1 site.
The AP-1 site is unmodifiable by the T/C variation.

2.3.2 *The role of GATA2, c-Jun, and c-Fos in the regulation of allele 2a and 2b enhancer activity*

To verify if the presence/absence of the putative GATA2 site is responsible for the different enhancer activity displayed by alleles 2a and 2b a series of transient co-transfection experiments in HeLa cells were carried out. In these experiments we used pGL3/allele2a and pGL3/allele2b constructs (Bellizzi et al, 2005) and expression vectors encoding GATA2 protein. These vectors were kindly provided by dr. Qiang Tong of the Children' s Nutrition Research Centre of the Baylor College of Medicine, Houston (Texas). In particular, pBabeGATA2 WT containing the full GATA2 cDNA cloned at the EcoRI site and pBabeGATA2 Del vector containing the GATA2 cDNA lacking of the DNA binding region were utilized. Furthermore, we also carried out co-transfection experiments with expression vectors encoding c-Jun and c-Fos factors which interact with the AP-1 site.

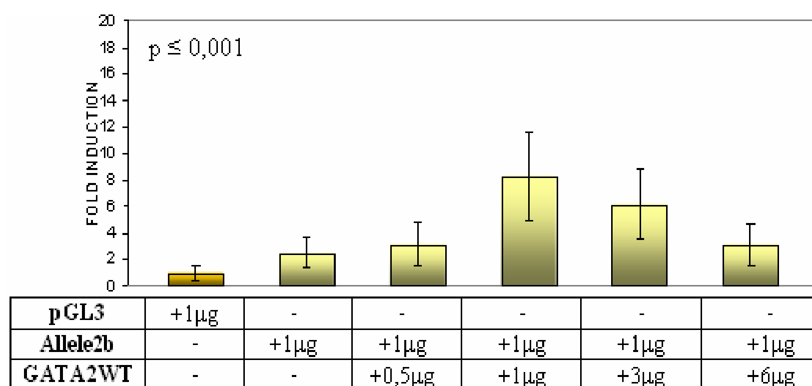
- *Co-transfections of pGL3/allele2a and pGL3/allele2b with pBabeGATA2 WT expression vector*

Firstly, in order to determine the amounts of pBabeGATA2 WT vector to use in the different experiments, we co-transfected 1μg of the pGL3/allele2a and 1μg of the pGL3/allele2b, respectively, with increasing amount of the pBabeGATA2 WT (0.5, 1, 3, 6, 10 μg). In Fig.(2.2) and Fig.(2.3) luciferase expression of pGL3/allele2a and pGL3/allele2b was reported as fold induction with respect to the pGL3-Promoter Vector. The values represent the means ± standard deviation of three independent experiments in triplicate



pGL3	+1μg	-	-	-	-	-
Allele2a	-	+1μg	+1μg	+1μg	+1μg	+1μg
GATA2 WT	-	-	+0,5μg	+1μg	+3μg	+6μg

Fig.(2.2) *Luciferase expression of pGL3/allele2a construct co-transfected with increasing amounts of pBabeGATA2 WT expression vector in HeLa cells. The statistical significance of the differences between construct activity was tested by ANOVA*



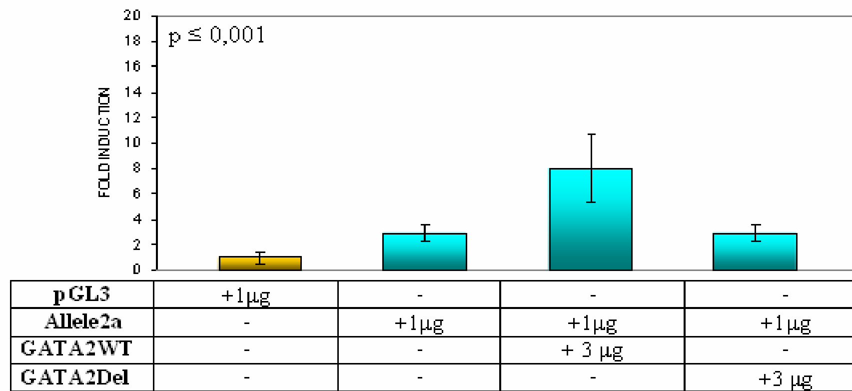
pGL3	+1μg	-	-	-	-	-
Allele2b	-	+1μg	+1μg	+1μg	+1μg	+1μg
GATA2WT	-	-	+0,5μg	+1μg	+3μg	+6μg

Fig.(2.3) *Luciferase expression of pGL3/allele2b construct co-transfected with increasing amounts of pBabeGATA2 WT expression vector in HeLa cells. The statistical significance of the differences between construct activity was tested by ANOVA*

The results showed that the GATA2 protein increases allele2a and allele2b enhancer

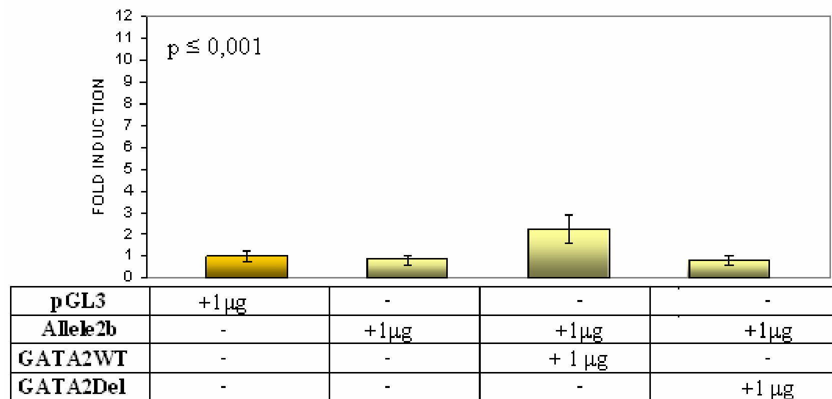
activity. This induction is related to the amount of pBabeGATA2 WT vector used in the co-transfection experiments and it is specific for the two alleles. In fact, the highest induction is obtained in co-transfections that use allele2a and pBabeGATA2 WT with 1:3 ratio respectively, and in co-transfections that use allele2b and pBabeGATA2 WT with 1:1 ratio respectively.

In order to determine whether the above allele-specific induction is really due to the GATA2 factor, pGL3/allele2a and pGL3/allele2b were co-transfected in separate experiments with pBabeGATA2 WT and pBabeGATA2 Del vectors, respectively. The results are shown in Fig. (2.4) and Fig. (2.5).



pGL3	+1µg	-	-	-
Allele2a	-	+1µg	+1µg	+1µg
GATA2WT	-	-	+ 3 µg	-
GATA2Del	-	-	-	+3 µg

Fig.(2.4) *Luciferase expression of pGL3/allele2a construct co-transfected with pBabeGATA2 WT or pBabeGATA2 Del expression vectors in HeLa cells. The statistical significance of the differences between construct activity was tested by ANOVA*



pGL3	+1µg	-	-	-
Allele2b	-	+1µg	+1µg	+1µg
GATA2WT	-	-	+ 1 µg	-
GATA2Del	-	-	-	+1 µg

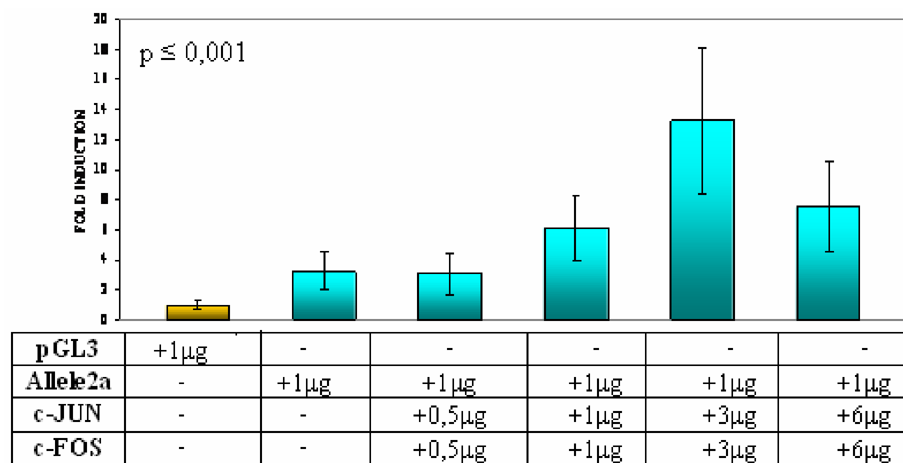
Fig.(2.5). *Luciferase expression of pGL3/allele2b construct co-transfected with pBabeGATA2 WT or pBabeGATA2 Del expression vectors in HeLa cells. The statistical significance of the differences between construct activity was tested by ANOVA*

Both for allele2a (Fig.2.4) and for allele2b (Fig.2.5) the induction of the luciferase

expression returns to the basal value when GATA2 WT is replaced by GATA2 Del.

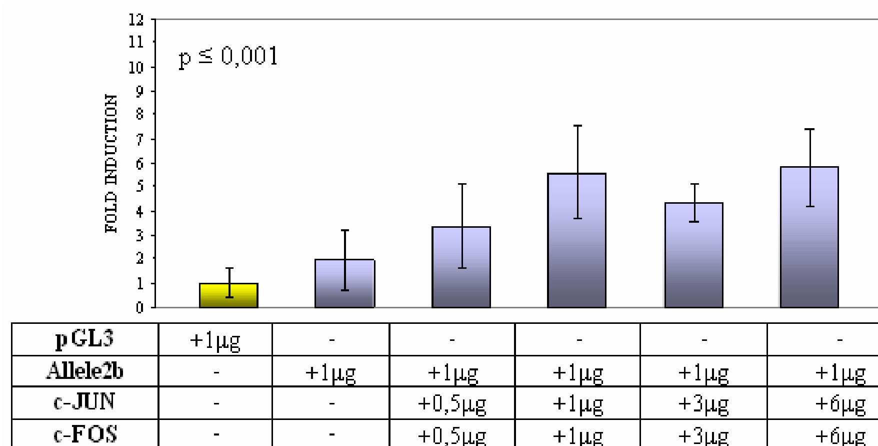
- *Co-transfections of pGL3/allele2a and pGL3/allele2b with c-Jun and c-Fos expression vectors*

Since GATA2 and AP-1 sites are partially overlapping, in order to determine if these factors were able to reveal a difference between the activity of alleles 2a and 2b co-transfection experiments were also carried out by using the c-Jun and c-Fos expression vectors. The results are shown in Fig. (2.6) and Fig.(2.7).



pGL3	+1μg	-	-	-	-	-
Allele2a	-	+1μg	+1μg	+1μg	+1μg	+1μg
c-JUN	-	-	+0,5μg	+1μg	+3μg	+6μg
c-FOS	-	-	+0,5μg	+1μg	+3μg	+6μg

Fig.(2.6) *Luciferase expression of pGL3/allele2a construct co-transfected with increasing amounts of c-Jun and c-Fos expression vector in HeLa cells. The statistical significance of the differences between construct activity was tested by ANOVA*



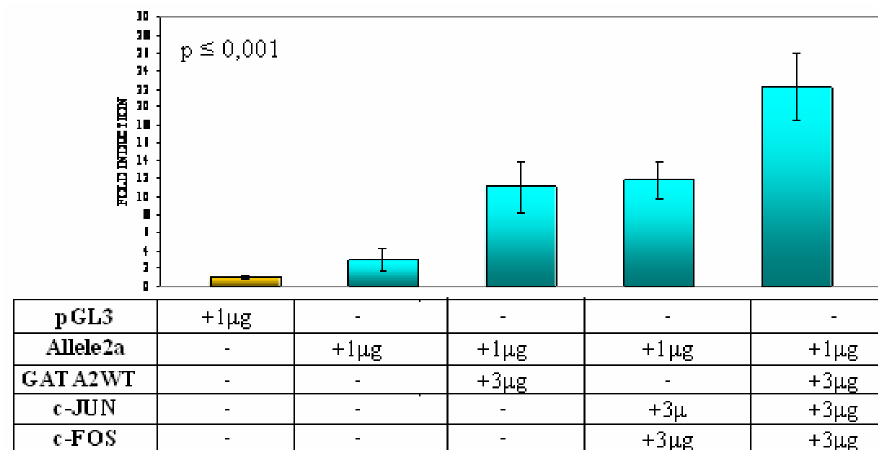
pGL3	+1μg	-	-	-	-	-
Allele2b	-	+1μg	+1μg	+1μg	+1μg	+1μg
c-JUN	-	-	+0,5μg	+1μg	+3μg	+6μg
c-FOS	-	-	+0,5μg	+1μg	+3μg	+6μg

Fig.(2.7) *Luciferase expression of pGL3/allele2b construct co-transfected with increasing amounts of c-Jun and c-Fos expression vector in HeLa cells. The statistical significances of the difference between construct activity was tested by ANOVA*

We observed that the c-Jun and c-Fos factors increase allele 2a and 2b activity in an allelic-specific way. In fact, in line with the results of Fig.s (2.6) and (2.7), the highest induction is obtained in the co-transfections that use allele2a and c-Jun/c-Fos vectors at ratio 1:3 and in the co-transfections that use allele2b and c-Jun and c-Fos vectors at ratio 1:1.

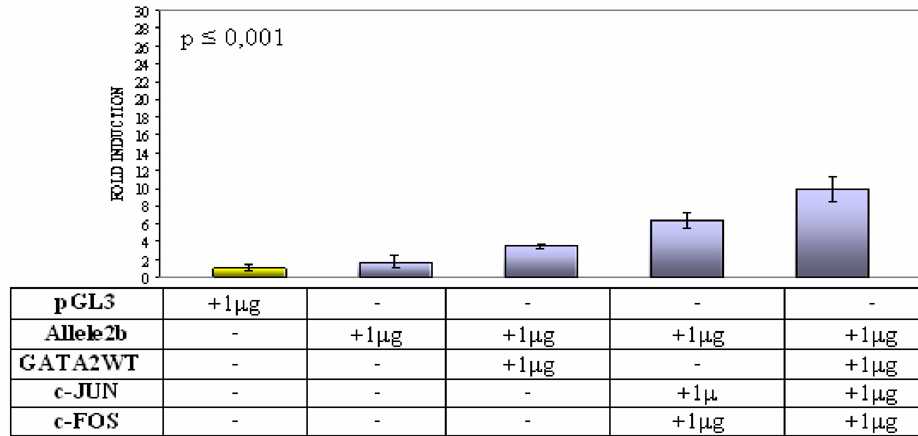
- *Co-transfections of pGL3/allele2a and pGL3/allele2b with pBabeGATA2 WT, c-Jun, and c-Fos expression vectors*

We wanted to investigate if a synergistic cooperation between GATA2 and c-Jun and c-Fos factors affects the activity of alleles 2a and 2b. In this regard, co-transfection experiments were carried out using the constructs pGL3/allele2a and pGL3/allele2b with pBabeGATA2 WT vector combined with c-Jun and c-Fos expression vectors. The results are reported in Fig.(2.8) and Fig.(2.9).



pGL3	+1μg	-	-	-	-
Allele2a	-	+1μg	+1μg	+1μg	+1μg
GATA2WT	-	-	+3μg	-	+3μg
c-JUN	-	-	-	+3μ	+3μg
c-FOS	-	-	-	+3μg	+3μg

Fig.(2.8) *Luciferase expression of pGL3/allele2a construct co-transfected with pBabeGATA2 WT and c-Jun and c-Fos expression vectors in HeLa cells. The statistical significance of the differences between construct activity was tested by ANOVA*



pGL3	+1µg	-	-	-	-
Allele2b	-	+1µg	+1µg	+1µg	+1µg
GATA2WT	-	-	+1µg	-	+1µg
c-JUN	-	-	-	+1µ	+1µg
c-FOS	-	-	-	+1µg	+1µg

Fig.(2.9) *Luciferase expression of pGL3/allele2b construct co-transfected with pBabeGATA2 WT and c-Jun and c-Fos expression vectors in HeLa cells. The statistical significance of the differences between construct activity was tested by ANOVA*

The results showed that GATA2 and the c-Jun/c-Fos complex are able to increase luciferase activity by a synergistic cooperation. In fact, the induction of allele2a and allele2b enhancer activity is higher in HeLa cells co-transfected with both the expression vectors (Fig.(2.8) and Fig.(2.9) last column) than in cells co-transfected with the single vectors (Fig.(2.8) and Fig.(2.9) third and fourth columns).

On the other hand, by comparing the data of the experiments we observe that the synergistic effect is enhanced when allele2a is used instead of allele2b (compare Fig.(2.8) and Fig.(2.9), last column).

On the basis of these results it was interesting to examine what happened when a sole *SIRT3-VNTR* repeat was present. Therefore we analysed the role of GATA2, c-Jun and c-Fos factors in the regulation of the enhancer activity of alleles 1a and 1b.

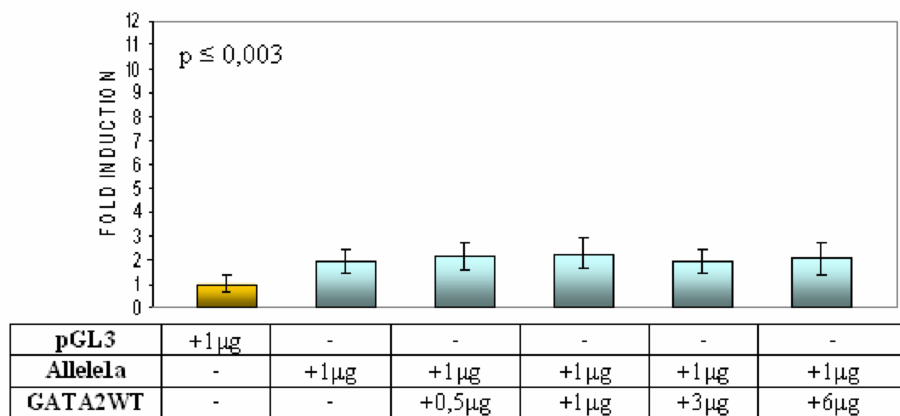
2.3.3 *The role of the GATA2, c-Jun, and c-Fos in the regulation of allele 1a and 1b enhancer activity*

In order to examine the effect of the GATA2-AP-1 sites on alleles 1, co-transfection experiments were carried out using allele1a and allele1b with pBabeGATA2 WT, pBabeGATA2 Del, c-Jun, and c-Fos expression vectors.

Allele1a was prepared from a biological sample (pGL3/allele1a) since all *SIRT3-VNTR* alleles with a sole repeat (allele 1) have the T nucleotide at the position 63nt. On the contrary, since allele1b does not exist in nature, it was synthesized it *in vitro*, amplified by PCR and then cloned at the SmaI site of the pGL3-promoter-vector (pGL3/allele1b).

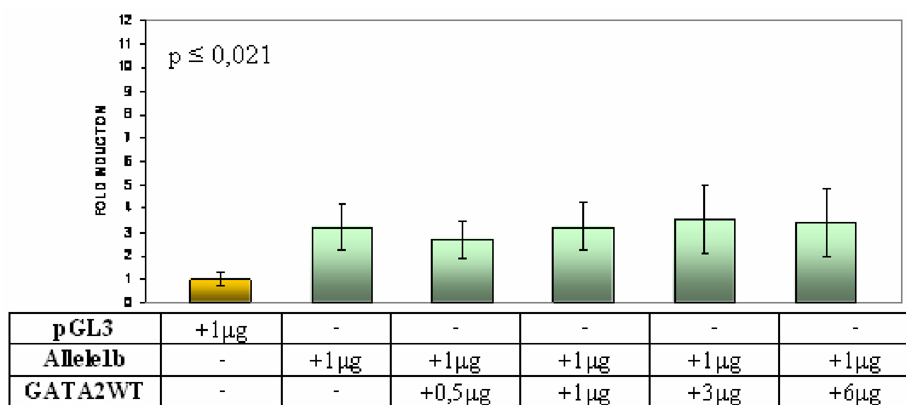
- *Co-transfections of pGL3/allele1a and pGL3/allele1b with pBabeGATA2 Wild Type expression vector*

Firstly, in order to establish the amount of pBabeGATA2 WT vector to use in the different experiments, we co-transfected 1µg of the pGL3/allele1a and 1µg of the pGL3/allele1b constructs, respectively, with increasing amount of the pBabeGATA2 WT (0.5, 1, 3, 6, 10 µg). In Fig.(2.10) and Fig.(2.11) luciferase expression of the constructs containing the alleles 1a and 1b was reported as fold induction with respect to the pGL3-Promoter Vector. The values represent the mean ± standard deviation of three independent triplicate experiments in triplicate.



pGL3	+1µg	-	-	-	-	-
Allele1a	-	+1µg	+1µg	+1µg	+1µg	+1µg
GATA2WT	-	-	+0,5µg	+1µg	+3µg	+6µg

Fig.(2.10) *Luciferase expression of pGL3/allele1a construct co-transfected with increasing amounts of pBabeGATA2 WT expression vector in HeLa cells. The statistical significance of the differences between construct activity was tested by ANOVA*

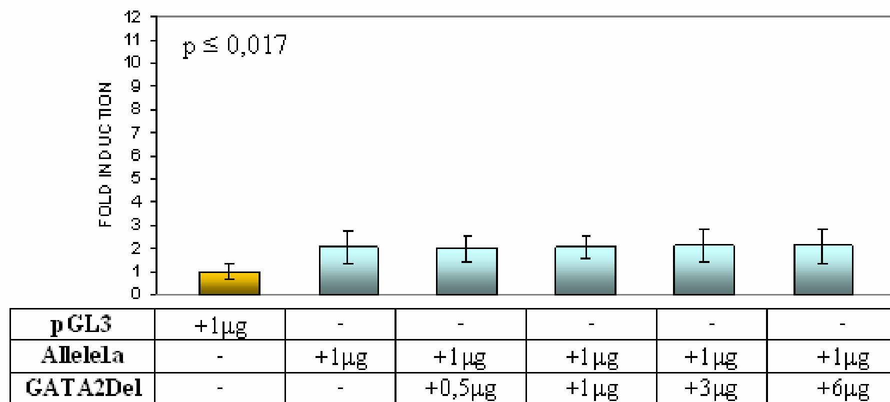


pGL3	+1µg	-	-	-	-	-
Allele1b	-	+1µg	+1µg	+1µg	+1µg	+1µg
GATA2WT	-	-	+0,5µg	+1µg	+3µg	+6µg

Fig. (2.11) *Luciferase expression of pGL3/allele1b construct co-transfected with increasing amounts of pBabeGATA2 WT expression vector in HeLa cells. The statistical significance of the differences between construct activity was tested by ANOVA*

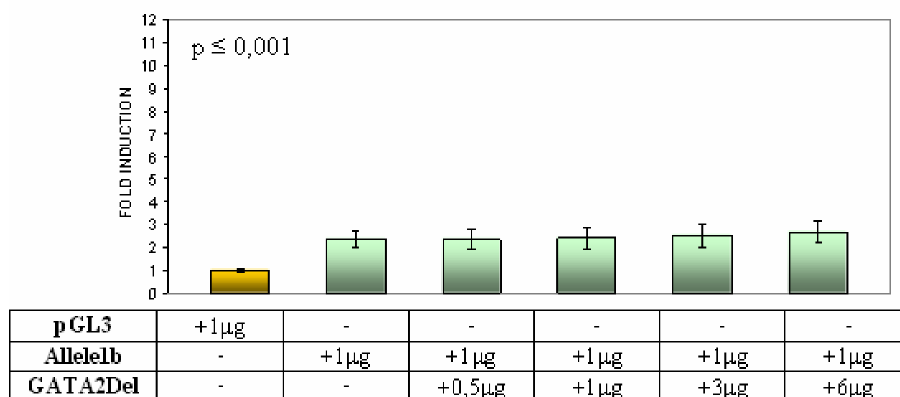
The results showed that GATA2 protein does not increase either allele1a or allele1b enhancer activity independently of the amount of pBabeGATA2 WT vector used in the co-transfection experiments.

These findings are confirmed by the experiments carried out using the pBabeGATA2 Del expression vector reported in Fig.(2.12) and Fig.(2.13).



pGL3	+1µg	-	-	-	-	-
Allele1a	-	+1µg	+1µg	+1µg	+1µg	+1µg
GATA2Del	-	-	+0,5µg	+1µg	+3µg	+6µg

Fig.(2.12). *Luciferase expression of pGL3/allele1a construct co-transfected with increasing amounts of pBabeGATA2 Del expression vector in HeLa cells. The statistical significance of the differences between construct activity was tested by ANOVA*



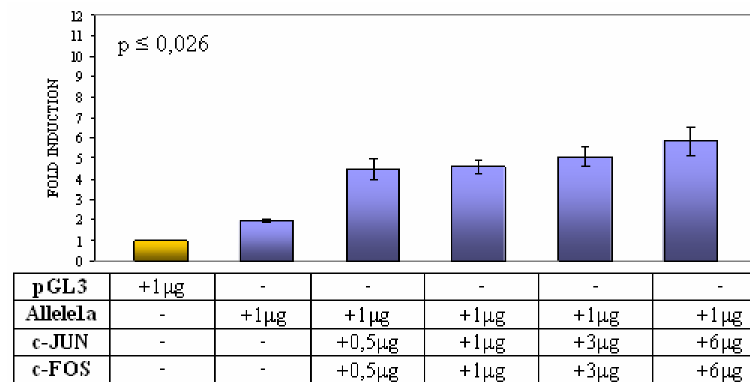
pGL3	+1µg	-	-	-	-	-
Allele1b	-	+1µg	+1µg	+1µg	+1µg	+1µg
GATA2Del	-	-	+0,5µg	+1µg	+3µg	+6µg

Fig.(2.13). *Luciferase expression of pGL3/allele1b construct co-transfected with increasing amounts of pBabeGATA2 Del expression vector in HeLa cells. The statistical significance of the differences between construct activity was tested by ANOVA*

For both allele1a (Fig.2.12) and allele1b (Fig.2.13) when GATA2 WT was replaced by GATA2 Del the expression pattern was not modifyied.

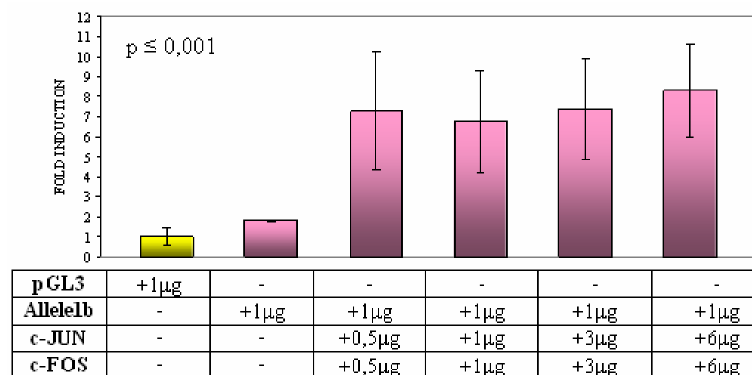
- *Co-transfections of pGL3/allele1a and pGL3/allele1b with c-Jun and c-Fos expression vectors*

As for Alleles 2a and 2b, we carried out co-transfection experiments using c-Jun and c-Fos expression vectors. The results are shown in Fig.(2.14) and Fig.(2.15).



pGL3	+1µg	-	-	-	-	-
Allele1a	-	+1µg	+1µg	+1µg	+1µg	+1µg
c-JUN	-	-	+0,5µg	+1µg	+3µg	+6µg
c-FOS	-	-	+0,5µg	+1µg	+3µg	+6µg

Fig.(2.14) *Luciferase expression of pGL3/allele1a construct co-transfected with increasing amounts of c-Jun and c-Fos expression vectors in HeLa cells. The statistical significance of the differences between construct activity was tested by ANOVA*



pGL3	+1µg	-	-	-	-	-
Allele1b	-	+1µg	+1µg	+1µg	+1µg	+1µg
c-JUN	-	-	+0,5µg	+1µg	+3µg	+6µg
c-FOS	-	-	+0,5µg	+1µg	+3µg	+6µg

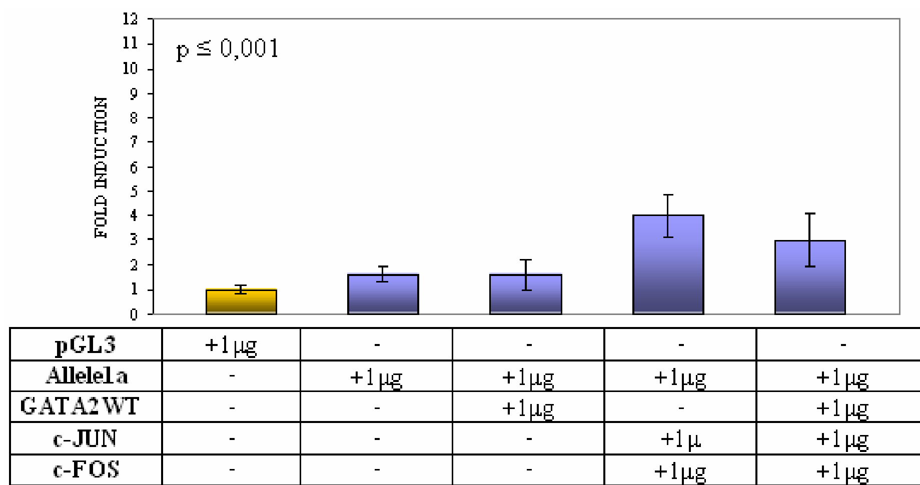
Fig.(2.15) *Luciferase expression of pGL3/allele1b construct co-transfected with increasing amounts of c-Jun and c-Fos expression vector in HeLa cells. The statistical significance of the differences between construct activity was tested by ANOVA*

The results showed that the c-Jun and c-Fos factors increased allele1a and allele1b enhancer activity. This induction was not related to the amount of expression vectors used in the co-transfection experiments and it was independent of the $(T/C)_{63}$ variability (compare Fig.(2.14) and Fig.(2.15).

- *Co-transfections of pGL3/allele1a and pGL3/allele1b with pBabeGATA2 WT, c-Jun, and c-Fos expression vectors*

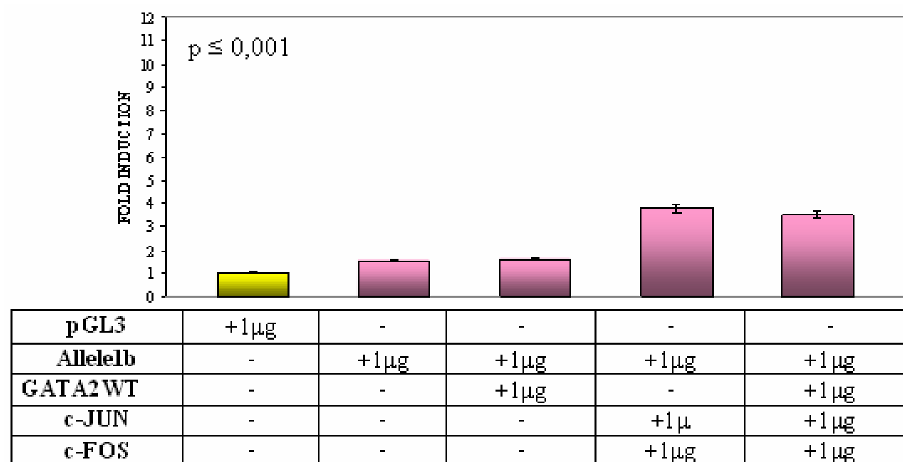
Furthermore, co-transfection experiments were carried out to investigate if a synergistic cooperation by GATA2, c-Jun and c-Fos factors exists in the regulation of allele 1a and 1b activity was observed for alleles 2a and 2b.

Therefore this, co-transfection experiments were carried out by using the constructs pGL3/allele1a and pGL3/allele1b with the pBabeGATA2 WT vector combined with c-Jun and c-Fos expression vectors. The results are reported in Fig. (2.16) and Fig.(2.17).



pGL3	+1 μg	-	-	-	-
Allele1a	-	+1 μg	+1 μg	+1 μg	+1 μg
GATA2 WT	-	-	+1 μg	-	+1 μg
c-JUN	-	-	-	+1 μ	+1 μg
c-FOS	-	-	-	+1 μg	+1 μg

Fig.(2.16) *Luciferase expression of pGL3/allele1a constructs co-transfected with pBabeGATA2 WT, c-Jun, and c-Fos expression vectors in HeLa cells. The statistical significance of the differences between construct activity was tested by ANOVA*



pGL3	+1 μg	-	-	-	-
Allele1b	-	+1 μg	+1 μg	+1 μg	+1 μg
GATA2 WT	-	-	+1 μg	-	+1 μg
c-JUN	-	-	-	+1 μ	+1 μg
c-FOS	-	-	-	+1 μg	+1 μg

Fig.(2.17). *Luciferase expression of pGL3/allele1b constructs co-transfected with GATA2, c-Jun, and c-Fos expression vectors in HeLa cells. The statistical significance of the differences between construct activity was tested by ANOVA*

Results of co-transfection experiments indicated that GATA2 plus c-Jun and c-Fos factors were able to increase the luciferase activity by synergistic cooperation but this activity was unchanged for allele1a and allele1b. What is more, the occurrence of the GATA2 factor seems to inhibit the effect of the c-Jun and c-Fos factors, at least in the case of allele1a.

2.4 Discussion

Many studies on the regulation of human sirtuins, a homologue of the *Sir2* gene, have been carried out to investigate their ability to contribute to longevity as has been previously observed in model organisms. In this context, the *SIRT3* gene appeared very promising. In fact, a marker of the *SIRT3* gene, a SNP located in exon 3, was found to be associated with survivorship in the elderly (Rose et al., 2003). In addition, we identified a VNTR located in the fifth intron of the gene with an allelic-specific enhancer activity, also correlated of a $(T/C)_{63}$ variation that modifies a GATA2 site in DeltaEF1 transcriptional site in the second repeat of the length-allele2. To further elucidate this matter, the aims of this study was to investigate the role of GATA2 and AP-1 sites and a their probable interaction in the regulation of the VNTR enhancer activity.

The transient co-transfection experiments in HeLa cells were performed with the constructs containing alleles 2a and 2b (Bellizzi et al., 2005) and expression vectors encoding GATA2, c-Jun, and c-Fos factors. The last two factors are thought to be the main factors that bind AP-1 sites as a heterodimer complex (Masatoshi et al., 1995; Bossis et al., 2005).

In titration experiments, where increasing amounts of the expression vectors were transfected in combination with alleles 2a and 2b, we observed that the highest enhancer activity for the construct containing allele2a is obtained when the vector encoding GATA2 is co-transfected with a 1:3 ratio. It was interesting to observe that this ratio was not maintained when allele2b was used for co-transfection instead of allele2a. In this case, the highest enhancer activity of allele2b is obtained when this allele and the vector encoding GATA2 are co-transfected with a 1:1 ratio. Therefore, these experiments provide evidence that GATA2 increases VNTR enhancer activity in an allele-specific way, suggesting that the amount of GATA2 required to obtain the best induction is higher in the presence of two GATA2 sites (allele2a), than in presence of a single GATA2 site (allele2b).

The evidence that the allele-specific induction reaction is due to the GATA2 factor came from the co-transfection experiments where the expression vector encoding deleted GATA2 was used. In fact, in these experiments enhancer activity of allele2a and allele2b equals the baseline value.

On the whole, the results presented above provide evidence that the $(T/C)_{63}$ polymorphism occurring in the second repeat of the *SIRT3-VNTR* allele2 affects the expression of the reporter gene by modifying the affinity of the GATA2 binding site.

Following, the observation that the GATA2 protein acts as positive regulator of *SIRT3-VNTR* enhancer activity, we explored the role of AP-1 sites whose sequence overlaps that of the GATA2 site. In line with the results obtained with GATA2 protein, we observed that the highest enhancer activity of allele2a is obtained when this allele and expression vectors encoding c-Jun and c-Fos factors at the ratio of 1:3:3 respectively were used. We can observe that this ratio was not maintained when allele2b was used for co-transfection instead of allele2a. In this case, the highest induction of the allele2b enhancer activity is obtained when this allele and expression vectors encoding c-Jun and c-Fos factors are co-transfected in a ratio of 1:1:1. These findings highlight that c-Jun and c-Fos factors bind to AP-1 sites and increase VNTR enhancer activity in an allele-specific way. Then we can assert that the $(T/C)_{63}$ polymorphism occurring in the second repeat of *SIRT3-VNTR* allele2 affects the expression of the reporter gene by affecting GATA2-AP-1 overlapping sites.

It is interesting to note that both for GATA2 and the c-Jun/c-Fos complex, we observed a surprising reduction in activity when the cotransfection of these transcript factors was over a certain value. This is likely to be caused by a phenomenon of “squelching”, that is the sequestering of a limiting cofactor by the over-expressed transcription factor.

Furthermore, we have investigated a possible interaction between GATA2 and c-Jun and c-Fos. Several studies have emphasized the importance of the interaction between factors in the regulation of gene expression (Masatoshi et al., 1995; Masuda et al., 2004). Co-transfection analysis revealed a synergistic activation of *SIRT3-VNTR* allele2a and allele2b when GATA2 and c-Jun and c-Fos expression vectors were combined. This effect is enhanced when allele2a is used instead of allele2b. It is likely that this cooperativity among GATA2, c-Jun, and c-Fos factors reflects protein-protein interaction. One possibility is that this interaction is due to the two factors associating and exerting a combined effect which is more effective than the effect of either one of the factors alone.

Next, we investigated the role of GATA2 and AP-1 sites in the regulation of *SIRT3*-VNTR alleles having a single repeat (allele 1). It is worth noting that we have previously observed

that all *SIRT3-VNTR* alleles having a single repeat (allele 1) show the T nucleotide at position 63nt (GATA2-AP-1 sites). Since allele1b does not exist in nature. We carried out co-transfection experiments using a reporter construct with allele1b synthesized *in vitro*. From an evolutionary perspective, the lack of allele1b may be explained by assuming that the mutation $(T/C)_{63}$ in the second repeat arose after a duplication which occurred on a two-repeat allele. As the $(T/C)_{63}$ SNP is present in all the other VNTR alleles, this implies that the two-repeat allele is older than the alleles with 3-6 repeats. To examine the effect of the GATA2 AP-1 sites in allele 1, we compared co-transfection experiments carried out using allele1a and allele1b. We observed that for any amount of the expression vectors used in titration experiments GATA2 did not increase the activity of alleles1a and 1b. in addition the induction of the luciferase expression observed is not specifically due to GATA2 factor. Therefore, when a single VNTR repeat is present, the $(T/C)_{63}$ polymorphism has no effect on the enhancer activity of the alleles. This finding is confirmed by experiments carried out with deleted GATA2 which showed that the use of deleted constructs did not modify the expression patterns. On the contrary, the co-transfection experiments carried out using c-Jun and c-Fos expression vectors revealed a functional response of the AP-1 site in alleles 1a and 1b. In fact, there is an increase of the enhancer activity of allele1 and this effect is independent of the $(T/C)_{63}$ variability. In agreement with the above observations, experiments carried out using both GATA2 and c-Jun/c-Fos constructs did not reveal any difference for the alleles having one repeat and furthermore the GATA2 protein seemed to inhibit the effect of the AP-1 factors, at least in the case of allele1a. Co-transfection analysis revealed a synergistic activation of *SIRT3-VNTR* allele1a and allele1b when GATA2, c-Jun, and c-Fos expression vectors were combined. However, this activity was not different for allele1a and allele1b. What is more, the occurrence of the GATA2 factor seemed to inhibit the effect of the c-Jun and c-Fos factors, at least in the case of allele1a.

It worth noting that the results presented probably can help to understand why the *SIRT3*-VNTR alleles having more than two repeats (alleles 3-6) do not show significant differences between a and b allelic categories (Bellizzi et al., 2005).

We supposed that the $(T/C)_{63}$ polymorphism occurring in the second repeat has an effect on the enhancer activity only when two (allele2a) instead of one (allele2b) GATA2 sites were present. When the number of repeats, and therefore number of GATA2 sites, was more than two (alleles 3-6) the enhancer activity could depend only on the number of repeats.

With respect to the absence of the VNTR-allele2b in centenarian, it interesting to think that the regulation carried out by GATA2, c-Jun, and c-Fos could be very important for the regulation of *SIRT3* gene expression. In particular, an under-expression of *SIRT3* can be detrimental for longevity in humans. Therefore allele2b is likely unfavourable for male longevity because it is not capable to support the enhancer activity needed for the optimal function of the *SIRT3* gene. In general, several studies have demonstrated that risk alleles can be present in a genetic pool (Bonafè et al., 1999; Garasto et al., 2003). In fact, one of the more paradoxical results emerging from the studies on centenarians is that survival up to and over 100 years is not necessarily related to the presence of "robust" genes (genes that confer an advantage to individual survival during life). The nature of the increment in human survival is complicated and could be due to pleiotropic effects (Toupance et al., 1998) and from a biological point of view the activity of allele2 can be interpreted as an example of "antagonistic pleiotropy".

3. Reprints of the published papers

A novel VNTR enhancer within the *SIRT3* gene, a human homologue of *SIR2*, is associated with survival at oldest ages (Genomics 85: 258-263, 2005)

Dina Bellizzi, Giuseppina Rose, Paola Cavalcante, **Giuseppina Covello**, Serena Dato, Francesco De Rango, Valentina Greco, Marcello Maggiolini, Emidio Feraco, Vincenzo Mari, Claudio Franceschi, Giuseppe Passarino, Giovanna De Benedictis

Characterization of a bi-directional promoter shared between two genes related to aging and longevity: *SIRT3* and *PSMD13* (In Press)

Dina Bellizzi, Serena Dato, Paola Cavalcante, **Giuseppina Covello**, Fausta Di Cianni, Giuseppe Passarino, Giuseppina Rose, Giovanna De Benedictis.

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A novel VNTR enhancer within the *SIRT3* gene, a human homologue of *SIR2*, is associated with survival at oldest ages

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Abstract

SIR2 genes control life span in model organisms, playing a central role in evolutionarily conserved pathways of aging and longevity. We wanted to verify whether similar effects may act in humans too. First, we searched for variability in the human sirtuin 3 gene (*SIRT3*) and discovered a VNTR polymorphism (72-bp repeat core) in intron 5. The alleles differed both for the number of repeats and for presence/absence of potential regulatory sites. Second, by transient transfection experiments, we demonstrated that the VNTR region has an allele-specific enhancer activity. Third, by analyzing allele frequencies as a function of age in a sample of 945 individuals (20–106 years), we found that the allele completely lacking enhancer activity is virtually absent in males older than 90 years. Thus the underexpression of a human sirtuin gene seems to be detrimental for longevity as it occurs in model organisms.
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Keywords: SIRT3; Human longevity; VNTR enhancer

Aging can be viewed as a lethal by-product of activities, such as reproduction and food intake, that are controlled by genes [1]. Since most of these genes are evolutionarily conserved, distant species may share common pathways of aging [2]. The insulin/insulin-like growth factor 1 (IGF1) signaling pathway could be one such common pathway, as it modulates aging in many species, including *Caenorhabditis elegans*, *Drosophila*, mice [3], and possibly humans [4]. An elegant study carried out in *C. elegans* by applying microarray techniques showed that a member of the SIR2-like protein family is regulated downstream of *DAF-16*, a FOXO-family transcription factor that affects the rate of aging in response to the insulin/IGF1 pathway [5]. SIR2 proteins constitute an evolutionarily conserved family of NAD-dependent deacetylases called sirtuins [6–8]. In model organisms the expression levels of *SIR2* modulate life span [9–11]. Since sirtuins are NAD^+ dependent these proteins through different routes may link energy metabolism, genome maintenance, and aging [11,12]. Thus *SIR2* genes may play a crucial role in conserved pathways of aging and longevity.

A human homologue of the *SIR2* genes, *SIRT3*, lies at the telomeric terminal on chromosome 11p15.5 (www.ncbi.nlm.nih.gov/omim; MIM 604481; contig NT_035113). The gene is ubiquitously expressed, particularly in metabolically active tissues, and the SIRT3 protein is targeted to mitochondria through an N-terminus signal necessary for

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mitochondrial localization [13,14]. By analyzing a large sample of individuals including people older than 100 years of age, we found an association between longevity and the silent marker G477T of *SIRT3* [15]. Owing to the silent nature of the SNP, we hypothesized a linkage disequilibrium between the G477T marker and an unknown functional variant of *SIRT3*. The aim of the present study was to identify the functional variant that could account for the association we observed between the silent marker of *SIRT3* and survival. Thus, first we searched for variability in the *SIRT3* gene in a large population sample; then we characterized the functional activity, and its association with longevity, of a VNTR polymorphism we identified in intron 5.

Results

To identify variability in the functional domains of *SIRT3*, we conducted DNA sequence analyses of PCR fragments around the FGE conserved motif (exon 5) in a panel of 50 unrelated subjects (25 males and 25 females randomly chosen from sample 1). We observed a VNTR polymorphism having a 72-bp core in the fifth intron of the gene (12,343 nt initial position referring to www.ncbi.nlm.nih.gov/omim; MIM 604481; contig NT_035113). After verification of Mendelian inheritance in 30 parent/offspring pairs collected in another study, we analyzed the VNTR polymorphism in all of sample 1. Six length-alleles were identified spanning one to six repeats (see Table 1); four alleles were common (alleles 1–4), while two were rare (alleles 5 and 6). Since the sample under study had been already typed for the silent variant G477T of *SIRT3* [15], we analyzed linkage disequilibrium between the markers G477T and VNTR. The disequilibrium was highly significant ($p < 0.001$), with 10 observed haplotypes of the 12 expected (estimated by MLE from both homozygous and heterozygous genotypes).

Then we checked whether nucleotide variability did occur within the VNTR alleles. By sequencing the PCR-DNA fragments of all the subjects homozygous for the alleles having 1 to 4 repeats (see Table 1) we identified a T/C variation located in the second repeat, 63 nt from its starting point. MatInspector V2.2 analysis carried out on the 72-bp core fragment revealed that the T/C variation transformed a GATA3 site into a DeltaEF1 site, potentially having different regulatory activities [17,18]. On the occurrence of the GATA3/DeltaEF1 site in the second repeat, the length-alleles having one to four repeats were resolved into seven alleles. In fact, with the exception of allele 1 (which lacked a second repeat and thus had the GATA3 core sequence in all cases), each of the remaining common length-alleles was resolved into two categories, one having the GATA3 site (alleles 2a, 3a, and 4a) and the other the DeltaEF1 site (alleles 2b, 3b, 4b). Fig. 1 shows the position of the VNTR with respect to the G477T silent marker we previously identified in exon 3 [15], the seven length-alleles spanning one to four repeats, and the GATA3/DeltaF1 variable sequence of the second allelic repeat.

We analyzed possible enhancer effects of specific VNTR alleles. By using constructs including the alleles lacking the DeltaEF1 site (alleles 1a, 2a, 3a, 4a) we found that these alleles act as an enhancer whose activity appears to be related to the number of VNTR repeats (Fig. 2A). One-way ANOVA showed a statistically significant difference among the luciferase activities observed in the whole experiment ($p \leq 0.001$). Then LSD post hoc tests showed that: (i) the luciferase activity sustained by each of the four alleles is significantly higher than that present in the control ($p < 0.05$ for every allele) and (ii) the luciferase activity is significantly different between alleles that differ for two repeats at least ($p = 0.04$ between alleles 1a and 3a, $p = 0.01$ between alleles 1a and 4a, $p = 0.02$ between alleles 2a and 4a). But, when the alleles harboring the DeltaEF1 site in the second repeat were assayed (alleles 2b, 3b, 4b) allele 2b showed a dramatic reduction in the enhancer activity with respect to allele 2a (Fig. 2B), although the enhancer activity of alleles 3b and 4b did not differ from that of 3a and 4a, respectively (results not shown).

By the above approach we demonstrated that the VNTR acts in vitro as an enhancer on a reporter gene. To explore a possible role of this enhancer in human longevity we analyzed the VNTR polymorphism in sample 2 (90- to 106-year-old subjects) and compared the genotypic and allelic frequencies with those of sample 1 (20- to 80-year-old subjects). The analyses were carried out according to sex because of gender effects in mortality of the oldest people and sex specificity in gene/longevity associations. On the basis of data in Table 2, we rejected the hypothesis of homogeneity between the genotypic pools of samples 1 and

Table 1
Genotypic and allelic frequencies of the VNTR polymorphism in sample 1 (20- to 80-year-old subjects)

Genotype	Count	Allele	Count
1.1	124 (17.6 ± 1.4)	1	587 (41.7 ± 1.3)
1.2	73 (10.4 ± 1.2)	2	166 (11.8 ± 0.9)
1.3	112 (15.9 ± 1.4)	3	291 (20.7 ± 1.1)
1.4	149 (21.2 ± 1.5)	4	351 (25.0 ± 1.2)
1.5	1 (0.1 ± 0.1)	5	6 (0.4 ± 0.2)
1.6	4 (0.6 ± 0.3)	6	5 (0.4 ± 0.2)
2.2	13 (1.9 ± 0.5)	Total	1406 (100.0)
2 3	27 (3.8 ± 0.7)		
2.4	38 (5.5 ± 0.9)		
2.5	2 (0.3 ± 0.2)		
3.3	40 (5.7 ± 0.9)		
3.4	69 (9.8 ± 1.1)		
3.5	2 (0.3 ± 0.2)		
3.6	1 (0.1 ± 0.1)		
4.4	47 (6.7 ± 0.9)		
4.5	1 (0.1 ± 0.1)		
Total	703 (100.0)		

Relative frequencies ± standard errors (×100) are given in parentheses. Allele nomenclature refers to the repeat number. Hardy–Weinberg equilibrium check (5000 permutations): $p = 0.185$.

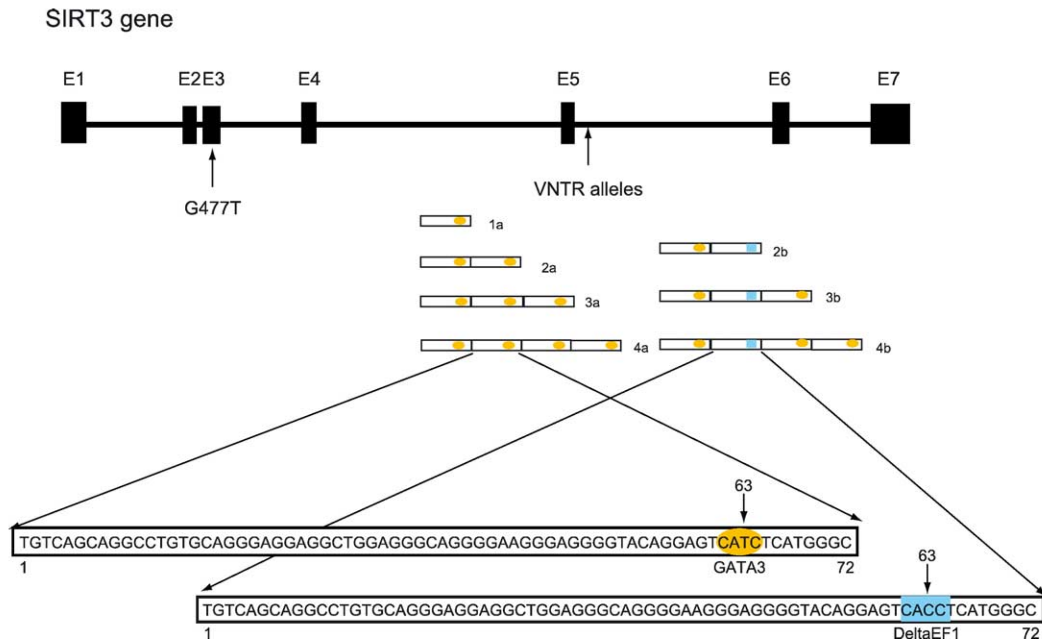

Fig. 1. A schematic representation of the *SIRT3* gene showing the positions of the G477T marker previously identified [15] and the VNTR discovered in the present study. The alleles including the GATA3 site in the second repeat (in yellow) are named 1a–4a, while the alleles including the DeltaEF1 site (in blue) are named 2b–4b. The variable sequence occurring in the second allele repeat is also shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2 in males ($p = 0.026$) but not in females ($p = 0.674$). Likewise, allele distribution frequencies were different between samples 1 and 2 in males ($p < 0.001$) but not in females ($p = 0.923$). In particular, in males, the frequency of allele 2 decreased from sample 1 to sample 2 (Table 2B). Since length-allele 2 included the sole allele lacking enhancer activity, we checked possible age-related variations of the gene pool in terms of alleles 2a and 2b by sequencing allele 2 in all the males. Of the 89 alleles found in the young group (sample 1, Table 2B) 49 were 2a and 40 were 2b, while all 5 alleles found in the oldest group (sample 2, Table 2B) were 2a. Therefore, in the entire gene pool, allele 2a decreased from 7.8 ± 1.1% in sample 1 to 2.9 ± 1.3% in sample 2 ($p = 0.025$ by Fisher exact test), while allele 2b decreased from 6.4 ± 1.0% in sample 1 to 0 in sample 2 ($p = 8 \times 10^{-5}$ by Fisher exact test).

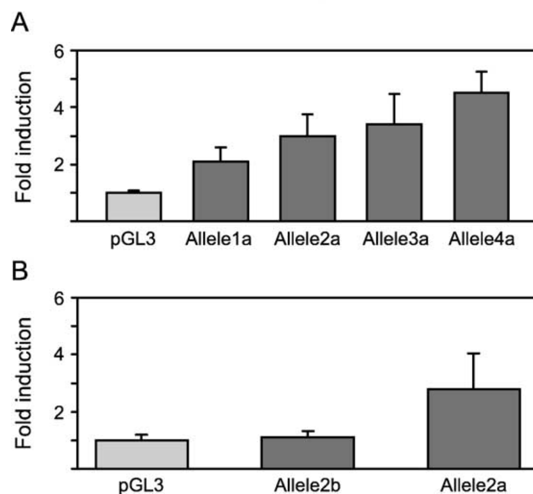

Fig. 2. (A) Luciferase expression of constructs including the 1a–4a alleles reported as fold induction (pGL3 negative control). (B) Luciferase expression of constructs including the allele 2b or the allele 2a reported as fold induction (pGL3 negative control). In both A and B the values reported for transfection experiments are the means ± SD of triplicate experiments.

Discussion

The aim of this study was to identify a putative functional variant occurring in *SIRT3* that could account for the association previously observed between longevity and a silent marker of this gene [15]. Transfection experiments demonstrated that the VNTR we identified in intron 5 of *SIRT3* is a functional polymorphism and that different VNTR alleles are able to modulate the expression of a reporter gene in an allele-specific way, according to number of repeats and occurrence of GATA3/DeltaEF1 site in the second repeat of the length-allele 2.

Is this polymorphism the functional variant we were searching for? Some hints indicate that this may be the case. First, a strong linkage disequilibrium occurs between the G477T silent marker and VNTR alleles; second, both genotypic and allelic frequency distributions differ between the group selected for longevity (male sample 2) and the younger control group (male sample 1).

It is intriguing that the sole allele lacking an enhancer effect in vitro, allele 2b, is completely absent in the oldest

Table 2
(A) Genotypic and (B) allelic counts of VNTR polymorphism in samples of males and females of different ages

	Males		Females	
(A) Genotype	Sample 1 (20–80 years)	Sample 2 (90–106 years)	Sample 1 (20–80 years)	Sample 2 (90–105 years)
1.1	46 (14.7 ± 2.0)	14 (16.3 ± 4.0)	78 (19.9 ± 2.0)	27 (17.3 ± 3.0)
1.2	36 (11.5 ± 1.8)	3 (3.5 ± 2.0)	37 (9.5 ± 1.5)	13 (8.3 ± 2.2)
1.3	42 (13.5 ± 1.9)	15 (17.4 ± 4.1)	70 (17.9 ± 1.9)	31 (19.9 ± 3.2)
1.4	69 (22.1 ± 2.3)	23 (26.7 ± 4.8)	80 (20.5 ± 2.0)	33 (21.2 ± 3.3)
1.5	—	—	1 (0.3 ± 0.3)	—
1.6	—	—	4 (1.0 ± 0.5)	—
2.2	10 (3.2 ± 1.0)	—	3 (0.8 ± 0.4)	4 (2.6± 1.3)
2.3	12 (3.9 ± 1.1)	—	15 (3.8 ± 1.0)	6 (3.8 ± 1.5)
2.4	20 (6.4 ± 1.4)	2 (2.3 ± 1.6)	18 (4.6 ± 1.1)	5 (3.2 ± 1.4)
2.5	1 (0.3 ± 0.3)	—	1 (0.3 ± 0.3)	—
3.3	18 (5.8 ± 1.3)	4 (4.7 ± 2.3)	22 (5.6 ± 1.2)	10 (6.4 ± 2.0)
3.4	37 (11.9 ± 1.8)	11 (12.8 ± 3.6)	32 (8.2 ± 1.4)	13 (8.3 ± 2.2)
3.5	1 (0.3 ± 0.3)	2 (2.3 ± 1.6)	1 (0.3 ± 0.3)	—
3.6	—	—	1 (0.3 ± 0.3)	—
4.4	19 (6.1 ± 1.4)	10 (11.6 ± 3.5)	28 (7.1 ± 1.3)	11 (7.1 ± 2.0)
4.5	1 (0.3 ± 0.3)	1 (1.2 ± 1.2)	—	2 (1.3 ± 0.9)
4.6	—	1 (1.2 ± 1.2)	—	1 (0.6 ± 0.6)
Total	312 (100.0)	86 (100.0)	391 (100.1)	156 (100.0)
(B) Allele				
1	239 (38.3 ± 1.9)	69 (40.1 ± 3.7)	348 (44.5 ± 1.8)	131 (42.0 ± 2.8)
2	89 (14.3 ± 1.4)	5 (2.9 ± 1.3)	77 (9.9 ± 1.1)	32 (10.3 ± 1.7)
3	128 (20.5 ± 1.6)	36 (20.9 ± 3.1)	163 (20.8 ± 1.5)	70 (22.4 ± 2.4)
4	165 (26.4 ± 1.8)	58 (33.7 ± 3.6)	186 (23.8 ± 1.5)	76 (24.4 ± 2.4)
5	3 (0.5 ± 0.3)	3 (1.8 ± 1.0)	3 (0.4 ± 0.2)	2 (0.6 ± 0.5)
6	—	1 (0.6 ± 0.6)	5 (0.6 ± 0.3)	1 (0.3 ± 0.3)
Total	624 (100.0)	172 (100.0)	782 (100.0)	312 (100.0)

Relative frequencies ± SE (×100) are given in parentheses.
(A) Genotypic homogeneity tests between samples 1 and 2: $p = 0.026$ in males, $p = 0.674$ in females.
(B) Allelic homogeneity tests between samples 1 and 2: $p < 0.001$ in males, $p = 0.923$ in females.

men while it is present in the younger group ($p = 8 \times 10^{-5}$). This finding suggests that allele 2b is detrimental for male longevity. On the other hand as the sample's age increases the frequency of allele 2a decreases as well ($p = 0.025$), thus suggesting that this allele is also unfavorable to male longevity. It follows that the GATA3/DeltaEF1 variability is not the only factor affecting the age-related variation of the gene pool and that other regulatory sites present in the 72-bp core do not act independent of the number of the VNTR repeats. We are now carrying out appropriate molecular studies to verify what is the exact portion of the VNTR repeat that links nuclear proteins, what are the proteins interacting with the repeat core, and, most importantly, how do these proteins interact according to the number of repeats.

Although the effect of the VNTR alleles as enhancer sequences has been demonstrated on a reporter gene, on the whole our findings are in line with the possibility that the VNTR acts in vivo on *SIRT3* and that variations in the expression of a *SIR2*-like gene may modulate life span in mammals [19]. Since the SIRT3 protein could deacetylate mitochondrial proteins involved directly in apoptosis [14], possibly by mimicking SIRT1 in inhibiting apoptosis by p53 deacetylation [20,21], the level of expression of *SIRT3* may affect longevity by acting on apoptotic mitochondrial patterns [22].

The association between VNTR alleles and longevity was restricted to males, because no difference was observed, either in the genotypic or in the allelic pool, between female samples 1 and 2 (Table 2). This finding confirms that longevity factors are sex-specific [23–25] and that males and females probably follow different trajectories to attain longevity [16].

Although cross-sectional data have been used for estimating the association between VNTR alleles and longevity, the present study should be reliable and not affected by bias caused by population stratification. In fact the population under study is highly homogeneous because of historical and geographical factors; furthermore, we did not find any stratification in this sample (unpublished results) by analyzing a panel of neutral markers spanning the genome [26].

In conclusion, our study has discovered a VNTR polymorphism in intron 5 of the *SIRT3* gene that has an allele-specific enhancer activity on a reporter gene. The frequency distribution of VNTR genotypes (and alleles) is significantly different between a sample of older men and a sample of younger men from the same population. Of course we need to confirm by appropriate longitudinal studies the effect of the VNTR on male longevity, and these studies are going on.

Materials and methods

Population samples

A population sample of 945 subjects was analyzed. All the subjects lived in Calabria (southern Italy) and their origin in the area was ascertained up to the grandparents' generation (interview). The sample consisted of two subsamples: sample 1 was made up of 20- to 80-year-old subjects (median age 59 years); sample 2 was made up of 90- to 106-year-old subjects (median age 102 years). Sample 1 comprised 703 subjects (312 males and 391 females); sample 2 comprised 242 subjects (86 males and 156 females). For collecting sample 1, an appropriate campaign was addressed to Calabria University students and staff, as well as to people who attended the University for the Elderly or used local thermal baths. For collecting sample 2, subjects older than 90 years were identified by consulting the population registers of the Municipalities of Calabria, contacted by phone, and then visited.

After a detailed explanation of the aims of the research, the subjects who agreed to participate donated a blood sample for routine laboratory analyses and DNA preparation; furthermore, people older than 60 years underwent a complete clinical and geriatric assessment. Subjects free of clinically overt pathologies and having blood and biochemical parameters in the normal age- and sex-specific range were enrolled in the study. In particular, the subjects enclosed in sample 2 belonged to health categories A and B previously described [16].

All the subjects analyzed in this study have given written informed consent for genetic studies on aging carried out by the present research group.

DNA samples

Six milliliters of blood was drawn from each subject. Plasma/sera were used for routine laboratory analyses, while DNA was extracted from blood buffy coats following standard procedures.

VNTR polymorphism identification

PCR amplification of genomic DNA was carried out in 25-μl final volume containing 100 ng of DNA; 0.08 μM each forward primer, 5′-TTCCTGAAGCTGGGTACA-3′ (5′ position 12,030 nt referring to www.ncbi.nlm.nih.gov/omim; MIM 604481; contig NT_035113), and reverse primer, 5′-CATTCACCTTCCCAAAGTGG-3′; 200 μM dNTP; 0.3 U Finnzymes *Taq* DNA polymerase; 1.5 mM $MgCl_2$; and 1× thermophilic buffer. The following thermal cycling conditions were used: 1 cycle at 95°C for 45 s and 25 cycles of denaturation for 1 min at 95°C, annealing for 1 min at 55°C, and elongation for 1 min at 72°C. An extra step was performed at 72°C for 5 min. PCR-amplified fragments were analyzed by automated sequencing in a 310 DNA sequencer with a BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems).

VNTR polymorphism typing

Both Gene Scan DNA fragment analysis (TAMRA 500 internal size standard) in a 310 DNA sequencer (PE Applied Biosystems) and electrophoresis in a 1.5% agarose gel (Marker Step Ladder 50 bp, Sigma, as molecular weight marker) were used on PCR-amplified products for VNTR genotyping.

Identification of alleles 2a and 2b

Allele 2 was analyzed by DNA sequencing in all the male carriers of the allele (10 homozygous plus 74 heterozygous subjects). After PCR amplification (see VNTR polymorphism identification) and electrophoresis, the DNA fragment containing two repeats was recovered from agarose gel in TAE buffer 1× (0.04 M Tris acetate, 0.001 M EDTA) by using the QIAquick gel extraction kit (Qiagen). The sequencing reactions were automatically carried out with the forward primer 5′-TCTTGCTGCATGTGGTTG-3′ (5′ position 12,275 nt).

Plasmid construction

The pGL3 promoter vector (Promega) containing an SV40 promoter was used in the construction of reporter plasmids for the analysis of allele-specific regulatory functions. Preliminarily, to discriminate between alleles having the same length but different nucleotide sequence (T/C at position 63 in the second repeat) the common alleles (one to four repeats) were amplified and automatically sequenced from subjects who were homozygous for the 1–4 length-alleles. The forward primer 5′-*CTCCCCGGG*TGGGAACTCCCTGGAGGTT-3′ (5′ position 12,324 nt referring to www.ncbi.nlm.nih.gov/omim; MIM 604481, contig NT_035113) and the reverse primer 5′-*CTCCCCGGG*TGGCACCAGCCCTGGAAG-3′ were used to have DNA fragments containing the VNTR sequence only (the italicized sequence was added to include the *Sma*I site for cloning). The PCR conditions were the same as described above. The PCR products of subjects having (CC) or (TT) homozygous genotype were purified by agarose gel in TAE buffer by using the QIAquick gel extraction kit (Qiagen) and digested with *Sma*I restriction enzyme. After purification by phenol extraction, the fragments were cloned into the *Sma*I-digested and dephosphorylated pGL3 vector, upstream of the *Luc*-promoter transcriptional unit. The sequence of each insert (and its orientation in the reporter vector) was confirmed by restriction mapping and automated DNA sequencing. Cloned vectors were purified by Qiagen Plasmid Maxi Kit and used for subsequent experiments.

Transfection assays

HeLa cells were cultured in DMEM (Sigma Aldrich) supplemented with 5% fetal bovine serum, 2 mM L-glutamine, 1% penicillin/streptomycin. Cells were transferred to 24-well plates with 500 μl of regular growth medium/well the day before transfection. Transfections were performed with the Fugene 6 reagent as recommended by the manufacturer (Roche Diagnostics) with a mixture containing 1 μg of each reporter plasmid and 2 ng of *Renilla* luciferase (Promega). Luciferase activity was measured after 24 h by using the Dual Luciferase Kit (Promega) in a Lumat LB9507 luminometer (EG&G Berthold). The luciferase activity of the reporter plasmids was normalized to the activity of *Renilla* luciferase. All transfections were performed in triplicate and repeated at least three times.

Statistical analyses

VNTR allele frequencies in samples 1 and 2 were estimated by counting genes from the observed genotypes, after verification of Hardy–Weinberg equilibrium by allele shuffling (5000 random permutations). The null hypothesis of homogeneity between the genotypic (or allelic) pools of samples 1 and 2 was checked by permutation tests (1000 permutations). The statistical significance of the differences among the enhancer activity of different VNTR alleles in transfection experiments was tested by ANOVA and LSD post hoc test. The MATLAB statistical textbook was used for all the statistical analyses, excepted for linkage disequilibrium analysis between the markers G477T [15] and VNTR, for which the Arlequin software was used; this software utilizes a maximum likelihood estimation procedure by using genotypic data from both homozygous and heterozygous subjects.

Acknowledgments

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Characterization of a bidirectional promoter shared between two human genes related to aging: *SIRT3* and *PSMD13*

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Abstract

The human *SIRT3* gene contains an intronic VNTR enhancer whose variability is correlated with life span. The *SIRT3* 5′ flanking region encompasses the *PSMD13* gene encoding the p40.5 regulator subunit of the 26S proteasome. Proteasome is a multicatalytic proteinase whose function declines with aging. *SIRT3* and *PSMD13* are linked in a head-to-head configuration (788-bp intergenic region). The molecular configuration of two genes that are both related to aging prompted us to search for shared regulatory mechanisms between them. Transfection experiments carried out in HeLa cells by deletion mutants of the *PSMD13–SIRT3* intergenic region showed a complex pathway of coregulation acting in both directions. Furthermore, linkage disequilibrium (LD) analyses carried out in a sample of 710 subjects (18–108 years of age) screened for A21631G (marker of *PSMD13*), and for G477T and $VNTR_{intron5}$ (markers of *SIRT3*), revealed high LD, with significantly different *PSMD13–SIRT3* haplotype pools between samples of centenarians and younger people.
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Keywords: *SIRT3*; *PSMD13*; bidirectional promoter; human longevity

Although aging is not regarded as an adaptive process, gene pathways affecting aging and life span are evolutionarily conserved [1,2]. Usually the conservation of aging-related paths is interpreted in terms of pleiotropic phenomena [3]; however, the significance of the evolutionary conservation of the paths is still under discussion [4,5]. In this frame, the evolutionarily conserved sirtuin 2 (*SIR2*) gene family is especially interesting. First, in model organisms the expression levels of *SIR2* modulate life span [6,7]; second, low-calorie diets that extend life span also promote sirtuin activity, showing that sirtuins may connect metabolism and aging [8]; third, Sir2 proteins exhibit nicotinamide dinucleotide phosphate (NAD^+)-dependent deacetylase activity that could account for the broad range of biological processes in which such proteins play a role, including gene expression, metabolism, and aging [9].

The *SIRT3* gene (11p15.5) is a human homologue of *SIR2* genes [10] that is expressed mainly in metabolically active tissues and is targeted to mitochondria through an N-terminal peptide sequence signal for mitochondrial localization [11–13]. The Ensembl database (http://www.ensembl.org) reports a list of putative orthologues of *SIRT3* (ENSG00000142082). For most of them a NAD-dependent deacetylase activity is reported in the database. Interestingly, it has been recently shown that murine Sirt3 activates mitochondrial functions and plays a crucial role in adaptive thermogenesis in brown adipocytes [14]. Taking into account the central role played by mitochondria [15] and lipid metabolism [16] in aging and longevity this finding suggests that in mammals *SIRT3* may play a role in the life span similar to that of its homologous *sir2* in yeast [6], the protozoan parasite *Leishmania* [17], worm [7], and fly [18]. According with this hypothesis, we found that *SIRT3* variability is associated with human longevity, likely through the enhancer activity of a VNTR (Variable Number of Tandem Repeats) occurring in intron 5 of the gene [19,20].

The *SIRT3* gene shows a head-to-head orientation (GenBank Accession No. NT_035113) with the proteasome 26S subunit non-ATPase 13 (*PSMD13*) gene, which encodes the p40.5 regulator subunit of the 26S proteasome. The 26S proteasome is

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a multicatalytic proteinase complex with a highly ordered structure composed of two complexes, a 20S core and a 19S regulator, working as natural machinery for the degradation of damaged proteins. Also for *PSMD13* (ENSG00000185627) the Ensembl database reports a list of putative orthologues that are involved chiefly in the degradation of ubiquitinated proteins, thus playing a role in cell cycle, cell cycle check point, and DNA replication, according to Reactome, a "knowledgebase" of biological pathways (http://www.reactome.org). The pivotal role played by proteasome in the degradation of abnormal proteins (for example oxidized proteins) links proteasome function to cellular senescence [21] and aging [22]. On the whole, we can infer that two genes that have a pivotal role in a number of cell pathways and lie head to head within a short chromosomal region are both related to aging and, possibly, to life span.

The unexpected configuration of *SIRT3* and *PSMD13* prompted us to investigate if the *PSMD13–SIRT3* intergenic region could act as a bidirectional promoter, capable of coordinating the expression patterns of the two genes. By transfection experiments we discovered not only that the region interposed between the two genes can regulate the transcription in both directions, but also that a common core rich in Sp1 sites plays a critical role in a shared regulatory mechanism. In addition, we investigated whether the gene/longevity association previously observed for *SIRT3* also involved *PSMD13*. The linkage disequilibrium studies presented here show that the haplotype pool defined by *PSMD13–SIRT3* variability differs in centenarians and in younger people. Our data provide the first evidence that two genes, both involved in aging and longevity, could share a common regulatory mechanism.

Results

Computer-assisted analyses of the PSMD13–SIRT3 intergenic region

Fig. 1 shows the two genes with their head-to-head orientation and the nucleotide sequence of the 788-bp intergenic region. Computer-assisted analyses revealed that the region has a high G+C content (67.19%) and contains two putative CpG islands (CpGPlot software); furthermore, the region lacks the typical TATA box sequence and its homologues (MatInspector software). As shown in Fig. 1B, the region contains multiple potential DNA motifs for AP-1, GATAs, NF-κB, and ZF5 and multiple binding sites for Sp1 factor clustered in proximity of the transcription start site of the *SIRT3* gene (+1 position in Fig. 1B, in which putative transcription factor binding sites are marked with arrows indicating the functional direction).

Molecular analyses of the PSMD13–SIRT3 intergenic region

By inspection of the *PSMD13–SIRT3* genomic region we observed that the *PSMD13* and *SIRT3* transcription start sites are located −72 and −32 nucleotides, respectively, from the translation initiation codons of the two genes. Therefore, the closeness of the transcription start sites suggested to us that the *PSMD13–SIRT3* intergenic region might act as a bidirectional promoter. Thus, to check our hypothesis and investigate the regulation of two divergently transcribed genes, we first verified the promoter activity of the entire intergenic region, then we searched for the core region that was essential for transcription in the direction of either *PSMD13* or *SIRT3*.

The PSMD13–SIRT3 intergenic region acts as a bidirectional promoter

We analyzed the promoter activity of the 788-bp intergenic region by using firefly luciferase as the reporter gene. This region was PCR-amplified from genomic DNA by using the primers SirtFor and SirtRev (see Fig. 1B). Then, the fragment was ligated in both directions into the promoterless pGL2-Basic vector upstream of the luciferase coding region. Transient transfection assays in HeLa cells revealed that the genomic fragment of 788 bp was sufficient for the expression of the firefly luciferase gene regardless of its orientation. Compared to the activity of the pGL2 vector alone (Fig. 2), the *PSMD13–SIRT3* intergenic region increased the luciferase activity by a factor of about 33 in the *SIRT3* direction ($p < 0.0001$) and about 49 in the *PSMD13* direction ($p < 0.0001$). On the whole, the transfection results showed that the regulation of the *SIRT3* and *PSMD13* gene expression could be coordinated through a bidirectional promoter.

Deletion analyses of the regulatory promoter

To check whether *SIRT3* and *PSMD13* genes share a common regulation pattern, we assembled partially deleted constructs and checked for their promoter activity in transient transfection experiments. In particular, we analyzed the functional effect of the multiple Sp1 sites located close to the transcription start site of *SIRT3* (Fig. 1B) in either the *SIRT3* or the *PSMD13* orientation. Deletion constructs were generated by cloning promoter PCR fragments in both directions upstream of the firefly luciferase gene into the pGL2-Basic vector. The reporter plasmids were transiently transfected into HeLa cells. The constructs, their promoter insert positions, and the cloning primer sequences are reported in Table 1. Promoter activity of the deletion fragments in either the *SIRT3* or the *PSMD13* direction was compared to that of the constructs containing the entire bidirectional promoter (pGL2/788/SIRT3 and pGL2/788/PSMD13 constructs). The results obtained by checking the entire group of constructs are shown in Fig. 3 and summarized in Table 2. In short, we see that Sp1 sites alone enhance the activity of the promoter in the *SIRT3* direction (A/SIRT3 construct) but not in the *PSMD13* direction (A/PSMD13 construct). In this orientation, ZF5/NF-κB/AP-1/GATA1-2 binding sites (D/PSMD13 construct) are required to restore the activity of the entire bidirectional promoter. However, in both directions, the absence of Sp1 sites (B/SIRT3 and B/PSMD13 constructs) causes a significant decrease in the promoter activity with respect to the entire intergenic region. On the whole, the results reported in Fig. 3 and summarized in

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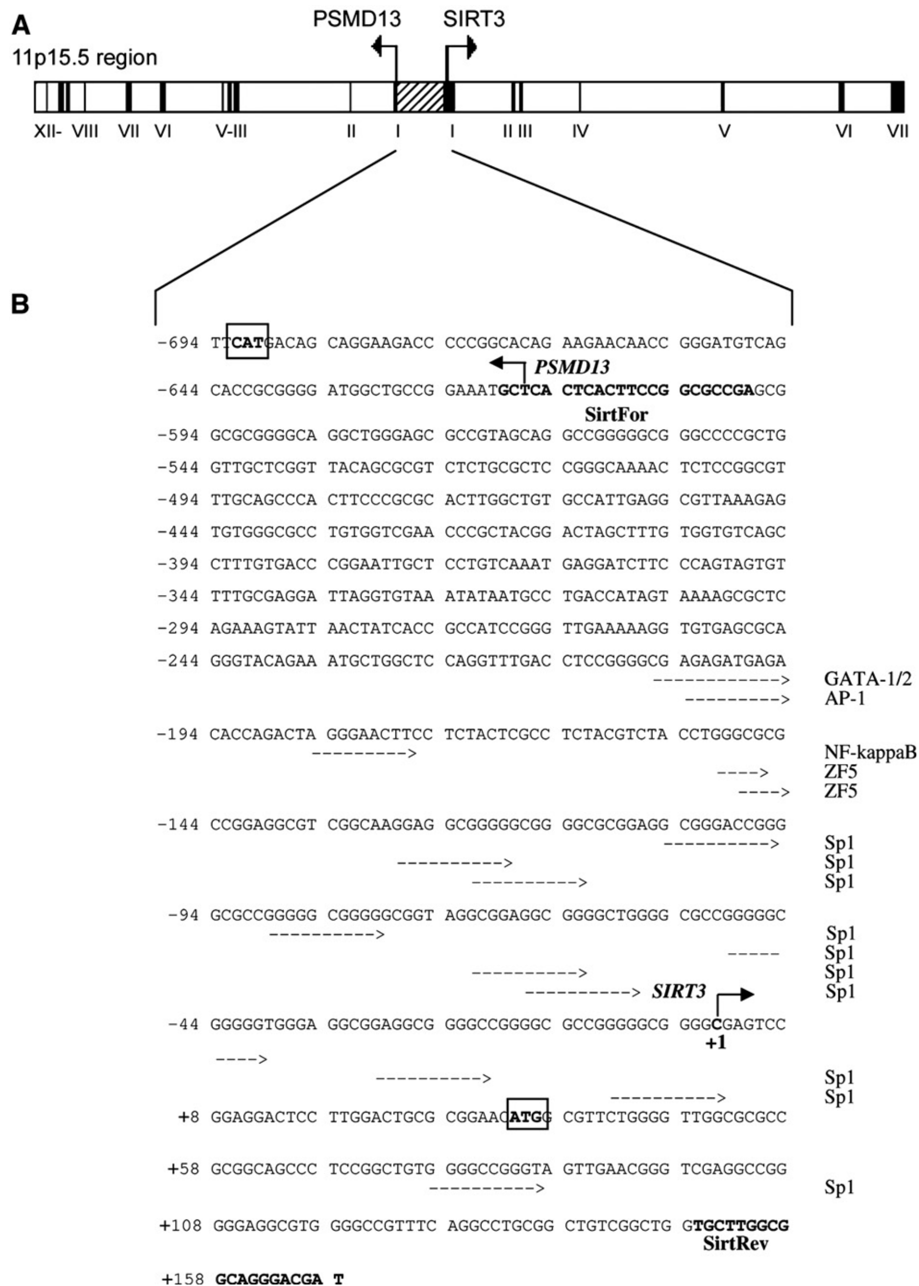

Fig. 1. (A) Structure of human *PSMD13* and *SIRT3* genes in the head-to-head orientation. The exons of the two genes are presented as filled boxes and indicated with roman numbers. The *PSMD13*–*SIRT3* intergenic region is presented as a hatched box. The bent arrows indicate the transcription direction. (B) Nucleotide sequence of the 788-bp intergenic region. Translation start codons of *SIRT3* and *PSMD13* genes are boxed. Transcription start sites are indicated by the bent arrows. The transcription start site of *SIRT3* is designated as "+1". Positive (negative) numbers are assigned to nucleotides downstream (upstream) of nucleotide +1. The analyzed putative transcription factor binding sites are shown with straight arrows. SirtFor and SirtRev primers used to clone the 788-bp intergenic region are in bold.

Table 2 show that the Sp1 sites play a critical role for the transcription in both directions, although synergies with further sites are required in the *PSMD13* orientation.

Linkage disequilibrium (LD) analysis

The finding that the *PSMD13* and *SIRT3* genes share a common regulation path prompted us to check whether the association we observed between longevity and *SIRT3* variability was extended to the *PSMD13* gene.

By using the A21631G marker of *PSMD13,* and the G477T and $VNTR_{intron5}$ markers of *SIRT3,* we carried out pairwise LD analyses in a sample of 710 unrelated subjects (see Materials and methods). We found statistically significant LD values (likelihood-ratio test) for all the pairs of markers ($A21631G_{PSMD13}$ and $VNTR_{SIRT3}$ $p=0.001$; $A21631G_{PSMD13}$ and $G477T_{SIRT3}$ $p=0.001$; $G477T_{SIRT3}$ and $VNTR_{SIRT3}$ $p=0.002$). Therefore, the linkage disequilibrium spans across the entire region depicted in Fig. 1A and involves the markers of both *PSMD13* and *SIRT3*.

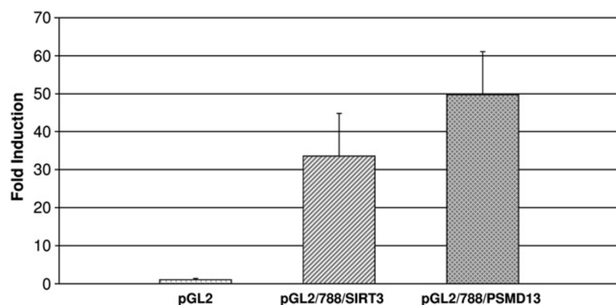

Fig. 2. Luciferase expression of pGL2/788/SIRT3 and pGL2/788/PSMD13 constructs reported as fold induction with respect to the pGL2-Basic vector. pGL2/788/SIRT3 and pGL2/788/PSMD13 contain the intergenic region in the *SIRT3* and *PSMD13* orientation, respectively. The values reported for transfection experiments are the means ± standard deviation of three independent duplicate experiments. The statistical significance of the differences between the promoter activity of the two constructs was tested by ANOVA and LSD post hoc tests.

To verify if the pool of *PSMD13–SIRT3* haplotypes changed in the population according to age, the sample was divided into two subsamples, the first made of 18- to 90-year-old individuals ($n=615$), the second made of 91- to 108-year-old individuals ($n=95$). The cut-off between age classes was chosen on the consideration that such a cut-off corresponds approximately to the surviving upper 0.1% of the population under study (Calabria, southern Italy). Table 3 shows the haplotype distribution in the two age groups. The exact test of population differentiation between the two subsamples revealed a significant difference between the haplotype pools ($p=0.019$). Therefore, as the population ages and survival selection operates, some haplotypes are preferentially lost from the haplotype pool (see for example the haplotype GG2 in Table 3), while others increase their frequency (see for example the haplotype AG4 in Table 3). On the whole, the haplotype analysis revealed that the chromosomal region associated with longevity comprises also the *PSMD13* gene.

Discussion

By exploring the neighborhood of the *SIRT3* gene we observed that the *PSMD13* gene lies very close to it (788 bp) in a head-to-head configuration. It was recently suggested that closely located bidirectional gene pairs whose transcription start sites are separated by less than 1 kb are common in the human genome and that such a bidirectional organization may control genes functionally related to each other [23–25]. Accordingly, the genes *PSMD13* and *SIRT3* could be functionally related. What is more, according to GenBank (http://www.ncbi.nlm.nih.gov/MapViewer) the head-to-head organization of the two genes is evolutionarily conserved in bird, rat, mouse, dog, chimpanzee, and human. Interestingly, the distance between the two genes increases throughout evolution (14 bp in *Gallus gallus,* 56 bp in *Rattus norvegicus,* 86 bp in *Mus musculus,* 157 bp in *Canis familiaris,* 555 bp in *Pan troglodytes,* where the regulatory sites display the same pattern as in humans, and 788 bp in *Homo sapiens*). This observation is intriguing, as it suggests an increasing complexity throughout evolution of a putative coregulation machinery shared between the two genes.

The aim of our work was: (a) to verify the bidirectional promoter activity of the *PSMD13–SIRT3* 788-bp intergenic region, (b) to identify a common regulation core in the promoter region, and (c) to verify whether the association with the longevity trait previously observed for *SIRT3* [19,20] involved *PSMD13*, too.

Bioinformatics analyses showed that the region is characterized by a GC content of 67% and comprises two CpG islands. These structural features are consistent with the findings that almost all the bidirectional promoters in mammalian genomes have a median GC content of 66%, and 77% of them are located in CpG islands [23,24,26]. The results of transfection experiments carried out by expression constructs containing the entire 788-bp intergenic region cloned in both *PSMD13* and *SIRT3* directions (Fig. 2) confirmed that such a region acts as a bidirectional promoter. Therefore we can give a positive answer to question (a).

As shown in Fig. 1B, the intergenic region contains a number of putative transcription factors binding sites such as AP-1, GATAs, NF-κB, ZF5, and Sp1. In particular, the GC boxes that bind Sp1 factors are clustered in proximity of the *SIRT3* transcription start site. A TATA box is absent. The lack of a TATA box is characteristic of the so-called housekeeping genes, but it was also observed in bidirectional promoters [26]. On the other hand, the presence of GC boxes close to the transcription start site is characteristic not only of TATA-less promoters, for which Sp1 factors are generally responsible for fixing the transcription start site, but also of bidirectional promoters [27,28]. By carrying out a series of transient transfection experiments with a set of deletion constructs (Table 1) cloned in both orientations, we discovered that Sp1 sites play a critical role in regulating gene expression in both directions (Fig. 3 and Table 2), although they act alone in the *SIRT3* orientation but in association with other transcription factors in the *PSMD13* orientation. In line with this observation, it has been documented that Sp1 factors may act in combination with other coactivator or corepressor factors to modulate transcription by physical or functional interaction [29,30]. On the whole, the entire set of results summarized in Table 2 suggests that *PSMD13* and *SIRT3*

Table 1
Deletion constructs, their promoter inserts, and cloning primers

Deletion construct	Promoter insert positions	Cloning primer sequences (5′→3′)
A/SIRT3	−145 to +169	ctcggtaccGCCGGAGGCGTCGGCAAG
A/PSMD13	−169 to +145	ctcggtaccATCGTCCCTGCCGCCAAGCA
B/SIRT3	−619 to −128	ctcggtaccGCTCACTCACTTCCGGCGCCGA
B/PSMD13	+128 to +619	ctcggtaccCTTGCCGACGCCTCCGGC
C/SIRT3	−174 to +169	ctcggtaccTCTACTCGCCTCTACGTC
C/PSMD13	−169 to +174	ctcggtaccATCGTCCCTGCCGCCAAGCA
D/SIRT3	−201 to +169	ctcggtaccGATGAGACACCAGACT
D/PSMD13	−169 to +201	ctcggtaccATCGTCCCTGCCGCCAAGCA

The 9-bp cloning adaptor is represented by lowercase characters. Promoter insert positions are related to the intergenic sequence in the *SIRT3* (A/SIRT3, B/SIRT3, C/SIRT3, D/SIRT3) and *PSMD13* (A/PSMD13, B/PSMD13, C/PSMD13, D/PSMD13) orientations.

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are coregulated. Conclusive evidence of the role played by Sp1 and other regulatory factors (for example ZF5) in the co-regulation of the two genes will be provided by appropriate further experiments (for example gel-shift and mutagenesis experiments), which we are planning right now. In any case, on the basis of the results here presented, we can say that a fine

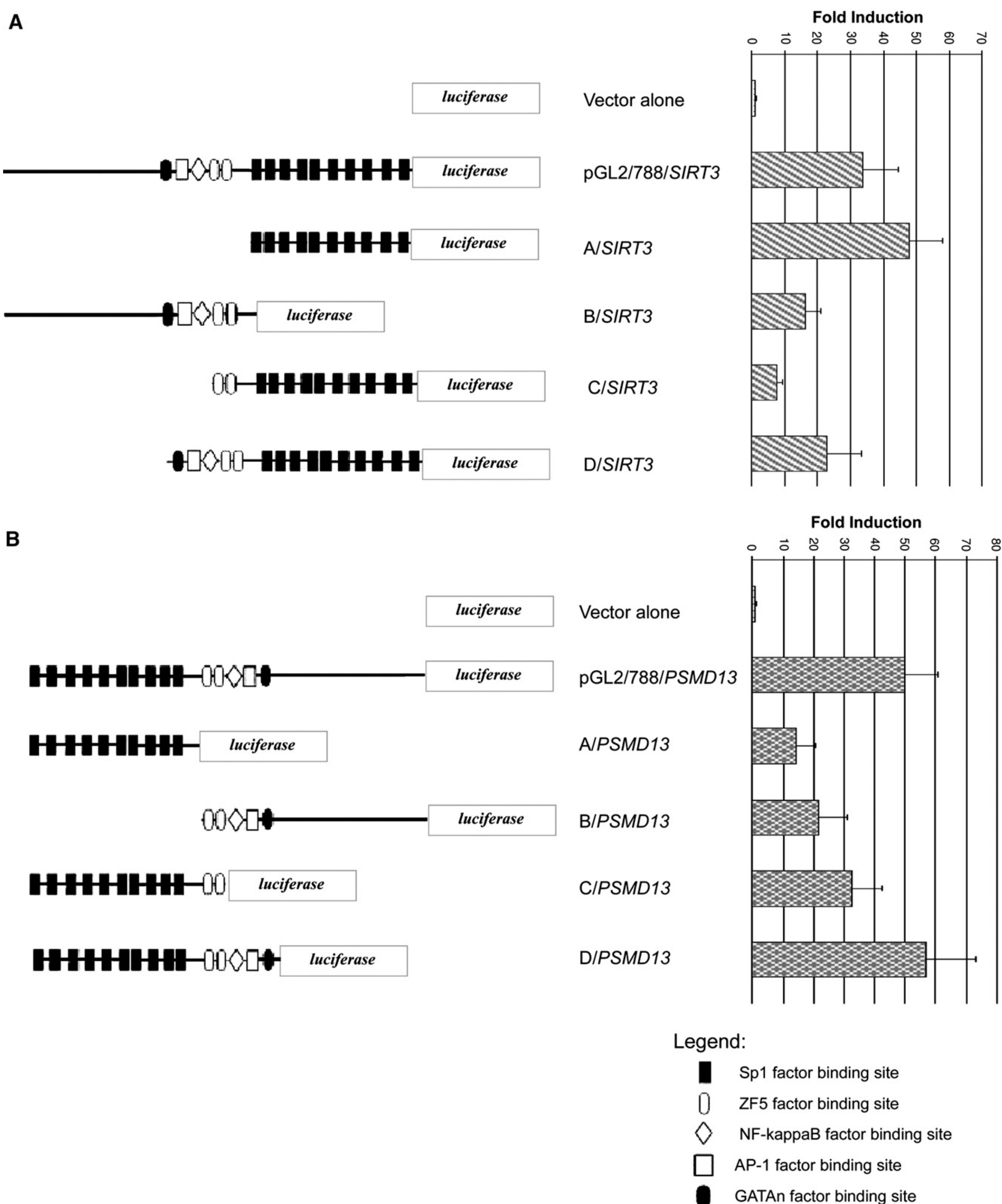

Fig. 3. Deletion analysis of the human *PSMD13–SIRT3* bidirectional transcription control region. On the left, the construct containing the whole 788-bp intergenic region and the constructs containing promoter fragments in the (A) *SIRT3* and (B) *PSMD13* orientation are shown with putative transcription factor binding sites. On the right, the activity of each construct is shown as fold induction with respect to the pGL2-Basic vector. The values reported for transfection experiments are the means ± standard deviation of three independent duplicate experiments.

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Table 2
Synoptic table summarizing the results of the transfection experiments shown in Fig. 3

Deletion construct	Transcription regulatory elements	Transcription activity with respect to the entire promoter	*p* value
A/SIRT3	Multiple Sp1 binding sites only	Up-regulation	0.001
A/PSMD13		Down-regulation	0.000
B/SIRT3	Absence of multiple Sp1 binding sites	Down-regulation	0.000
B/PSMD13		Down-regulation	0.000
C/SIRT3	Multiple Sp1 binding sites plus two ZF5 binding sites	Down-regulation	0.001
C/PSMD13		Down-regulation	0.001
D/SIRT3	Multiple Sp1 binding sites plus two ZF5 binding sites and	Down-regulation	0.013
D/PSMD13	NF-κB/GATA1 binding sites	Same activity	0.156

The *p* values refer to the null hypothesis of no difference between the transcription activity of the entire 788-bp promoter and the transcription activity of the deletion construct (ANOVA and LSD post hoc tests).

game of coactivators and corepressors likely modulates a concerted expression of the two genes (question (b)).

Finally, through pairwise linkage disequilibrium analyses carried out between markers of *PSMD13* and *SIRT3* in a population sample including centenarians (Table 3), we showed that the entire chromosomal region encompassing the two genes is associated with the longevity phenotype (question (c)).

The observation that *PMSD13* and *SIRT3* share a bidirectional promoter (and that this architecture is conserved along evolution) is very interesting given that both genes are involved in aging. Usually, when more genes are clustered in the genome, their organization is selected relative to a possible complex phenotype to which such genes contribute. It would be surprising if this phenotype was aging, since several theories support the idea that aging is not a programmed and adaptive process, but a process due mainly to accumulation of stochastic mutations and that longevity is due, at most, to antagonist pleiotropic phenomena [3,4]. On the other hand, recent studies show that gene order in eukaryotic genomes is not completely random, but that genes with comparable and/or coordinated expression tend to be clustered together [31,32]. On the basis of this, the evidence for a common regulation between *SIRT3* and *PSMD13* genes is very intriguing considering that the two genes contribute to the same phenotype. However, it is still under discussion whether coexpressed genes have been linked together by natural selection to facilitate their expression or whether the genes are coexpressed simply because of their physical proximity in the genome. Several studies show that natural selection acts to preserve linked pairs of coexpressed genes [33,34]. Indeed, adjacent pairs of essential genes are preferentially conserved along evolution. The close proximity of the genes could be an adaptation that facilitates the coregulation of their transcription. It was observed that coexpressed genes remain linked more often than expected, which indicates that selection might favor their retention as a pair. It was also found that clusters of essential genes are in regions of low recombination and that larger clusters have lower recombination rates [35]. The selection could act to modify both the intragenomic variation in the recombination rate and the distribution of genes, thus determining the coevolution of gene order and recombination rate. A landmark in this field could be a recently published paper that reports results on head-to-head gene organization [36]. The authors carried out a systematic investigation of bidirectional gene pairs, focussing on structural features, evolutionary conservation, expression correlation, and functional association. The conclusion was that the head-to-head gene organization is ancient and conserved and may provide a fine mechanism of transcriptional coregulation based on gene organization. In particular, by comparing 42 head-to-head human gene pairs to their orthologue pairs in chicken and *Fugu,* the authors identified 10 pairs (20 genes) for which important conserved functions could be assumed: the *PSMD13–SIRT3* gene pair was one of them.

These considerations suggest an intriguing question: why did “Mother Nature” conserve a common pathway of regulation between two genes involved in a process that is believed to have come out of natural selection? It has been recently proposed that a programmed and altruistic aging may occur in higher eukaryotes [5]. Our findings are in line with this idea, although the deep evolutionary force that has driven such an architecture along evolution needs to be explored.

Table 3
PSMD13–SIRT3 haplotype pools in 18- to 90- and 91- to 108-year-old subjects

Haplotype	18- to 90-year-old subjects (n=615)		91- to 108-year-old subjects (n=95)	
	RF (%)	SE	RF (%)	SE
AG1	2.0	0.4	1.1	0.8
GG1	11.3	0.9	10.1	2.2
AT1	0.4	0.2	0.0	0.0
GT1	28.4	1.3	29.3	3.2
AG2	0.3	0.2	1.6	1.0
GG2	10.1	0.9	4.7	1.6
GT2	0.4	0.2	0.5	0.6
AG3	2.0	0.4	2.6	1.2
GG3	19.6	1.2	18.3	2.9
GT3	0.1	0.1	0.6	0.6
AG4	22.8	1.2	28.6	3.3
GG4	0.9	0.3	1.6	0.9
AT4	0.2	0.1	0.0	0.0
GT4	0.2	0.1	0.0	0.0
AG5	0.4	0.2	1.0	0.7
GG5	0.2	0.1	0.0	0.0
AG6	0.7	0.2	0.0	0.0

The markers used for haplotype analysis are the following (in order): A21631G for *PSMD13*, G477T and 1–6 $VNTR_{intron5}$ for *SIRT3*. Haplotype relative frequencies (RF) and standard errors (SE) are ×100.

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Materials and methods

Molecular analyses of the PSMD13–SIRT3 intergenic region

Bioinformatic analyses

The GC content of the 788-bp common 5′ region shared by the *SIRT3* and *PSMD13* genes and detection of areas rich in CpG islands were performed using CpGPlot software (http://bioweb.pasteur.fr/seqanal/interfaces/cpgplot/html). Prediction of putative transcriptional factor binding sites was performed using MatInspector software (http://www.genomatix.de/matins).

Construction of reporter gene plasmids

The 788-bp *PSMD13–SIRT3* intergenic region was PCR amplified from human genomic DNA by using the forward primer SirtFor and the reverse primer SirtRev (Fig. 1B). The primers contained a 9-bp cloning adaptor with a *Kpn*I restriction site. The PCR was carried out in 100 μl of a mix consisting of 1× buffer, 100 mM dNTPs, 500 nM primers, 1.5 mM $MgCl_2$, and 5 U of DNAzyme (Finnzyme). The reaction consisted of 25 cycles, each cycle consisting of a denaturation step (94°C for 60 s), an annealing step (55°C for 60 s), and an extension step (72°C for 60 s). The first cycle was preceded by a denaturation step of 1 min at 94°C and the last one was followed by an extension step of 3 min at 72°C. The resulting 788-bp fragment was purified by agarose gel electrophoresis (Wizard SV Gel; Promega) and digested with *Kpn*I enzyme (Promega) as recommended by the manufacturer. After enzyme digestion, the fragment was inserted using T4 DNA ligase (Promega) in both orientations into the *Kpn*I site upstream of the firefly luciferase reporter gene in the pGL2-Basic vector (Promega), yielding two plasmids, pGL2/788/SIRT3 (intergenic region in *SIRT3* orientation) and pGL2/788/PSMD13 (intergenic region in *PSMD13* orientation). DNA was transformed into Top10 *Escherichia coli* cells by electroporation according to standard protocols. pGL2/788/SIRT3 and pGL2/788/PSMD13 constructs were prepared using the Wizard Plus SV Minipreps DNA purification system (Promega). The constructs were sequenced with the BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems) to check the correct insert orientation and to confirm that the sequences matched the original genomic sequences without PCR-generated errors.

Deletion analyses

Deletion constructs were generated by PCR amplification of promoter fragments by using as template the reporter plasmids pGL2/788/SIRT3. The constructs, their promoter inserts, and the cloning primers are reported in Table 1. The primers contained a 9-bp cloning adaptor with a *Kpn*I restriction site. PCR was carried out as described above. After enzyme digestion, PCR products were purified by agarose gel electrophoresis and then inserted in both orientations into the *Kpn*I-linearized pGL2-Basic. All the plasmids were sequenced to check the correct insert orientation and to confirm the sequence.

Promoter analysis by luciferase assay

HeLa cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) containing 4.5 g/L glucose and 2 mM L-glutamine supplemented with 5% fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin (Invitrogen). The cells were cultured in a water-humidified incubator at 37°C in 5% CO_2/95% air. HeLa cells (1×10^5) were transferred into 24-well plates with 500 μl of regular growth medium/well the day before transfection. Transfections were performed with the Fugene6 reagent as recommended by the manufacturer (Roche Molecular Biochemicals) with a mixture containing 1 μg of each reporter plasmid and 2 ng of pRL-CMV (Promega), a plasmid that contains the *Renilla* luciferase gene under the cytomegalovirus promoter and is utilized as an internal control to normalize the effects of transfection efficiencies. Cells were lysed 24 h after transfection by applying 50 μl Passive Lysis Buffer of the Dual Luciferase Reporter Assay Kit (Promega) into each well of the 24-well plate. Twenty microliters of cell lysate was used for luciferase reporter assay by using the same kit according to the manufacturer's protocol. Light intensity was quantified in a Lumat LB9507 luminometer (EG&G Berthold). The luciferase activity of the reporter plasmids was normalized to the *Renilla* luciferase activity. Each transfection experiment was carried out three times in duplicate. ANOVA and LSD post hoc tests were used to check the significance of the difference between the fold induction value of the deleted construct and that of the construct containing the entire 788-bp *PSMD13–SIRT3* intergenic region.

Population genetic analyses

Population sample

A population sample of 710 subjects was analyzed. All the subjects lived in Calabria (southern Italy) and their origin in the area had been ascertained up to the grandparents' generation (interview). The sample consisted of two subsamples: one included 18- to 90-year-old subjects (615 subjects, 262 males and 353 females), the other 91- to 108-year-old subjects (95 subjects, 31 males and 64 females). The younger group had been collected between 2000 and 2003 by an appropriate campaign addressed to Calabria University students and staff and to people who attended the University for the Elderly or used local thermal baths. The older group had been collected in the same period by consulting the Population Registers of the Municipalities of Calabria, contacting the potential probands by phone, and then visiting them in the case of positive answer to a first contact. After a detailed explanation of the aims of the genetic studies on aging carried out by our research group, the subjects who agreed to participate gave us a written informed consent and donated a blood sample for routine laboratory analyses and DNA preparation. People older than 60 years underwent a complete clinical and geriatric assessment. Subjects free of clinically overt pathologies and having blood and biochemical parameters in the normal age- and sex-specific range were enrolled in the study. In particular, the subjects in the older group belonged to the health categories A and B previously described [37].

DNA analyses

All the genotype data relevant to the G477T marker of *SIRT3* [14] and to the $VNTR_{intron5}$ marker of *SIRT3* [15] were already included in our database. Therefore DNA analyses were carried out only for genotyping *PSMD13* variability.

The A21631G marker located in exon 1 (position 21631) of the *PSMD13* gene (GenBank Accession No. AC136475) was analyzed by PCR and *Sac*I restriction analysis. A 465-bp fragment was amplified from genomic DNA (extracted from blood buffy coats) in 25 μl of reaction mixture containing 200 mM each dNTP, 0.5 μM both forward (5′-GACATCCCGGTTGTT-CTTCTG-3′, nt 21551, GenBank Accession No. AC136475,) and reverse (5′-CTACTCCTGAACCGTTTTAGT-3′, nt 22015, GenBank Accession No. AC136475,) primers, 1.5 mM $MgCl_2$, 1× polymerase buffer, 1 unit of EuroTaq DNA polymerase (EuroClone). The PCR consisted of 30 cycles, each cycle consisting of a denaturation step (95°C for 60 s), an annealing step (55°C for 60 s), and an extension step (72°C for 60 s). The first cycle was preceded by a denaturation step of 45 s at 95°C and the last one was followed by an extension step of 7 min at 72°C. A 20-μl amount of amplified DNA was digested by the restriction enzyme *Sac*I (5 U) for 3 h at 37°C as recommended by the manufacturer. The fragments were separated by electrophoresis on a 2.5% agarose gel and stained with ethidium bromide. The 465-bp PCR fragment contains two *Sac*I restriction sites, one of them is not polymorphic and produces two fragments of 341 and 124 bp. The *Sac*I polymorphic site is located within the 124-bp fragment. The presence of the G base in this site (GA*G*CTC) produces two fragments of 43 and 81 bp.

Haplotype analyses

Allele frequencies of single markers were computed by gene counting from the observed genotypes, and Fisher's exact test was applied to verify Hardy–Weinberg equilibrium [38]. Pairwise LD analyses were carried out on marker A21631G of *PSMD13* and markers G477T and $VNTR_{intron5}$ of *SIRT3* [19,20]. LD between pairs of markers was tested by a likelihood-ratio test. As for genotype data with unknown haplotype phase, an empirical distribution of haplotype frequencies obtained by a permutation procedure was utilized [39].

Population haplotype frequencies were estimated by maximum-likelihood estimation [38,40]. The null hypothesis of no difference between haplotype pools in the two age groups (18- to 90- and 91- to 108-year-old subjects) was checked by ad hoc exact test [41]. All the statistical analyses were carried out by means of Arlequin 2.0 software.

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4. Concluding Remarks and Perspectives

During my PhD appointment I was involved in the study of *SIRT3* gene regulation and on defining the molecular basis of the reported association between the variability of this gene and human longevity. Starting from the association study previously carried out on the *SIRT3* gene by Rose et al., 2003, we identified a VNTR that acts as a functional variant for this gene that could account for the association previously observed. In particular, we explored a possible role of this VNTR in human longevity using a case-control approach and then the functional activity of this VNTR by transient transfection experiments was carried out using reporter constructs containing the common *SIRT3*-VNTR alleles. The studies showed that:

1. This polymorphism, as well as the one identified by Rose et al., (2003), is associated with human longevity. In particular, we observed that the distributions of *SIRT3*-VNTR alleles differ for different age groups.
2. The different *SIRT3*-VNTR alleles were shown to be able to modulate the expression of a reporter gene in an allele-specific way according to the number of repeats and to the occurrence of the T/C variation that modifies a GATA2 transcription site in the DeltaEF1 site within the second repeat of the length-allele 2. The enhancer activity of DeltaEF1-allele2 (allele2b) resulted to be significantly lower than that of the GATA2-allele2 (allele2a). This observation suggests that the presence/absence of the GATA2 site is responsible for the different enhancer activities displayed by the two alleles. In addition, as allele2b (lacking of enhancer activity) is absent in males older than 90 years, this suggests that a link exists between the expression level of the gene and survival at old age.

Furthermore, bioinformatic analysis has shown that the GATA2 site partially overlaps with an AP-1 site. It is worth noting that the two sites have been found to be integral parts of enhancers associated with a number of eukaryotic genes (Wisdom et al., 1999: Bossis et al., 2005).

Therefore, further studies were carried out to investigate whether a GATA2 site and the overlapping AP-1 site are responsible for the different enhancer activities displayed by alleles 1 and 2 of the *SIRT3-VNTR*. For this purpose, we carried out a series of transient cotransfection experiments using reporter constructs containing the different alleles (1a, 1b, 2a, and 2b) and expression vectors encoding for GATA2, c-Jun, and c-Fos factors.

The results obtained provided the evidence that:

1. The $(T/C)_{63}$ polymorphism occurring in the second repeat of the *SIRT3*-VNTR allele 2 affects the expression of the reporter gene through the GATA2 site (Figs.2.2-2.5). This effect was confirmed by co-transfection experiments where pBabeGATA2 WT is replaced by pBabeGATA2 Del. In this last case the induction of the luciferase reporter gene is equal to the basal value.
2. The $(T/C)_{63}$ polymorphism modulates the *SIRT3*-VNTR enhancer activity only when two repeats are present (allele 2) (Figs.2.10-2.15). In fact, in co-transfection experiments carried out by using allele1a and allele1b we observed that the induction of the luciferase expression was independent of the SNP $(T/C)_{63}$ variability.
3. C-Jun and c-Fos factors, which recognize and bind the AP-1 site, affect the enhancer activity of alleles 2a and 2b in an allelic-specific way (Figs.2.6-2.7). Therefore, the induction of the luciferase expression observed is not only specifically due to the GATA2 factor alone.
4. In co-transfection experiments we observed that c-Jun and c-Fos factors increase the allele1a and allele1b enhancer activity. The luciferase reporter gene expression was independent of the SNP $(T/C)_{63}$ variability present in the repeat sequences.
5. GATA2 and AP-1complex factors show a synergistic effect on the enhancer activity of both allele 2a and 2b, but the synergistic effect is enhanced when allele2a is used (Figs.2.8-2.9).
6. The effect of synergistic cooperation is similar between allele 1a and 1b, although the occurrence of the GATA2 factor seems to inhibit the effect of c-Jun and c-Fos factors at least in the case of allele1a.

Moreover, we discovered the *SIRT3* 5’ flaking region encompasses the *PSMD13* gene which encodes for the p40.5 regulator subunit of the 26S proteasome, a multicatalytic complex whose function declines with aging. *SIRT3* is in head-to head organization with *PSMD13* gene, spaced by a 788 bp intergenic region. This unexpected configuration of *SIRT3* and *PSMD13* prompted us to investigate if this intergenic region could act as a bidirectional promoter, to identify a common regulation core in the promoter region and finally to investigate if the gene/longevity association observed for *SIRT3* also involved *PSMD13*.

The results obtained in the present research showed that:

1. Transfection experiments carried out by expression constructs containing the intergenic region (788 bp) cloned in both *PSMD13* and *SIRT3* directions confirmed that such a region acts as bi-directional promoter.
2. Transfection experiments carried out by deletion mutants of the *SIRT3-PSMD13* intergenic region showed that the Sp-1 transcriptional factors, discovered in these sequences, plays an important role in regulating gene expression in both the directions, although they act alone in the *SIRT3* orientation but in association with other transcription factors in the *PSMD13* orientation. Therefore the expression of these genes seems modulated by the concerted activity of co-activators and co-repressors.
3. Finally, by linkage disequilibrium analyses carried out between markers of *SIRT3* and *PSMD13* in a population sample including centenarians, we discovered that the chromosomal region encompassing the two genes is associated with the longevity phenotype.

On the whole, the results obtained provide evidence that the *SIRT3* and *PSMD13* genes have a coordinated although differential regulation and contribute to the same phenotype. With regards to the data reported in different studies and this result it seems that a common pathway of regulation between these genes is conserved through evolution and that also in higher eukaryotes a programmed and altruistic aging may occur which, for several theories, is believed to be out of natural selection.

Overall the results commented here clearly attest a deep contribution of genes to longevity. In particular, we observed to understanding the fine mechanisms that regulate gene expression help us to clarify the intriguing interplay that links expression levels of genes and survival at old age.

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