Dottorato in Oncologia e patologia sperimentale

MECCANISMI DI SENESCENZA CELLULARE: P21 E RISPOSTA AI DANNI AL DNA NELL'INVECCHIAMENTO E LONGEVITA'

MECHANISMS OF CELL SENESCENCE : P21 AND DNA DAMAGE RESPONSE IN AGING AND LONGEVITY

Silvia Gravina
Relatore Claudio Franceschi

SUMMARY 1

INTRODUCTION

Chapter 1: Human aging 2
1.1 Definition of aging 2
1.2 Main theories of aging 3
Chapter 2: Microarrays studies in aging 6
2.1 Trascriptional profiles as markers for human aging 6
2.2 Mitotic misregulation and human aging 7
2.3 DNA damage and human aging 7
2.4 Common pathways for aging in diverse tissues 9
2.5 Comparison of aging patterns across species 10
Chapter 3: the p21 gene 12
3.1 P21 roles and regulation 12
3.2 P21 trascriptional regulation 13
3.2.1 P53 dependent induction of p21 trascription 14
3.2.2 P53 indipendent induction of p21 trascription 14
3.3 p21 and aging 16

Chapter 4: Longevity 17
4.1 Lessons from centenarians 17
4.2 Genetics of longevity 18

AIMS OF THE WORK 20

Chapter 5: Materials and Methods
5.1 Cell Cultures
5.2 Samples 24
5.3 RNA extraction 24
5.4 cDNA synthesis 24
5.5 Real-time quantitative PCR 27
5.5.1 URT primers strategy 27
5.5.2 Rq PCR Protocol 28
5.6 MICROARRAYS
5.6.1 Microarrays: general characteristics 28
5.6.2 Software for microarrays analysis: genespring 28
5.7 P21-PCR-Sequencing 31
5.7.1 Sequencing: generalities 31
5.7.2 PCR sequencing on the p21 gene protocols 33
5.8 Constructs 35
5.8.1 pGL4 Reporter Vectors: Description 35
5.9 Cloning of the p21 promoter centenarian haplotype region 37
5.9. 1 HAPLO- p21 promoter PCR 39
5.9.2 Ligation 39
5.9.3 Bacterial transformation 42

5.10 Transfections and luciferase reporter gene assays
5.10. 1 Transient Transfection-Fugene 6 43
5.10.2 Preparation of Cell Lysate for Luciferase assay 44
5.11 Sequenom 44

CHAPTER 6: Haplotype Analysis 48

RESULTS

CHAPTER 7: Project #1 Results

7.1. Identification of polymorphic variants in the p21 gene and promoter regions 49
7.1.1 Sequencing of the 3 exons: identification of a polymorphic variant in the exon 2 region 49

7.1.2 P21 promoter sequencing: identification of 29 polimorphic variants 50

7.2 Comparison of the frequencies of the polymorphic variants: Centenarians versus General Population 57

7.3 Sequenom analysis on adjuntive DNA samples 60
7.4 Functional studies on the p21 promoter haplotype 61
7.4.1 Cloning of the promoter region containing the haplotype 61
7.4.2Luciferase assays carried out on cells HCT116 (p53 WT and p53 null) 62

CHAPTER 8: Project #2 Results 65

8.1.1 Microarrays analysis on changes in gene expression in aging: elaboration of a Centenarian-gene signature 65
8.1.2 List of the centenarian signature-genes 66

8.2 Correlation of age-specific difference in gene expression with transcriptional effects of p21 68
8.2.1 The centenarian signature in the p21 (Wi38) model 68
8.2.2 The p21-centenarian signature genes 69

8.3 DNA damage response of the p21-centenarian signature genes 70
8.3.1 in Young subjects 70
8.3.2 in old people subjects 71
8.3.3Utrophin : an age-dependent response to DNA damage 72
8.3.4 A brief overview of utrophin functions 73
8.4 Basal levels of the p21-centenarian signature genes 74
8.5 Microarrays analysis on additional set of young, elderly, old samples 75

DISCUSSION 76

GENERAL CONCLUSION 82

BIBLIOGRAPHY

SUMMARY

Theory of aging postulates that aging is a remodeling process where the body of survivors progressively adapts to internal and external damaging agents they are exposed to during several decades.
Thus , stress response and adaptation mechanisms play a fundamental role in the aging process where the capability of adaptating effects, certainly, also is related the lifespan of each individual.

A key gene linking aging to stress response is indeed p21, an induction of cyclin-dependent kinase inhibitor which triggers cell growth arrest associated with senescence and damage response and notably is involved in the up-regulation of multiple genes that have been associated with senescence or implicated in age-related .

This PhD thesis project that has been performed in collaboration with the Roninson Lab at Ordway Research Institute in Albany, NY had two main aims:
-the testing the hypothesis that p21 polymorphisms are involved in longevity
-Evaluating age-associated differences in gene expression and transcriptional response to p21 and DNA damage

In the first project, trough PCR-sequencing and Sequenom strategies, we we found out that there are about 30 polymorphic variants in the p21 gene.
In addition, we found an haplotpype located in -5kb region of the p21 promoter whose frequency is ~ 2 fold higher in centenarians than in the general population (Large-scale analysis of haplotype frequencies is currently in progress).

Functional studies I carried out on the promoter highilighted that the "centenarian" haplotype doesn't affect the basal p21 promoter activity or its response to p53. However, there are many other possible physiological conditions in which the centenarian allele of the p21 promoter may potentially show a different response (IL6, IFN,progesterone, vitamin E, Vitamin D etc).

In the second part, project #2, trough Microarrays we seeked to evaluate the differences in gene expression between centenarians, elderly, young in dermal fibroblast cultures and their response to p21 and DNA damage.
Microarray analysis of gene expression in dermal fibroblast cultures of individuals of different ages yielded a tentative "centenarian signature".
A subset of genes that were up- or downregulated in centenarians showed the same response to ectopic expression of p21, yielding a putative "p21-centenarian" signature.

Trough RQ-PCR (as well Microarrays studies whose analysis is in progress) we tested the DNA damage response of the p21-centenarian signature genes showing a correlation stress/aging in additional sets of young and old samples treated with p21-inducing drug doxorubicin thus finding for a subset of of them , a response to stress age-related.

CHAPTER 1:

1.1 Definition of Aging

Aging is a complex process involving the additive effects of many genetic pathways (Kirkwood and Austand, 2000). It can be also defined as a progressive functional decline, , a gradual deterioration of physiological function, including a decrease in fecundity (Partridge and Mangel, 1999), or the intrinsic, inevitable, and irreversible process of loss of viability and increase in vulnerability (Comfort, 1964).

Clearly, human aging is associated with a wide range of physiological changes that not only make us more susceptible to death but that limit our normal functions and render us more susceptible to a variety of diseases.

Over the years, many theories have emerged to explain what changes lead to aging (reviewed in Medvedev, 1990): different theories of aging are not mutually exclusive and may adequately describe some or all features of the normal aging process alone or in combination with other theories.

Theories formultated to explain aging processes have been grouped into several categories some of the most widely used being the programmed and error theories of aging (reviewed in Weinert and Timiras, 2003). The table belove summarize the main characteristics of the major theories on aging.(from Winert and Timiras, 2003)

Biological Level/Theory	Description
Evolutionary	
Mutation accumulation*	Mutations that affect health at older ages are not selected against.
Disposable soma*	Somatic cells are maintained only to ensure continued reproductive success; after reproduction, soma becomes disposable.
Antagonistic pleiotropy*	Genes beneficial at younger age become deleterious at older ages.
Molecular	
Gene regulation*	Aging is caused by changes in the expression of genes regulating both development and aging.
Codon restriction	Fidelity/accuracy of mRNA translation is impaired due to inability to decode codons in mRNA.
Error catastrophe	Decline in fidelity of gene expression with aging results in increased fraction of abnormal proteins.
Somatic mutation	Molecular damage accumulates, primarily to DNA/genetic material.
Dysdifferentiation	Gradual accumulation of random molecular damage impairs regulation of gene expression.
Cellular	
Cellular senescence-Telomere theory*	Phenotypes of aging are caused by an increase in frequency of senescent cells. Senescence may result from telomere loss (replicative senescence) or cell stress (cellular senescence).
Free radical*	Oxidative metabolism produces highly reactive free radicals that subsequently damage lipids, protein and DNA.
Wear-and-tear	Accumulation of normal injury.
Apoptosis	Programmed cell death from genetic events or genome crisis.
System	
Neuroendocrine*	Alterations in neuroendocrine control of homeostasis results in aging-related physiological changes.
Immunologic*	Decline of immune function with aging results in decreased incidence of infectious diseases but increased incidence of autoimmunity.
Rate-of-living	Assumes a fixed amount of metabolic potential for every living organism (live fast, die young).

Table n.1.1
table n.1 main characteristics of major theories on aging (from Winert and Timiras , 2003)

1.2 Brief overview of main theories og aging

According to the ***"programmed" thoeries***, aging depends on biological clocks regulating the timetable of the life span trough the stages of growth, development, maturity, and old age.: this regulation would depend on genes sequentially switching on and off signals to the nervous , endocrine, and immune systems responsible for maintenance of homeostasis.
The***"error" theories*** identify enviromental insults to living organisms that iduce progressive damage at carious levels (e.g. mitochondrial DNA damage, oxygen radicals accumulation, cross-linking).

Evolutionary theories argue that aging results from a decline in the force of natural selection. Because evolution acts primarily to maximize reporductive fitness in an individual, longevity is a trait to be selected only if it beneficial for fitness.
Among the evolutionary theories it has to be highlighted the Disposable Soma and the theory of antagonistic pleiotropy.
The basic idea of the first one argues that somatic organism is effectly mainained only for reproductive success: afterward it is disposable. Inherent in this theory is the idea that somatic maintenance, in other word, longevity has a cost : the balance of resouces invested in longeviti vs reproductive fitness determines the life span.

The Antagonistic pleiotropy theory suggests that some genes may be selected for beneficial effects early in life and yet have unselected deleteroius effects with age, thereby contributing directly to the aging phenotype.

The ***Molecular theories*** argue that the gene regulation theory of aging proposes that aging results from changes in gene expression (Kanungo MS, 1975). Life span is influenced by the selection of genes that promotes longevity: recently DNA microarrays have been used to assay genome-wode trascriptional changes qith age in seveal models. In the section belove, Microarrays and aging, I will discuss it more into detail.

The main ***cellular theories*** are the cellular/senescence one and the free radical.
The first one was formulated when cell senescence was described as the process that limits the number of cell divisons in normal human cells can undergo in culture (Hayflick L, 1965). This imit in replicative capacity occurs afer a characteristicc number of cell divisions and results in terminally arrested cells with altered physiology. (Campisi J., 2003).
Cellular senescence can also occur in response to distict molecular events such as in response to a variety of stressors, including but not limited to 1) DNA damage2) modications in heterochromatin structure and 3)strong mitogenic signals resulting from oncogene expression (Campisi J., 2003)
Cell expressing stress induced markers found in senescent cells accumulate with age in many tissues (Dimri GP, 1995 ; Krtolica A, 2002). Althoug it remains uncelar whether this indicates the presence of senescent cells in vivo. Several studies suggest that atherosclerosis results from senescent changes in arterial endothelial cells (Chang E et al., 1995 ; Fenton M, et al., 2001; Krtolica A and Campisi J., 2001).
The altered physiology of senescent cells might contribute to aging and cancer through secondary effects on neighboring cells in tissues (Krtolica A and Campisi J., 2001).

P21, as I will discuss in the next session, is a CDK inhibitor and stress responsive gene which plays and important role in the senescent phenotype.

The free theory of aging is one of the best known theories and remains controversial to this day. All organisms live in an environment that contains free radical-containing reactive oxygen species (ROS): mitochondrial respiration, the basis of energy production, generates ROS by leaking intermediate from the electron transport chain (Finkel T and Holbrook NJ., 2000).

This theory supposes that free radical reactivity results in cumulative damage and senescence.

In fact, elevated levels of both oxidant-damaged DNA and protein are found in aged organisms (Beckman KB and Ames BN, 1998; Shringarpure R and Davies KJ, 2002). An age dependent reduction in the ability to degrade oxidatively modified proteins may contribute to the build-up of damage, dysfunctional molecules in the cells (Shringarpure R and Davies KJ, 2002).

Also mutation in mitochondrial DNA accelerates free radical damage by introducing altered enzyme components into the electron transport chain. Faulty electron transport results in elevated free radical leakage and ultimately more mitochondrial DNA mutation and exacerbated oxidant production. This cycle of mutations and oxidant production eventually leads to cellular catastrophe, organ failure, and senescence (Mandavilli BS et al., 2002).

System based theories of aging" neuroendocrine and immune theories

This theories argues that aging process is related to the decline of the organ system essential for 1)central and maintenance of the other systems within an organism and 2)the ability of organisms to communicate and adapt to the environment in which they live.

The nervous, endocrine and immune system play a key role in their interactive and defensive responsiveness to eternal and internal stimuli.

For example the Neuroendocrine theory proposes that aging is due to changes in neural and endocrine functions that are crucial for 1)coordinating communication and responsiveness of all body systems with the external enviroment 2) programming physiological responses to enviromental stimuli and 3) maintaining an optimal functional state for reproduction and survival while responding to enviromental demands.

Inflammaging

The term "Inflammaging" has been coined by Claudio Franceschi to explain the now widely accepted phenomenon that ageing is accompanied by a low-grade chronic, systemic up-regulation of the inflammatory response and that the underlining inflammatory changes are also common to most age-associated diseases (Franceschi C, et al., 2000; Vasto S et al., 2006)

The inflammatory scenario that characterizes inflammaging constitutes a highly complex response to various subtle internal and environmental inflammatory stimuli mediated mainly by the increased circulating levels of pro-inflammatory cytokines. Inflammaging also generates Reactive Oxygen Species (ROS) causing both oxidative damage and eliciting an amplification of the cytokines' release, thus perpetuating a vicious cycle resulting in a chronic systemic pro-inflammatory state where tissue injury and healing mechanisms proceed simultaneously and damage slowly accumulates asymptomatically over decades and is a major determinant both of the ageing process and of the development of age-associated diseases (De Martinis M, et al., 2005; Franceschi C et al., 1995)

Moreover, Claudio Franceschi, Tom Kirkwood, and Calogero Caruso as well as other authors, postulate that both the ageing process and age-associated diseases are late consequences of evolutionary programming for a pro-inflammatory response mainly selected to resist infections and for a successful response to wound healing in early age, a view that has been discussed in the light of the antagonistic pleiotropy theory as explained above (Van Den Biggelaar AHJ, et al., 2004; Vasto S,et al., 2006)

CHAPTER 2: Microarrays studies in aging

To embrace the complexity of aging, an attractive approach is to use DNA microarrays to scan the entire genome for genes that change expression as a function of age or under conditions when longevity is extended.
Expression data from DNA microarrays are quantitative, and thus it is possible to compare age related transcriptional profiles between different tissues, between different conditions that affect longevity and even between diverse species.
Combining DNA microarrays results on aging from multiple labs, will be a powerful Insight into the complex mechanism of aging and longevity.

Many DNA microarrays experiments have analyzed aging in various human tissues. (reviewed by Kim, 2007)
Table belove contains references that profile gene expression changes in eight human tissues and two human progerias.

Human	
Hutchison-Guilford	
Progeria syndrome	**Cao et al. (2001)**
Werner's syndrome	**Ly et al. (2000); Kyng et al. (2003)**
Brain	**Blalock et al. (2004); Lu et al. (2004); Ricciarelli et al.**
(2004);	
	Fraser et al. (2005); Liang et al. (2006)
fibroblasts	**Kim et al. (2003)**
	Rodwell et al. (2004); Melk et al. (2005)
kidney	
	Welle et al. (2001, 2003, 2004); Giresi et al. (2005); Zahn
muscle	
et al. (2006)	
	Thomas et al. (2002)
liver	
	Yoshida et al. (2002); Hawse et al. (2004); Segev et al. (2005)
eye	
	Lener et al. (2006)
skin	
	Visala Rao et al. (2003); Tan et al. (2005)
blood	

Table 2: references for microarrays studies on aging

2.1 Trascriptional profiles as markers for human aging

Rodwell et al.m (2004) in their analysis of kidney aging showed that age-regulated markers could predict physiological age.
They used Affymetrix genechips to measure levels of gene expression in kidney samples from 72 patients between 27 and 92 years old age, and they identified a set of

447 age regulated genes. Next, they tested whether the over-all level of expression of the 447 genes was a signature of physiological age for different individuals.
The relative amount of physiological aging for each of the patients used in the study was measured by examining the morphological and histological appearance of the kidney. A remarkable similarity was found between the transcriptional aging profiles and the physiological states of the kidney in different individuals.
Some elderly persons displayed an aging signature similar to that of people who were 30-40 years younger, and these individuals also had relatively healthy kidneys. Others displayed an aging signature similar to people who were 10-20 years older, and these patients showed poor kidney health.

A similar experiment was performed by Zahn et al., to analyze aging of human muscle. They identified a set of 250 age-regulated genes in skeletal muscle from 81 patients ranging from 16 to 89 years of age. As muscle ages, there is a shift in the relative numbers of type II (slow twitch) to type I(fast twitch) muscle fibers , and the type II/type I muscle fiber ratio can be used as a rough estimate of the physiological age of the muscle. Similar to the aging expression profile for the kidney, the overall expression behavior of this set of age-regulated muscle genes correlated with the physiological as well chronological age of the muscle sample (Zahn et al. 2006).
In summary these results indicate that the overall expression behavior of the age regulated kidney and muscle genes is a biomarker that depicts the physiological age in human kidney or muscle.

2.2 Mitotic misregulation and human aging

Ly et al., studied gene regulation of normal and premature aging in actively dividing cells. Messanger RNA levels were measured in actively dividing fibroblasts isolated from young, middle-age, and old-age humans and humans with progeria. The data obtained suggest that an underlying mechanism of aging process involves increasing errors in the mitotic machinery of dividing cells in the post reproductive stage of life. Ly et al., proposes that this dysfunction leads to chromosomal pathologies that result in misregulation of genes involved in aging process.

Summarizing their Microarrays data revealed a set of gene that correlate with, and hence likely contribute to, age related phenotypes and diseases and suggest that mitotic errors in dividing cells may lead to the altered expression of these collection genes.

2.3 DNA damage and human aging

A long –held view is that accumulation of somatic mutations plays a a key role in decreasing cellular function in old age.
Recent genomic studies by Lu et al. (2004) have provided intriguing new evidence for the role of DNA damage in aging of the human brain.

In their study, they used post-mortem samples of the frontal polo of 30 individuals ranging in age from 26 to 106 and found about 440 genes that change expression with age.
They then measured the rate of aging by examining changes in gene expression at different points during life. Interestingly they found that most of the age-related changes occurred in people between 43 and 73 years old. Gene expression patterns in the brain were more consistent in the young and the old, but showed large variability in people were middle-aged.

Next Lu. Et al. asked whether DNA damage may have a role in age related changes in gene expression. They devised a PCR-based assay to measure levels of damaged DNA in brain samples from old and young patients. They isolated genomic DNA from brain samples ad then treated it with formamidopyrimidine-DNA glycosylase (FPG), which is an N-glycosidase and AP-lyase that selectively releases damaged bases from DNA.
Damaged DNA would be selectively cleaved by FPG and would show lower levels of amplification in a PCR assay. They examined the promoters of 30 different genes and they observed that DNA damage targeted the promoter of genes that are repressed with age but did not affected or that increase expression with age.
Lu. Et al. suggested that selective DNA damage gene promoter sequences is a potential mechanism whereby <u>the expression of specific genes could register the passage of time.</u>

These findings also suggest that accelerated DNA damage may contribute to reduced gene expression in the human brain after age 40.
There may be sites in the genome that are exceptionally prone to DNA damage, based on chromatin accessibility and GC content. These sites would be expected to include promoter regions which have high –GC content and are not protected by transcription –coupled repair. Genes that are located near hot spots for DNA damage may show decreased expression levels in old age due to high levels of stochastic DNA damage.

2.4 Common pathways for aging in diverse tissues

Some aspects of aging only affect specific tissues; examples include progressive weakness of muscle, declining synaptic function in the brain, and decreased filtration rate in the kidney. Other aspects of aging occur in all cells regardless of their tissue type, such as the accumulation of oxidative damage, DNA damage, and telomere shortening.

Genome-wide searches for gene expression changes during aging would include both types of expression changes, and it would be interesting to discern which expression changes are tissue-specific and which are common to all tissues.

Genes that are age-regulated in all tissues would reveal genes involved in core mechanisms that underlie cellular aging.

Several papers have found similarity in the transcriptional aging profiles of different parts of the same human tissue. Rodwell et al. (2004) found that the transcriptional profiles for aging in the cortex and medulla of the kidney were very similar. Likewise, Fraser et al. (2005) found that age-related gene expression changes were similar between five different regions of the human cerebral cortex. However, they did not find any evidence for similarity between age-related expression changes in the cerebral cortex and in the cerebellum.

Recently, Zahn et al. (2006) have discovered genetic pathways that show common age regulation in kidney, brain, and muscle. They used Affymetrix GeneChips to analyze expression in 81 skeletal muscle samples from patients aged 16 to 86 years and found 250 age-regulated muscle genes. Next, they compared their muscle-aging results to previously published data on kidney and brain aging.

Although most of the age-related changes were tissue specific, they found evidence for common age regulation of five genetic pathways in all three tissues. Specifically, there is an overall increase in expression of the extracellular matrix genes, the cell growth genes, and complement activation genes in all three tissues.

Conversely, there is an overall decrease in expression of chloride transport genes and electron transport genes in all three tissues.

2.5 Comparison of aging patterns across species

DNA microarray experiments have defined molecular profiles for aging for worms, flies, mice, rats, monkeys, and humans. An important question to answer is which parts of the aging transcriptional profile are shared between species (public) and which are specific for only one species (private). On the one hand, many biological processes involved in aging are shared across diverse species.

For example, oxidative stress and DNA damage have been postulated to play a role in aging in nearly all animals. Caloric restriction or mutations in genes such as the insulin-like receptor gene (*daf-2*) or *sir2* affect longevity in diverse species from yeast to mice. These common themes for aging suggest that there might also be genes that show common patterns of age regulation in different species.

On the other hand, evolutionary theory postulates that there should be little or no overlap in the particular degeneration pathway of one species with that of distantly related species. Unlike most other biological processes, there is strong reason to believe that the aging process is not evolutionarily conserved per se (Kirkwood and Austad 2000). As a rule, wild animals do not live long enough to grow old. For example, more than 90% of mice die in their first year in the wild, and thus, only a very small fraction of wild mice achieve their maximal life span of 3 years (Kirkwood and Austad 2000). The median human life span in the United States was 47 years in 1900 (Statistics 2006), and relatively few individuals lived to maximal life span during human evolution (Martin 2002). In the wild, life span is limited by disease and predation, not by aging Events that occur late in life escape the force of natural selection, and the aging process is unlikely to be evolutionarily conserved per se.
Thus, there is little reason to believe that genetic and molecular events which occur late in life in model organisms such as the mouse would be conserved in humans.

Early work by McCarroll et al. (2004) characterized gene expression profiles for normal aging in flies and worms.
These investigators reported several biological processes that seemed to share a similar overall pattern of change with respect to age in flies and worms. However, the shared similarity between flies and worms is probably not related to aging, as most of the expression changes occur in young adults rather than old animals. Furthermore, the analysis used in the paper was flawed becauseit greatly overestimated the statistical significance of the results (Melov and Hubbard 2004). Nevertheless, this early paper contained a number of insightful ideas about ways to analyze large-scale gene expression data and to compare emergent properties from transcriptional profiles of aging across different species.
Zahn et al. (2006) compared transcriptional profiles for aging in human to aging profiles for mice, flies, and worms. They asked whether any of the biological processes that showed age-dependent changes in expression in human also showed similar age regulation in mice, flies, or worms.
Of the five genetic pathways that were age-regulated in human, one (the electron transport chain genes) showed similar age regulation in the other three species (Zahn et

al. 2006) Compared to humans, mice age 20 to 30 times faster, flies age 400 times faster, and worms age 2000 times faster.

Given this large difference in life span, it is interesting that there is a similar twofold decrease in overall expression of the electron transport chain in each species. Expression of the electron transport chain may be a particularly interesting biomarker for aging because it scales with life span in species that are distantly related.

CHAPTER 3:THE p21 GENE

3.1 P21 roles and regulation

p21$^{WAF1/CIP1}$ is a cyclin-dependent kinase (cdk) inhibitor, and is a key mediator of p53-dependent cell cycle arrest after DNA damage (Gartel at al., 1996; Gartel at al., 1998). Induction of the cyclin-dependent kinase (CDK) inhibitor p21$^{Waf1/Cip1/Sdi1}$ is a common mechanism of growth arrest in different physiological situations.
p21 is transiently induced in the course of replicative senescence, reversible and irreversible forms of damage-induced growth arrest, and terminal differentiation of postmitotic cells; as discussed above, its induction is regulated through p53-dependent and -independent mechanisms (Gartel at al., 1996).
The N-ammino domain is necessary to inhibit cylin/cdk activity while the , the C-terminal portion of p21, associates with the PCNA and can inhibit DNA replication directly or inhibits apoptosis through binding procaspase 3 and blocking its processing and activation , thus protecting cells against *fas* induced apoptosis.
p21 was also reported to bind c-Jun amino-terminal kinases, apoptosis signal-regulating kinase 1 and Gadd45 (Gartel at al., 1996, Gartel 2002.).

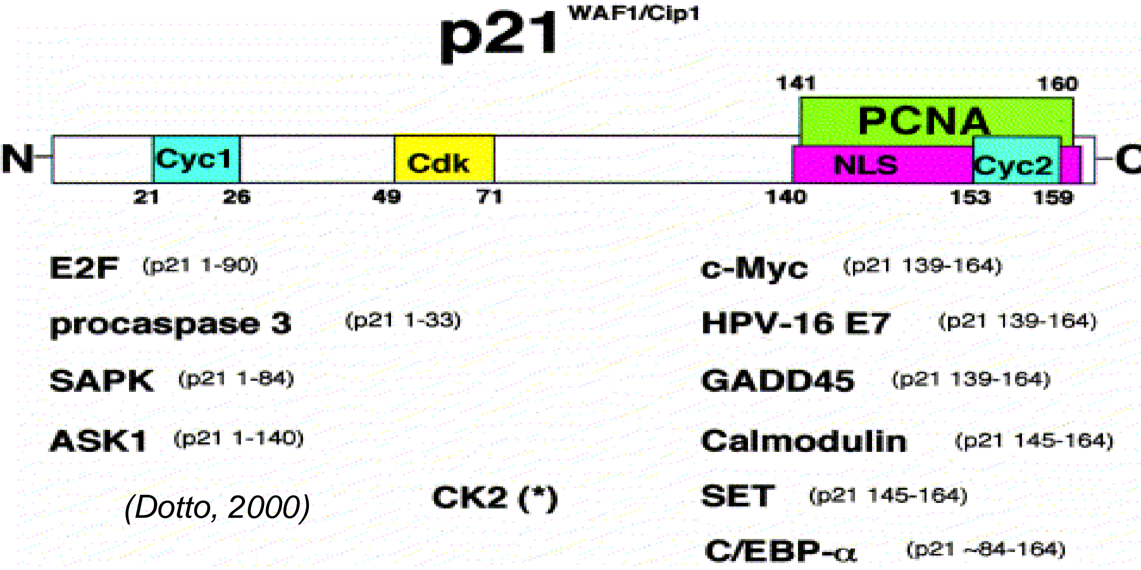


Figure 1: p21 gene rapresentation

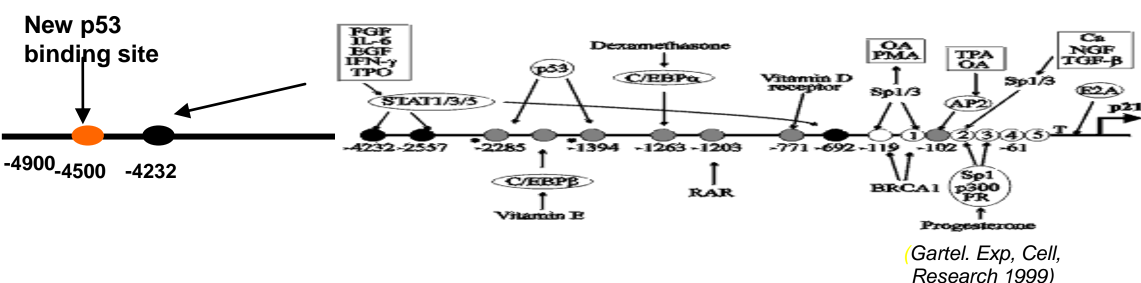
Although p21 is not a transcription factor, it is conceivable that some of its functions may be mediated by indirect effects of p21 on cellular gene expression.

3.2 P21 trascriptional regulation

P21 is a target for diverse signals that induce growth arrest and differentiation. Its expression is usually controlled at the transcriptional level by both p53-dependent and independent mechanisms. (reviewed in Gartel and Tyner, 1999)

3.2.1 P53 dependent induction of p21 trascription: p53 tumor suppressor protein is required for the transcription of a number of genes involved in growth control.
While p53 is not required for induction of p21 trascription during development and in most tissues of the adult mouse, gamma irradiation of p53 deficient and normal mice suggested that p53-dependent regulation of p21 is critical for the response to DNA damage (Macleod at al.,genes dev, 1995).

Until last year due to the fact that most analyses of the p21 promoter were restricted to the first 3 kb, it has been thought that the p21 promoter contained only two p53 responsive elements at approximate positions -1400 and -2300.
Only recently Saramaki et al., identified a novel p53 sites at position -4500 thus suggesting that the p21 promoter were bigger than believed so far.
Their results indicated a 10-fold lower binding affinity of this novel site, compared to the site at position -2300. In contrast this novel p53 binding region demonstrated a significant induction after 5-fluoracil treatment.
Since p21 promoter contains high and low affinity sites, it may support a hypothesis that affinity dictates the choice between cell cycle arrest and apoptosis, with high affinity sites regulating cell cycle arrest and low affinity pro-apoptotic effects (Vousden et al., 2002)



(Gartel. Exp, Cell, Research 1999)

Figure 2: p21 promoter and cis acting binding sites

3.2.2 p53 independent regulation of p21 gene expression

A variety of agents that promote differentiation activate p21 trascription by p53 independent mechanisms. These agents induce binding of different transcription factors to specific cis-aciting elements located with the p21 promoter.
The region between -119 bp and start of transcription of the human p21 gene contains six Sp1 binding sites and plays a major role in the regulation of p21 trascription. The tumor suppressor protein BRCA1 transactivates p21 in p53 independent fashion via the region from -143 to -93 bp which contains the Sp1-1 and Sp1-2 sites. The sp1-3 site in the p21 promoter has been shown to be required for p21 induction by transforming growth factor –B , Ca, butyrate , lovastatin, the histone deacetylase inhibitor, trichostatin A (TSA) and NGF (reviewed in Gartel and Tyner, 1999)
TGF and butyrate inhibit proliferation and induce G1 cell cycle arrest in various cell types, whereas Calcium induces differentiation of cultured keratinocytes.
The transcriptional coactivators p300 and CBP possess histon acetyl transferase activity and interact with other transcription factors to regulate gene expression.

p300/CBP can cooperate with sp1 and/or sp3 to induce expression from the p21 promoter. Addition of nerve growth factor to PC12 cells induces neuronal differentiation and p21 expression.

A variety of other transcription factors such as AP2, E2Fs, STATs, C/EBP alfa and beta and the homeobox transcription factor gax can induce p21 trascription n response to different signals. (see table n.3) E2F1 and E2F3 but not E2F4 , strongly transactivate p21 transcription through cis-acting elements located between nucleotides -119 to +16 of the p21 promoter.
In addition EGF and IFN induced growth arrest and differentiation in a variety of cell lines by increasing levels of p21mRNA but the mechanisms responsible for this induction have not been established.
The hematopoietic growth factor thrombopoietin induces megakaryocytic differentiation and trascription activation of p21 by STAT5 trough two STAT binding sites.

Il6 induced differentiation of an osteoblast-like human osteosarcoma cell line and can stimulate activity of a 2.4 kb segment of the p21 promoter.

The hormonal vitamin D activates the p21 and leads to the expression of terminal differentiation markers in the myelomonocytic U937 cell line.
A functional vitamin D3 responsive element has been identified at -771 bp of the p21 promoter.
P21 is also transcriptionally activated by retinoic acid in U937 cells and a functional RA responsive element was localized between -1212 and -1194 in the p21 promoter.

The table n.3 belove summarize the main p53-indipendent activators of p21 transcription.

p53-Independent Activators of p21 Transcription

Signal	Transcription factors	*cis*-acting elements
Butyrate	Sp1, Sp3	−82/−77 bp; −69/−64 bp
Lovastatin	Sp1, Sp3	−82/−77 bp
Trichostatin A	Sp1, Sp3	−82/−77 bp; −69/−64 bp
TGF-β	Sp1, Smad3/4, Smad2/4, p300	−82/−77 bp
NGF	Sp1, Sp3, p300	−82/−77 bp
Calcium (keratinocytes)	Sp3	−82/−77 bp
Progesterone	Sp1, p300	−82/−77 bp; −69/−64 bp
Okadaic acid	Sp1, Sp3 (?)	−119/−114 bp; −109/−104bp; −82/−77 bp; −69/−64 bp
	Ap2	−102/−94 bp
TPA	Ap2	−102/−94 bp
PMA	Sp1, Sp3 (?)	−119/−114 bp; −109/−104 bp
—	BRCA1	−143/−93 bp
—	E2A (E47)	−162/−157 bp; −20/−15 bp; −5/+1 l
Vitamin D3	VDR	−779/−765 bp
Retinoic acid	RAR	−1212/−1194 bp
Dexamethasone	C/EBPα	−1270/−1256 bp
Vitamin E	C/EBPβ	−1928/−1920 bp
EGF	STAT1, STAT3	−4236/−4228 bp; −2561/−2553 bp; −696/−688 bp
IFN-γ	STAT1	−4236/−4228 bp; −2561/−2553 bp; −696/−688 bp
TPO	STAT5	−4236/−4228 bp; −2561/−2553 bp
IL6	STAT3	−696/−688 bp

Table n.3 p53-indipendent activators of p21 transcription.(Gartel et al., 1999)

In conclusion a variety of very different extracellular signals can regulate the p21 promoter, often trough the same transcription factors and cis-acting elements. At the same time transmission of a specific extracellular signal may be delivered by a diverse set of transcription factors in different cellular environments.
The p21 promoter appears to represent a unique sensor that is able to integrate many extracellular stress signals into cellular decisions that lead to proliferation or cell cycle arrest.(Gartel et al., 1999)

3.3 p21 and aging

p21 is an intriguing gene integrating stress response mechanisms to aging
Some evidences suggest that the effects of p21 induction on gene expression in senescent cells may contribute to the pathogenesis of cancer and age-related diseases. Also p21 up-regulates multiple genes that have been associated not only with senescence but also implicated in age-related diseases, including atherosclerosis, Alzheimer's disease, amyloidosis, and arthritis thus suggesting that p21 may play a causal role or can be linked to different age-related diseases.(Chang at al., 2001)

Particularly Chang et al., showed that p21-induced APP gives rise to β-amyloid peptide, the main component of Alzheimer's amyloid plaques. p21 strongly induces the inflammatory protein SAA, deposition of which causes amyloidosis and contributes to atherosclerosis, osteoarthritis, and rheumatoid arthritis (Jensen, L. E. et al1998). p21 also induces t-TGase, which has been described as a pleiotropic mediator of cell differentiation, carcinogenesis, apoptosis, and aging and which plays a role in plaque formation in both Alzheimer's disease and amyloidosis (Dudek, S. M. et al., (1994) Park S. et al., 1999)

p21-induced CTGF and galectin-3 have been implicated in atherosclerosis (Oemar, B. et al., 1997 ; Nachtigal, M et al., 1998), whereas p21-up-regulated complement C3 and AMP deaminase were suggested to play a role in Alzheimer's disease (Sims, B., et al., 1998; Howie, A. J., et al., 1985).

In addition, expression of p21-induced proteins cathepsin B, PAI-1, fibronectin, *N*-acetylgalactosamine-6-sulfate sulfatase, and Mac2-BP has been associated with osteoarthritis and/or rheumatoid arthritis (Cerinic, M. M., et al., 1998; Seki, T., et al., 1998)

The elucidation of the mechanisms and the specificity of the effects of p21 on gene expression may suggest new approaches to the prevention of different diseases that are associated with human aging.

CHAPTER 4 : longevity

4.1 Lessons from centenarians

Centenarians can be considered as a model to address the biological basis of aging and longevity.
One of the main characteristic of centenarians is having a history of aging very slowly and either markedly delayed or entirely escaped diseases normally associated with aging such as Alzheimer's disease, cancer, stroke and heart disease

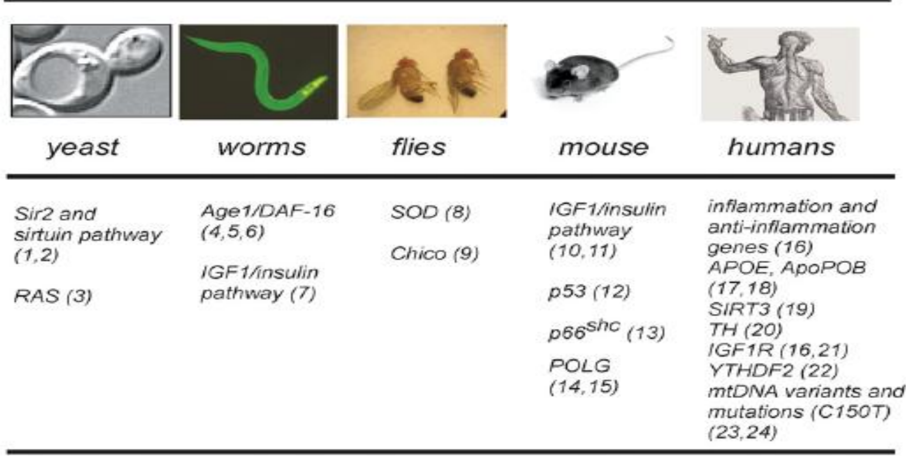
- . survivors": diagnosis of age-related disease before age 80, survived it. 24% of males, 43% of females.
- "delayers": diagnosis of age-related disease after age 80.
    - 44% of males, 42% of females.
- "escapers": live to 100 without any age-related disease.
    - 32% of males, 15% of females.

(Evert et al., 2003)
Indeed, despite the increasing numbers of very old people, centenarians are very unique in the population infact they are still few from a demographic point of view; in economically developed countries their number approx. 1 in 5000 to 10 000 of the population.
However, at the same time, centenarians are by definition extremely old people, and show all the signs and the characteristics of a prolonged aging process.
All the phenotypic characteristics of nonagenarians and centenarians fit the hypothesis that aging is a remodeling process where the body of survivors progressively adapts to internal and external damaging agents they are exposed to during several decades (Capri et al., 2006)

As proposed by Franceschi et al., inflammaging which caracherize aging is explained by an imbalance between inflammatory and anti-inflammatory networks, which results in the low grade chronic pro-inflammatory status.
Within this perspective, healthy aging and longevity are likely the result not only of a lower propensity to mount inflammatory responses but also of efficient anti-inflammatory networks, which in normal aging fail to fully neutralize the inflammatory processes consequent to the lifelong antigenic burden and exposure to damaging agents. Such a global imbalance can be a major driving force for frailty and common age-related pathologies.

4.2 Genetics of longevity

How much of human longevity is due to genes? Many epidemiological data indicate the presence of a stong familiar component of longevity that is largely determined by genetics , and a number of possible associations between longevity and allelic variants of genes have been described. Estimates of the heritability of human lifespan vary from 10-50% with the most common finding being that about a third of human lifespan may be heritable. Phenotypes that suggest slower aging, such as survival to 90+ years, may have an even stronger genetic basis, which explains why centenarians and near-centenarians tend to cluster in families.
In the table n.4 belove a brief summary of the major contributions to understand the genetics of longevity. The genes and genetic pathways which gave the most important insights on longevity are shown for each of the most used animals models and humans. Some of those Longevity- genes are inflammation and anti-inflammation genes (Franceschi at al., 2005), APOE, ApoPOB (Smith, 2000, De Benedictis at al., 1997), Other genes involve other pathways such as the Sirtuin and the insulin one that has been also linked to longevity in Yeast and worms. (Rose at al., 2003), IGF1R (Franceschi at al. 2005), Kim at al., 1999, Lin at al., 2001.Also studies on Mithocondrial DNA has suggested that mtDNA variants have an impact on the survival in the early stages of life as well in attaining extreme longevity. (De Benedictics at al., 1999, Zhang at al., 2003).



yeast	worms	flies	mouse	humans
Sir2 and sirtuin pathway (1,2) RAS (3)	Age1/DAF-16 (4,5,6) IGF1/insulin pathway (7)	SOD (8) Chico (9)	IGF1/insulin pathway (10,11) p53 (12) p66^{shc} (13) POLG (14,15)	inflammation and anti-inflammation genes (16) APOE, ApoPOB (17,18) SIRT3 (19) TH (20) IGF1R (16,21) YTHDF2 (22) mtDNA variants and mutations (C150T) (23,24)

(1) Kim et al., 1999; (2) Tissenbaum and Guarente, 2001; (3) Chen et al., 1990;
(4) Vanfleteren and De Vreese, 1995; (5) Lin et al., 1997; (6) Ogg et al., 1997;
(7) Lin et al., 2001; (8) Orr and Sohal, 1993; (9) Clancy et al., 2001; (10) Brown-Borg et al., 1996;
(11) Barbieri et al., 2003b; (12) Tyner et al., 2002; (13) Migliaccio et al., 1999; (14) Trifunovic et al., 2004;
(15) Kujoth et al., 2005; (16) Franceschi et al., 2005; (17) Smith 2000; (18) De Benedictis et al., 1997;
(19) Rose et al., 2003; (20) De Benedictis et al., 1998; (21) Bonafe et al., 2003;
(22) Cardelli et al., 2006; (23) De Benedictis et al., 1999; (24) Zhang et al., 2003.

The tablen.4 highlight the Major contribution to understand the genetics of longevity (from Franceschi et al.,)

In conclusion, centenarians are an ideal group of people for the study of specific genes that are either directly associated with longevity or enable people to escape or delay the effects of age-related diseases.

Identifying factors that help us remain healthy, vigorous and disability-free at older ages is one of our major research priorities. Identifying genes (and variations of those genes) that impact human aging and longevity is a promising novel strategy.
If such genes and their genetic pathways can be identified then novel therapies might be created that affect the biology of these pathways.

AIMS OF THE WORK

Project #1

Aim : to identify polymorphic variants in the p21 gene and their possible involvement in longevity

Induction of cyclin-dependent kinase inhibitor p21(Waf1/Cip1/Sdi1) triggers cell growth arrest associated with senescence and damage response.
It has been proved that p21 also up-regulates multiple genes that have been associated with senescence or implicated in age-related diseases, including atherosclerosis, Alzheimer's disease, amyloidosis, and arthritis. For example, when the gene APP is activated by p21, it produces the beta-amyloid peptide, which forms plaques in the brain that are a hallmark of Alzheimer's disease.(Chang et al., 2000)
The p21 gene is also linked to the activation of the inflammatory protein SAA, which is involved in the development of hardening of the arteries and arthritis according to the report published by Chang et al., in the April 11, 2000 issue of the Proceedings of the National Academy of Sciences.

The main goal of this study consists in the identification of polymorphisms in the p21 gene that may affect the induction of the p21 regulated genes involved in age-related disease and eventually implicated in longevity and healthy aging.
Infact centenarians not only exhibit extreme longevity but also have delayed, survived or escaped diseases associated with the aging process (such as cardiovascular disease, Alzheimer's disease, and some types of cancer). These characteristics make centenarians an ideal group of people for the study of specific genes that are either directly associated with longevity or enable people to escape or delay the effects of age-related diseases.

Discovering polymorphisms in the p21 gene that could impart the ability to live to old age while compressing the period of disability toward the end of life yields important insight into how the aging process increases susceptibility to diseases associated with aging and how this susceptibility might be modulated.

Strategy-Project #1

The study has been structured in 3 main steps:

-Sequence p21 gene (3 exons) and p21 promoter (5 kb) from 10 (european) centenarians; identify polymorphisms and their frequencies (Pilot study)

-Compare the frequencies of the polymorphisms identified in the centenarians with the known frequencies of the general population reported in NCBI

-Use Sequenom to determine the frequency of polymorphisms in ~100 individuals in the general population; identify polymorphisms that are present at a lower frequency in the general population

- (in progress). Verify centenarian-specific difference by using Sequenom to determine the frequency of polymorphisms in ~200 centenarians and additional ~200 individuals in the general population

-Functional studies : in case I found interesting difference in the frequencies
The aim of step 4 is to
-clone the region of interest in a Luciferase plamsid
- carry out functional studies in order to verify whether the polymorphic variant/s effect the activity of the p21 gene.

Project #2

Aim: to evaluate the differences in gene expression between centenarians, elderly, young in dermal fibroblast cultures and their response to p21 and DNA damage.

Aging is a remodeling process where the body of survivors progressively adapts to internal and external damaging agents they are exposed to during several decades.

This progressive remodeling process which involves a wide range of physiological changes and trough, mainly, gene expression changes is associated with a progressive functional decline, a gradual deterioration of physiological function, including a decrease in fecundity (Partridge and Mangel, 1999).

Many biological processes involved in aging are shared across diverse species. For example, previous studies have lead to postulate that oxidative stress and DNA damage play a role in aging and longevity in nearly all animals.

Some data from Lu et al., have suggested that DNA damage have a role in age related changes in gene expression. Their finding suggested that accelerated DNA damage may contribute to reduced gene expression in the human brain after age 40 and that DNA damage acts selectively on promoter of the age-downregulated genes .they argue that these phenomena may be a potential mechanism whereby the expression of specific genes could register the passage of time. However the mechanims by which DNA damage “select” the targeted promoter is but so far the mechanism is poorly understood.

In addition some experimental evidence suggests that epigenetic changes could also be critical determinants of cellular senescence and organismal aging.

The project#2 is focused on the investigation of the relationship between DNA damage response and age related changes in gene expression.
Microarrays analysis were performed on actively dividedly dermal Fibroblasts cultures from subject young, elderly and centenarians treated and untreated with the DNA damaging agent doxorubicin which in turn acts inducing oxidative stress (thus cell senescence) and p21.
We used the unique materials from centenarians who can be considered as the best model of healthy aging. Infact, centenarians are characterized by having a history of aging very slowly and either delayed of escaped diseases normally associated with aging.
At the same time, intriguitly enough, centenarians are also by definition extremely old people and show all the signs and the characteristics of a prolonged aging process.

The main aims of project #2 are:

- to identify genes showing interesting **age correlations**
- to generate a **centenarian** gene expression **“signature”**

- to evaluate the effect of **aging** in the **stress response**
- to highlight the different pathways involved in the **stress response** in **aged people** and **centenarians**
- to correlate stress response in aging with p21 induction

the strategy of this project is esemplifiacated belove:

-Microarray analysis of gene expression in 10 cultures from individuals of different ages in order to identify genes showing age correlations (pilot study)

-Correlate age-specific difference in gene expression with transcriptional effects of p21. To carry out this step we compared the data obtained from the Microarray analysis of gene expression from individuals of different ages, with the Microarrays data relative to fibroblast WI38 transfected with a lentivirus which induces an ectopic expression of p21.

-Determine the effects of DNA damage (which induces p21) on the expression of identified genes (trough RQ-PCR) in independent samples

-to extend the Microarrays studies to a set of fibroblast samples 15 (5 young, 4 old, 5 >90 y.o subjects) before and upon treatment with the DNA damaging agent Doxorubicin (in progress)

CHAPTER 5: MATERIALS AND METODS

5.1 Cell Cultures

Human fibroblasts (coriell bank, Bologna University) cells young, elederly, and > 90 y.o subjects, were grown in Eagle's MEM with Earle's salts with supplemented with 10% FCS and 2 mM L-glutamine in a humidified 95% air/5% CO2 incubator.
DFs from 6th to 14th passage were used for all the experiments.
The DFs were allowed to reach sub confluence prior of being treated to the chemioterapic treatment (500 ng/ml doxorubicin).
Cells were collected after 48 hours of treatment : in order to extract RNA about 3-4 10^6 cells (before and upon treatments) were washed in PBS, scrapered and collected.

5.2 Samples

Fibroblasts samples

NAME	AGE	PROVENIENCE
C5	17	ITALY-FRANCESCHI
AG11747	22	CORIELL BANK
AGO4054	29	CORIELL BANK
AGO5247	87	CORIELL BANK
AGO4356	74	CORIELL BANK
AG11795	76	CORIELL BANK
C1	63	ITALY-FRANCESCHI
CENT 3	100	ITALY-FRANCESCHI
CENT6	100	ITALY-FRANCESCHI
BELZ	100	ITALY-FRANCESCHI
AG07124	26	CORIELL BANK
BER	22	ITALY-FRANCESCHI
2PRAT	25	ITALY-FRANCESCHI
AG09975	25	CORIELL BANK
AG11748	22	CORIELL BANK
AG4054	29	CORIELL BANK
AG11735	25	CORIELL BANK
AG09860	26	CORIELL BANK

Fibroblasts samples

NAME	AGE	PROVENIENCE
AG11732	24	CORIELL BANK
AG07356	27	CORIELL BANK
AG11733	70	CORIELL BANK
AG11725	84	CORIELL BANK
AG5247	87	CORIELL BANK
AG11795	76	CORIELL BANK
AG4356	74	CORIELL BANK
AG07720	24	CORIELL BANK
AG08434	29	CORIELL BANK
AG09266	26	CORIELL BANK
AG06310	27	CORIELL BANK
AG06234	17	CORIELL BANK
AG07135	77	CORIELL BANK
AG11488	84	CORIELL BANK
AG013349	86	CORIELL BANK
AG04382	81	CORIELL BANK
AG07801	70	CORIELL BANK
AG04059	96	CORIELL BANK
AG09602	92	CORIELL BANK
AG07725	91	CORIELL BANK
AG08433	94	CORIELL BANK
AG12788	90	CORIELL BANK

DNA samples

Coriell Bank: 10 DNA samples (europeans) (DNA from 5 females and 5 males each of whom is over the age of 90) were obtained from the CORIELL bank. The DNA samples belong the NIA aging cell repository DNA panel.

AG12257	Male	95 YR
AG11716	Male	98 YR
AG11426	Female	101 YR
AG11398	Female	98 YR
AG11371	Male	95 YR
AG11354	Female	97 YR
AG11340	Female	96 YR
AG11467	Female	100 YR
AG14615	Male	93 YR
AG11400	Male	92 YR

DNA –Franceschi Lab:

~200 DNA from general population (europeans) and ~ 200 from centenarian population (europeans)

5.3 RNA extraction

Total RNA was extracted using the RNeasy Mini Kit according to manufacture's instructions (Quiagen)

RNA quality control

Before submitting RNA samples for labeling and hybridization, the quality was checked by :

1. measuring the optical density (OD) of total RNA and mRNA samples including OD_{260}, OD_{280}, OD_{260} /OD_{280}
2. Visualizing each total RNA sample by running on an agarose gel containing 1.1% formaldehyde

For each total RNA sample, 12 µg were sent at a concentration 1 µg/µl.

The quality of RNA was then double-checked by the Micorarrays facility because to obtain high quality data from a gene chip experiment, it is essential to start with high quality total RNA.

Micorarrys facility RNA Quality Control

Spectrophotometric Analysis:

A small amount of your sample will be assayed with an Eppendorf BioPhotometer(at least 1 mg/mL.) The OD 260/280 is calculated to estimate the purity of the RNA. A ratio close to 2.00 indicates a high percentage of ribonucleotide.

Electrophoretic Analysis:

To assess the integrity of your total RNA, the facility tests it with the Agilent Technologies 2100 Bioanalyzer Lab-on-a-chip system. This assay is similar to gel electrophoresis in concept, but it is cleaner, more efficient, and only requires a very small amount of sample.

Agilent's Lab-on-a-chip technology

A small amount of sample is loaded into the wells in the chip and electrodes cause the RNA to move through microchannels filled with a sieving polymer and fluorescent dye. Fluorescence signal is plotted against run-time to generate an electropherogram or translated to gel-like images.

5.4 cDNA synthesis

cDNA synthesis was performed with ~ 2.5 ug of total RNA using Superscript III (invitrogen) reverse transcriptase as follows. RNA template was added to 1 ul of RT primer (100 uM) Universal RT primer (URT) , 1 ul of dNTP mix (each base 10 uM) and DNase /RNase –free water to a colume of 12 ul. The solution was incubated at 65 C for 5 min and then cooled to 4 C. A master mix containing 4ul of 5X first-strand buffer (invitrogen) , 2 ul of 1 mM DTT and 1 uL DNase/RNase-free water per RT sample was prepared and added to each sample. The samples were incubated at 25 – 5min folowed by 50 – 60 min and finally 70° for 15 min.

5.5 Real-time quantitative PCR

In order to verify the behavior of the gene of the signature in an additional set of samples , real time PCR was performed using cDNAs prepared from the RNAs of a total of 15 of young and old human skin fibroblasts before and upon stress (Doxorubicin).

The experiment was perfomed a 7900 HT sequencer .

5.5.1 URT primers strategy

In order to assure a specifity of the RQ PCR, the following uRT primers strategy has been used.

A major confounding problem for standard reverse-trasciption-priming strategies is the presence of contaminating genomic DNA (gDNA) carried over from the original “RNA” extract into the RT and PCR steps. In order to avoid false RNA-Specific PCR primer pairs, I followed a new strategy proposed by Greg Hurteau currently working in the Roninson Lab (Hurteau at al., 2002).

A universal RT primer has been designed that targets the polyA) tail of mRNA and adds unique tag sequence not otherwise existing in the human genome.

Genomic DNA does not incorporate this RT-inserted unique tag. PCR is then performed using a transcript-specific forward primer and a reverse primer that is identical to the unique tag incorporated at RT.

Only cDNA made with this RT primer is compatible with this reverse PCR primer , thus eliminating confounding signal from contaminating gDNA. This methos performs RNA-specific qualitative and quantitative evaluation of gene expression, while preservinf the sensitivity of standart RT-PCR techniques.

5.5.2 Protocol

the cDNA templates were added to a mix containing Buffer 7.5 (Applied Biosystems),ul, Primer (PF+PR) 0.5ul, cDNA 1ul and RNase free water to a volume of 10 ul.

PCR cycling conditions were: 40 cycles of 30 s at 95°C, 30 s at 60°C and 30 s at 72°C.

RQ-PCR primers

il1-r	GTC GGA GAT TCG TAG CTG GAT
il1-f	CTC GCC AGT GAA ATG ATG GCT
mxr1-f	GTT TCC GAA GTG GAC ATC GCA
mxr1-r	GAA GGG CAA CTC CTG ACA GT
DUSP4-f	GAC ATC TGC CTG CTC AAA GG
DUSP4-r	CAC TGC CGA GGT AGA GGA AG
MBNL2-f	GTA CCA GCG CGG TCT TTA AC
MBNL2-r	AGC AGA AAC GGA ATG GAA TG
earlyendantigen1-f	AAG AGG CCA AAC CTG ATG GG
earlyendantigen1-r	GCA ATT TCA GTA GCA AGT TGG G
utrophin-f	GCA AGG CTC AAA CTT ATT TGG G
utrophin-r	TCA GGC GCT GTA CTC TGT ATA TT
radixin-f	AAA TCC CAG TAG GCT TTT ACC A
taok f2	ACT TAG TGA CCA TTT GGC ATC A
Soares xv	
35c04.x1-f2	GGG AGA TAC CAT GGT TTA ACG TA
NME5-f	CCT CGA AAG AAC AAA TCA TGA AC
HIST1H2BD-f	CAG ACC TAG TTA CTG GGA ATT CAT
AU156822-f2	CTG GGT CTT ATA AAA ATC AGG TAC AAA
lln3-f2	ATT AGG TGA GCT TTA TCC TCC TCT G
p16-f2	TTC ATG ACA AGC ATT TTG TGA ACT A

RQ-PCR primers	**sequences**
FLJ20637-f	TGG ACC AAG TGA CAC AAG CAA
FLJ20637-r	TGG CTA ATT CGG CTT ATT TCT GG
aldr1-f	GGC ATC CTC TGT ACC CCT TT
sxemidin F2	GGC ATG GGA AGA ATG AAA GA
Soares-qf93g07.x1 -f	GCT GTC TTC CCA AGT TGA CC
Herc6-f	GCC CTG TCT CTC TGT GGG A
Herc6-r	AGA GCG ATT GTC TCC AAA TGT G
universal	AAC GAG ACG ACG ACA GAC TTT

5.6 MICROARRAYS

5.6.1 Microarrays: general characteristics

It is widely believed that thousands of genes and their products (i.e., RNA and proteins) in a given living organism function in a complicated and orchestrated way that creates the mystery of life. However, traditional methods in molecular biology generally work on a "one gene in one experiment" basis, which means that the throughput is very limited and the "whole picture" of gene function is hard to obtain. In the past several years, DNA microarray, has attracted tremendous interests :this technology promises to monitor the whole genome on a single chip allowing to have a better picture of the interactions among thousands of genes simultaneously. With microarray technology, the transcription thousands of genes can be determined simultaneously.

An array is an orderly arrangement of samples. It provides a medium for matching known and unknown DNA samples based on base-pairing rules and automating the process of identifying the unknowns. An array experiment can make use of common assay systems such as microplates or standard blotting membranes, and can be created by hand or make use of robotics to deposit the sample. In general, arrays are described as *macroarrays* or *microarrays*, the difference being the size of the sample spots. Macroarrays contain sample spot sizes of about 300 microns or larger and can be easily imaged by existing gel and blot scanners. The sample spot sizes in microarray are typically less than 200 microns in diameter and these arrays usually contains thousands of spots. Microarrays require specialized robotics and imaging equipment that generally are not commercially available as a complete system.

There are two variants of the DNA microarray technology, in terms of the property of arrayed DNA sequence with known identity:

Format I: probe cDNA (500~5,000 bases long) is immobilized to a solid surface such as glass using robot spotting and exposed to a set of targets either separately or in a mixture. This method, "traditionally" called DNA microarray, is widely considered as developed at Stanford University.

Format II: an array of oligonucleotide (20~80-mer oligos) or peptide nucleic acid (PNA) probes is synthesized either *in situ* (on-chip) or by conventional synthesis followed by on-chip immobilization. The array is exposed to labeled sample DNA, hybridized, and the identity/abundance of complementary sequences are determined. This method, "historically" called DNA chips, was developed at Affymetrix, Inc. , which sells its photolithographically fabricated products under the *GeneChip*® trademark. Many companies are manufacturing oligonucleotide based chips using alternative in-situ synthesis or depositioning technologies.

Human Genome U133 Plus 2.0 Array-affimterix

In our pilot (project #2) study we use the U133 plus 2.0 array chips which are able to complete coverage of the Human Genome for analysis of over 47,000 transcripts

Human Exon 1.0-affimetrix

We have been using, in the second part of project #2, the GeneChip® Human Exon 1.0 ST Array that enables two complementary levels of analysis—gene expression and alternative splicing.

Multiple probes per exon enable "exon-level" analysis and allow to distinguish between different isoforms of a gene. This exon-level analysis on a whole-genome scale allow us to detecting also specific alterations in exon usage that may play a central role in disease mechanism and etiology.

The second level is "gene-level" expression analysis, in which multiple probes on different exons are summarized into an expression value of all transcripts from the same gene.

5.6.2 Software for microarrays analysis: genespring

the Roninson lab we has a site license for GeneSpring version 7 (www.silicongenetics.com). The software allows to visualize, organize and manipulate gene expression data.

Normalization steps can be applied in virtually any order and include operations such as dye swapping experiments and median polishing. Scenarios can be saved and applied in other experiments. GeneSpring offers visually intuitive filtering tools for both entry-level and advanced users. All visual filtering windows generate graphs of results in real-time. These filters allow to exclude particular conditions, set minimum and maximum values and choose specific gene lists to filter. The advanced filtering window allows you to create complex Boolean expressions to identify genes with a highly specific expression pattern.

It a lso provides various analysis tools, such as t-tests, 2-way ANOVA tests and 1-way post-hoc tests for reliably identifying differentially expressed genes. GeneSpring also has class prediction tools that can identify genes capable of discriminating between one or more experimental parameters or sample phenotypes. Groups of genes identified by expression profiling can be further characterized by performing sequence searches for potential regulatory elements.

GeneSpring provides sophisticated clustering methods to uncover patterns of gene expression data and the relationships between these patterns. Researchers can use one or a combination of clustering options to characterize their data: gene trees (hierarchical clustering), experiment trees, self-organizing maps, k-means, Principal Components Analysis (PCA) and QT clustering. QT clustering is an unsupervised technique that allows you to specify both the minimum size and maximum correlation coefficient of each cluster. Principal Components Analysis (PCA), allow s you to reduce the

complexity of your data by discovering a number of principal components that define most of the data variability. Pathways may also be explored with the pathway viewer, genes and their expression patterns can be visually characterized based on their location within a cellular pathway.

5.7 P21-PCR-SEQUENCING

5.7.1 Sequencing: generality

There are several methods available to determine the actual sequence of nucleotides in a segment of DNA. One procedure uses specially altered nucleotides called dideoxynucloetides which have been made either radioactive or fluorescent.

For sequencing the p21 promoter, I used the PCR-SEQUENCING strategy: when enough of the targeted DNA fragment has been amplified by PCR, the mix is put through a series of DNA sequencing reactions that are a variation of PCR.

A 3 ul of mix ontaining Taq DNA polymerase, and all four of the nucleotide bases (A, T, C, G.) was added to 1ul of primer that can hybridize at the desired location on only one complementary strand of the DNA (as opposed to both strands in PCR), to 1 ul of the amplified product from the PCR and water up to 10 ul total final volume.

In addition, small amounts of fluorescence labeled dideoxynucleotides (A, T, C, G) are added to the mixture. Dideoxynucleotides are human-made nucleotides whose sugar component is slightly different from that of the nucleotides that make up DNA. (There is no OH on the 3' carbon.) Dideoxynucleotides can be picked up and added to a growing DNA chain. However, as a result of this structural difference another nucleotide cannot be added at its 3' end. Consequently, if one of the dideoxynucleotides is added to a growing chain of nucleotides, the strand will be terminated. Each dideoxynucleotide is labeled with a different fluorescent compound so that it will give off an identifying color in a laser beam.

After 20 - 30 cycles of the PCR heating and cooling, the resulting mixture will contain a series of fragments of different lengths depending on how many bases had been added to the chain before one of the dideoxynucleotides sneaked in and blocked further growth.

The mix of billions of short fragments from the sequencing reactions is loaded into glass capillary tubes that contain a gel solution that serves as a sieving matrix During electrophoresis, a voltage is created across the gel so that one end is made positive and the other negative. Since DNA is slightly negative, its fragments will move to the positive end of the gel. Not surprisingly, the different length DNA strands migrate at different rates and therefore separate from each other according to size. The smallest strand travels the fastest.

As each DNA fragment reaches the bottom of the capillary tube, its fluorescence-labeled end dideoxynucleotide is excited by a laser beam that is directed at the bottom of the tube

Each of the four dideoxynucleotides fluoresces a different color when illuminated by a laser beam. An automatic scanner records each wavelength of light and a computer generates an electropheorgram with colored peaks representing each wavelength in the sequence it passes through the beam.

3730 DNA Analyzer

For our sequencing I have been using the 3730 DNA analyzer , which is the Gold Standard in medium-to-high throughput genetic analysis.

5.7.2 PCR SEQUENCING on the p21 gene

The p21 gene consists in 3 exons (68, 450, 1600 bp) and a 5 kb promoter.

In order to sequence the whole gene , the exons and the promoter were amplified in overlapping fragments.

Primers design for PCR sequencing

A protocol of PCR each amplifying ~ 400 bp of the p21 gene was optimized with the following primer pairs (where p21pro.1 represents the farer from the transcription site)

Primers were designed using the primer 3 software available on http://frodo.wi.mit.edu/. The products size was set to ~400 bp, the primers were sized between 22-24 bases with a Tm of 69-71 and a GC content of 40-60 %. The primers were checked for loops, hairpins and 3' complementarity. The best primers pair was ordered from idtDNA.

Once amplified, the fragments were purified with Zymo columns (Zymo research) and then sequenced.

PCRs protocol

The DNA templates were added to a master mix containing 2ul of each primer (10 uM), 5 ul Buffer (New England biolabs) , 2 ul od Taq (), and water to a volume of 50 ul. the following PCR profile was used: preincubation for 2 min at 96°C, 40 cycles of 30 s at 95°C, 30 s at a primer-specific annealing temperature (see table belove) and 15 s at 72°C and one final incubation for 5 min at 72°C.

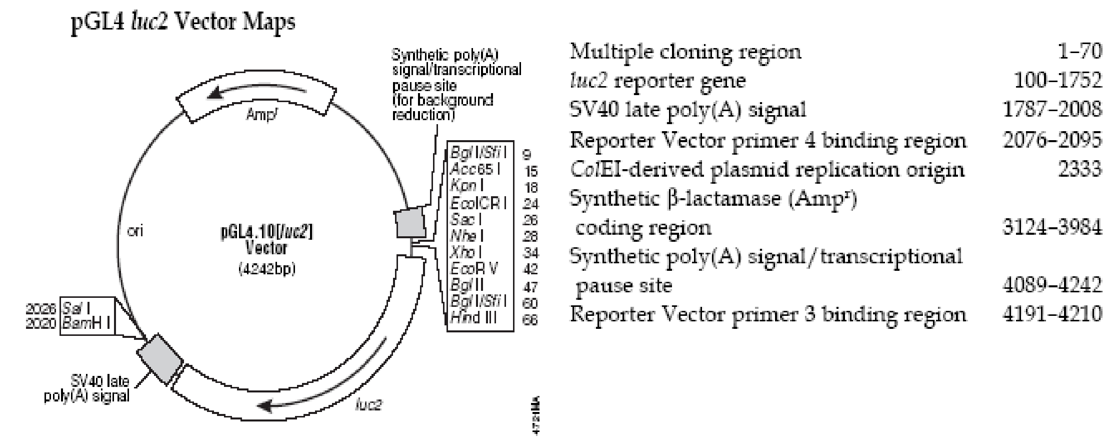
Name	Sequence	Tm
p21.exon1.R	AAGGCGAGCTCCCAGAAC	60°
p21seq.exon1.F	ACTGGGGGAGGAGGGAAGT	
p21seq.exon2.F	ACCAGCTGGAAGGAGTGAGA	60°
p21seq.exon2.R	GTCTTTGCTGCCTACTTGC	
p21seq.exon3.F1	TGCGGTGATGGATAAAATCA	58°
p21seq.exon3.R1	GAAAAGGAGAACACGGGATG	
p21seq.exon3.F2	TCCTAAGAGTGCTGGGCATT	60°
p21seq.exon3.R2	GCCCTTCTTCTTGTGTGTCC	
p21seq.exon3.F3	TCTTCTCCAGCTGGGCTCT	58°
p21seq.exon3.R3	CCCAAAAGCCCATTTATTTG	

Name	Sequence	Tm
p21pro1.r	GGGGCTGCCTATGTAGTGAA	58° + dmso
p21pro1.F.new	GTGCCACAGTTCACAAGTGC	
p21pro2.f	TTTGCTTCTGGGCAGAACTT	58°
p21pro2.r	CAGAGCCAGGATGAATTGGT	
P21.pro.3.f	GATGTT GTT AGA GCC AGG AAC AG	54°
P21.pro.3.r	ATC AAG GCA TAA AAA TTT CAT TGTG	
P21pro4f	AAA AGG TTT TTG AAT GAA TGG ATG	58.5° +dmso
P21pro4r.new	AGA AGA GGC GGA ACA AAG ATA GAA	
P21pro5f.	CAC GCC CGG CCA GTA TAT ATT TTT	58 °+ dmso
P21pro5r.	GAC AAA ATA GCC ACC AGC CTC TTC T	
p21pro6.f	CACCTTTCACCATTCCCCTA	58°
p21pro6.r	AGGGCTGGTTGTCAAATGTC	
p21pro7.f	TGCATGGTTGCAAACTTTTT	54°
p21pro7.r	TCACCTTTGCCTCCTTTCTG	
p21pro8.f	AGGTCAGCTGCGTTAGAGGA	58°
p21pro8.r	GGAAGGAGGGAATTGGAGAG	
p21pro9.f	GGAGGCAAAAGTCCTGTGTT	54°
p21pro9.r	ACATTTCCCCACGAAGTGAG	
p21pro10.f	TCTAGGTGCTCCAGGTGCTT	58° +dmso
p21pro10.r	CTGTGAACGCAGCACACAC	
p21pro11.f	CCGAAGTCAGTTCCTTGTGG	54°
p21pro11.r	GCTTCCTTGGGAACAAACTG	

5.8 CONSTRUCTS

5.8.1 pGL4 Reporter Vectors: Description

The p21 centenarian haplotype area was cloned in a p21-Luc vector (p21-pgl4.10-Luc2 containing the general population-haplotype and gently provided by A. saramaki) . The pGL4 Vectors are state-of-the-art Luciferase Reporter Vectors. This vector family features codon optimized synthetic genes with or without Rapid Response™ technology, and clean backbones with dramatically reduced numbers of cryptic regulatory elements.



pGL4.10[luc2] Vector sequence reference points:

Base pairs	4242
Multiple cloning region	1-70
luc2 reporter gene	100-1752
SV40 late poly(A) region	1787-2008
Reporter Vector primer 4 (RVprimer4) binding region	2076-2095
ColE1-derived plasmid replication origin	2333
Synthetic Beta-lactamase (Ampr) coding region	3124-3984
Synthetic poly(A) signal/transcriptional pause site	4089-4242
Reporter Vector primer 3 (RVprimer3) binding region	4191-4210

5.9 Cloning of the p21 promoter centenarian haplotype region in a P21-PGL4.10 vector

5.9. 1 HAPLO- p21 promoter PCR

The p21 centenarian-haplotype area of the promoter was amplified by PCR from human genomic DNA (containing the centenarian haplotyphe) previously sequenced and fused with the p21 PGL.4 Luc (plasmid containing the 5 kb promoter with the normal population haplotype and a luciferase reporter gene).

In order to increase the specificity of the PCR a double round of PCR was carried out using a high proofreading polymerase (New England Biolabs)

The Phusion DNA Polymerase used in order to limitate the introduction of possible errors, generates templates with an accuracy and speed previously unattainable with a single enzyme. The unique structure and characteristics of this polymerase make Phusion DNA Polymerase a superior choice for cloning, and sets a new standard for PCR performance: its error rate is 50-fold lower than that of Thermus aquaticus, and 6-fold lower than that of Pyrococcus furiosus - making it the most accurate of available thermostable polymerases. Phusion DNA Polymerase possesses processivity 10-fold greater than Pyrococcus furiosus and twice that of Thermus aquaticus.

The primed template was pre-formed in the presence of 5x Phusion GC buffer (New England Biolabs) and 200 µM of each dNTPs , 0,5 uM primers , 3% dmso ,1 U of Phusion DNA polymerase and water to a final volume of 20 ul.

Being the template GC rich the dmso was added in order to optimize the product yield

The sample were incubated as following:

preincubation for 30 sec at 98°C, 30 cycles of 10 s at 98°C, 30 s at 66°C and 3 min at 72°C and one final incubation for 5 min at 72°C.

p21-4997F	TAC AAA CAT TGG GTG GGG CGA GTC
p21-R-44	CTC CGG CTC CAC AAG GAA CTG ACT T

HAPLO- p21 promoter PCR (PCR product about 2.1 Kb)

The 5kb PCR product of the p21 promoter amplification was used as a template for the following PCR (same condition as above)

p21-4497F	TAC AAA CAT TGG GTG GGG CGA GTC
p21-5R	GAC AAA ATA GCC ACC AGC CTC TTC T

DNA DIGESTION and GEL PURIFICATION

The p21 cent-HAPLO DNA and the plasmid pGL4.10 were digested O/N at 37 using the enzyme AatII/Sph (New England Biolabs)
The AatII/ Sph enzymes cut about 2.1 kb of the pGL4.10 in the haplotype area (where we will ligate the cent-haplotype one) ~600 bp in the p21-cent-Haplo PCR product

GEL PURIFICATION

The product of digestion was run on a gel (1 %) and the bands of interest (7.142 kb for the p21-PGL4.10 and 2.1 for the p21-cent-haplopromoter) were cut and purified using the quiagen kit according to the manufacturer's protocol.

5.9.2 LIGATION

The ligation was performed O/N at 37. a mix containing of Buffer 10X (New England Biolabs) , 1 ul of ligase (New Englad Biolabs) was added at ~50-100 ng of vector and insert.

The ligation was performed using two different ration vector: insert (1:3, 1:5) in order to Increase the chance of ligation.

The calculation was performed using the promega calculator available at the http://www.promega.com/biomath/ , based on the assumption that

$$\frac{\text{length of insert (in kb)}}{\text{length of vector (in kb)}} \times \text{ng of vector} = \text{ng of insert needed for a 1:1 ratio}$$

5.9.3 BACTERIAL TRANSFORMATION

Transformation is a technique to introduce DNA into bacterial cells. For cells to uptake exogenour DNA they must first be made permeable tso the DNA can enter the cells. Some bacteria (i.e. Coli) became competente due to environmental stresses.
Some of those are treatments with chloride salts of metal cations such as calcium, rubidium or magnesium and cold treatment. These changese affect the structure and permeability of the cells wall and membrano so that DNA can pass through.
In my case I used an HEAT shock treatment on E.Coli.
E.coli bacteria are normally poisoned by the antbiotic ampicillin. Amipicillin acts to damage the membranes of e. coli by inhibiting the crosslinking of the bacterial membrane. This results in bacteria shich are very structurally weak. The plasmid has an additional gene that codes for a B lactamase that is excreted by cells and in a local area will hydrolyze ampicillin. Therefore by adding ampicillin, only bacteria that contain the plasmid will survive.

Bacterial Transformation

The first step of bacterial transformation consist in adding 1-10 l of the DNA (Experimental reaction or positive/negative control) to a vial (20-200 l) of competent *E. coli* cells (Invitrogen) and then mix gently.
The step is followed by an Incubation on ice for 30 min. then the cells are Heat shock for 30 sec at 42˘C
After heat shock, the cells are Immediately transfer the tubes to ice and incubate for 2 min then 100 ul of nutrient broth are added. (room temperature).
This step is followed by shaking the tubes at 37˘C for 60 min.
Finally the cells are Spread 50-500 l from each transformation on a L-broth agar plates containing antibiotics at the appropriate concentration (AMP) .
The plates are then overnight at 37˘C..

5.10 Transfections and luciferase reporter gene assays

Luciferases are the most commonly used genetic reporters because luciferase activity assays have broad dynamic range and high sensitivity. This makes them ideal for processing many samples without the need to test multiple sample dilutions or to prepare large quantities of cells. For vector normalization, activity of two luciferases, firefly and Renilla, can be measured in the same lysate . The internal vector control (Renilla)(Promega) must encode a reporter other than that used in the test construct. As common, I used the firefly luciferase as the test reporter and Renilla luciferase as the control reporter .

5.10. 1 Transient Transfection-Fugene 6

Fugene 6 Transfection Reagent (Roche) is a nonliposomal, multi-component reagent that forms a complex with DNA, then transports it into animal cells.

Compared to other transfection methods, FuGENE 6 Transfection Reagent gives more consistent results, saves time and effort, and has minimal effect on cell physiology.

HCT116 cells plated in a 12 well- plate the night before to give 60-80% confluence in a phenol red-free DMEM. In order to normalize the firefly signal , I carried out a co-trasfection with the internal vector (a ratio of 10:1 test vector:control vector is used)

1)47µl of serum-free medium and add 3µl of FuGENE 6 (Roche) directly into the tubes with out allowing contact with the walls of the plastic tubes.

2)A co-trasfection was carried out adding a total 1µg experimental DNA (Cent p21-Luc or Normal-p21Luc plus Renilla-Vector) into the sterile tube, tap the tube or vortex for one second to mix the contents.

3) Incubate the transfection reagent:DNA complex for minimum of 15 min at room temperature.

4) add this mixture directly to the cells drop wise through the medium. Making sure to do not evenly sprinkle the droplet over the entire area. There is no need to remove and replace with fresh medium.

5)Incubate for 48 h.

5.10.2 Preparation of Cell Lysate for Luciferase assay

For the preparation I have been using the following protocol:

1. Remove growth medium from cultured cells.

2. Rinse cells in 1x PBS. Do not dislodge cells. Remove as much of the final wash as possible.

3. Dispense a minimal volume of 1x lysis reagent (RLB; Promega) into each culture vessel

4. scrape attached cells from the dish, and transfer the cells and solution to a micro centrifuge tube.

5. Pellet debris by brief centrifugation, and transfer the supernatant to a new tube.

6. Mix 20-30 µl of cell lysate with same volume l of Luciferase Assay Reagent (first Dual-Glo™ and then Stop solution-see above) (Promega)

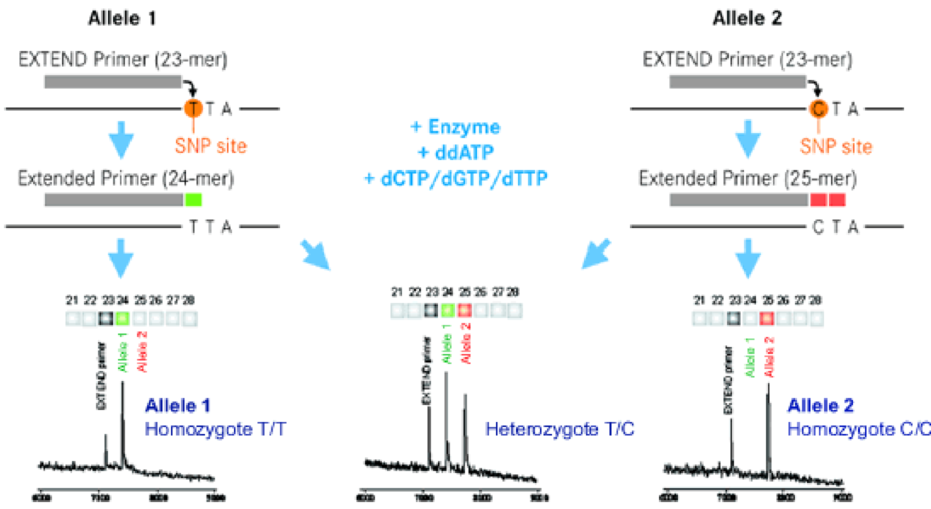
The Dual-Glo™ Luciferase Reagent can be added directly to cells in growth medium without washing or preconditioning. This reagent induces cell lysis and acts as a substrate for firefly luciferase, producing a stable luminescent signal that can be read over a period of two hours, with >60% of the luminescent signal retained. Addition of Dual-Glo™ Stop & Glo® Reagent quenches the luminescence from the firefly reaction by at least 10,000-fold and provides a substrate for *Renilla* luciferase in a reaction that can also be read within 2 hours (with similar signal retention).

Each sample is normalized by dividing the test reporter activity by the control reporter activity. Triplicate samples are then averaged .

5.11 SEQUENOM

The Company's proprietary MassARRAY® system is a high-performance DNA analysis platform that efficiently and precisely measures the amount of genetic target material and variations therein. The system is able to deliver reliable and specific data from complex biological samples and from genetic target material that is only available in trace amounts.

Sequenom's MassARRAY technology is used to perform allele specific primer extension reactions which allow differentiation of homozygous normal, heterozygous mutant and homozygous mutant samples (hME assay). The MassEXTEND primer anneals up to the polymorphic site and is extended dependent on the polymorphism. The allele products consist of either a one or two base extension and their masses are easily distinguished on the mass spectrometer.



CHAPTER 6: HAPLOTYPE ANALYSIS

Haplotype Analysis

The haplotype analysis has been performed with two different methods:

1) a EM method (Estimated Maximum likelihood), implemented in Haploview, to calculate pairwise linkage disequilibrium (i.e. the association between markers) and then to build the LD blocks and estimate the phase from unrelated individuals (Barrett et al., 2005).

The default algorithm is taken from Gabriel et al, Science, 2002. 95% confidence bounds on D prime are generated and each comparison is called "strong LD", "inconclusive" or "strong recombination". A block is created if 95% of informative (i.e. non-inconclusive) comparisons are "strong LD". This method by default ignores markers with MAF < 0.05. The default behavior is to sort the list of all possible blocks and start with the largest and keep adding blocks as long as they don't overlap with an already declared block.

To the identified haplotypes, a chi-square similar statistics is applied to compare estimated haplotype frequencies in cases versus controls.

Even to this results, a permutation test can be applied to correct the obtained p-values for multiple testing.

2) another two-stage method, implemented in the WHAP software.

The primary test is a regression-based analysis of association of haplotype and trait (quantitative or qualitative) with one regression coefficient per haplotype (Sham et al., 2004).

The secondary test analyses the relationship between haplotype similarity and regression coefficient similarity.

The secondary test is implemented as a multivariate weighted least squares regression of effect similarity on genetic similarity. An empirical significance value can be combined with the primary omnibus significance value, by Fisher's method of log-transformation.

In each case permutation testing can be applied to generate empirical significance values.

The unit of analysis can be a window/block, or a specific haplotype, so the analysis can be done using sliding windows, i.e. testing all possible haplotypes of a certain size (irrespectively of the blocks calculation), or testing directly certain haplotypes highlighted with the Haploview softare.

This second approach is very useful since it is possible to combine together the results of the primary omnibus test together with the secondary local test which is able to add positional information and increase power.

LD structure

The Haploview software allows also to build a graphical nice view of the LD structure of the population, in order to identify visually the block definition.

CHAPTER 7: RESULTS PROJECT #1

7.1 Identification of polymorphic variants in the p21 gene and promoter regions.

The sequencing was performed first on the 3 exons of the p21 gene which are respectively 68, 450, 1600 bp.
Then I sequenced the whole p21 promoter which is approximately 5 kb. So far only 2.5 kb of the p21 promoter has been extensively studied.

7.1.1 Sequencing of the 3 exons: identification of a polymorphic variant in the exon 2 region.

- A transition A->C at codon codon 31 which leads to Arg-> Ser

The polymorphic variant has been already characterzed.

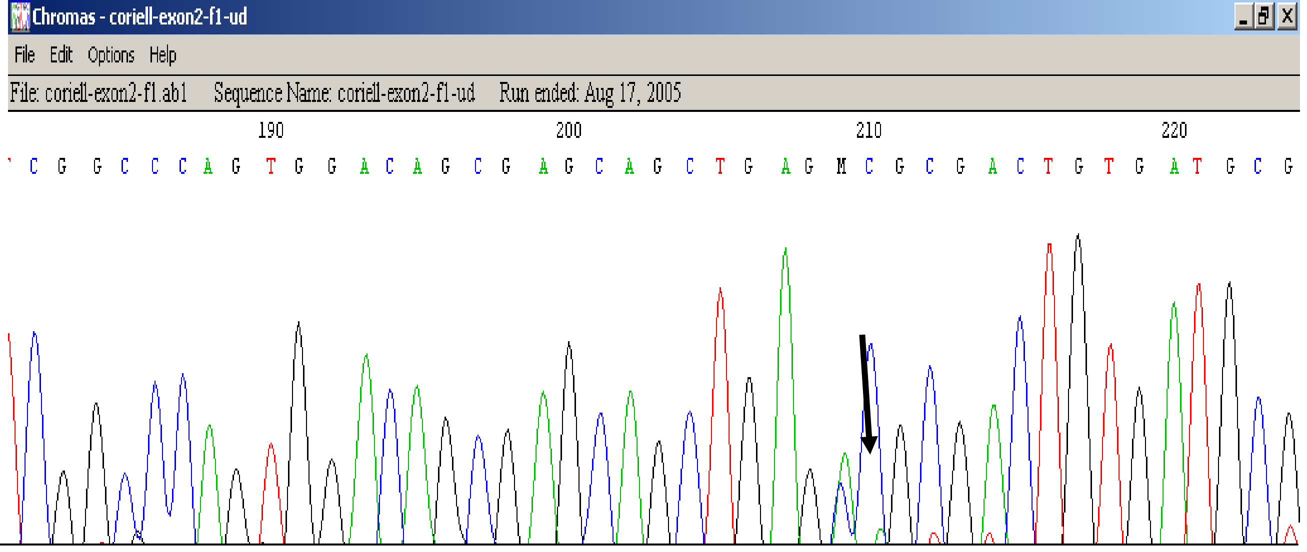


Figure 1: example of Sequence , exon 2, heterogosis A/C
The frequencies of the polymorphic variant has been reported in NCBI :
Average Allele Frequency in the general population:
A; 0.261
C; 0.739
Frequencies in the centenarian population A: 0.28
C:0.71

7.1.2 P21 promoter sequencing: identification of 29 polimorphic variants

The sequencing of the p21 promoter lead to the identification of 29 polimorphic variants.
up to 29 polimorphic variants(see tables pages):
-6 polymorphic variants were uncaractherized
-23 previously charachterized
up ot the 23 already charactherized only 6 had known frequencies(reported in NCBI)

Figure 2: Examples of polymorphic variants identified in the p21 promoter of centenarians:

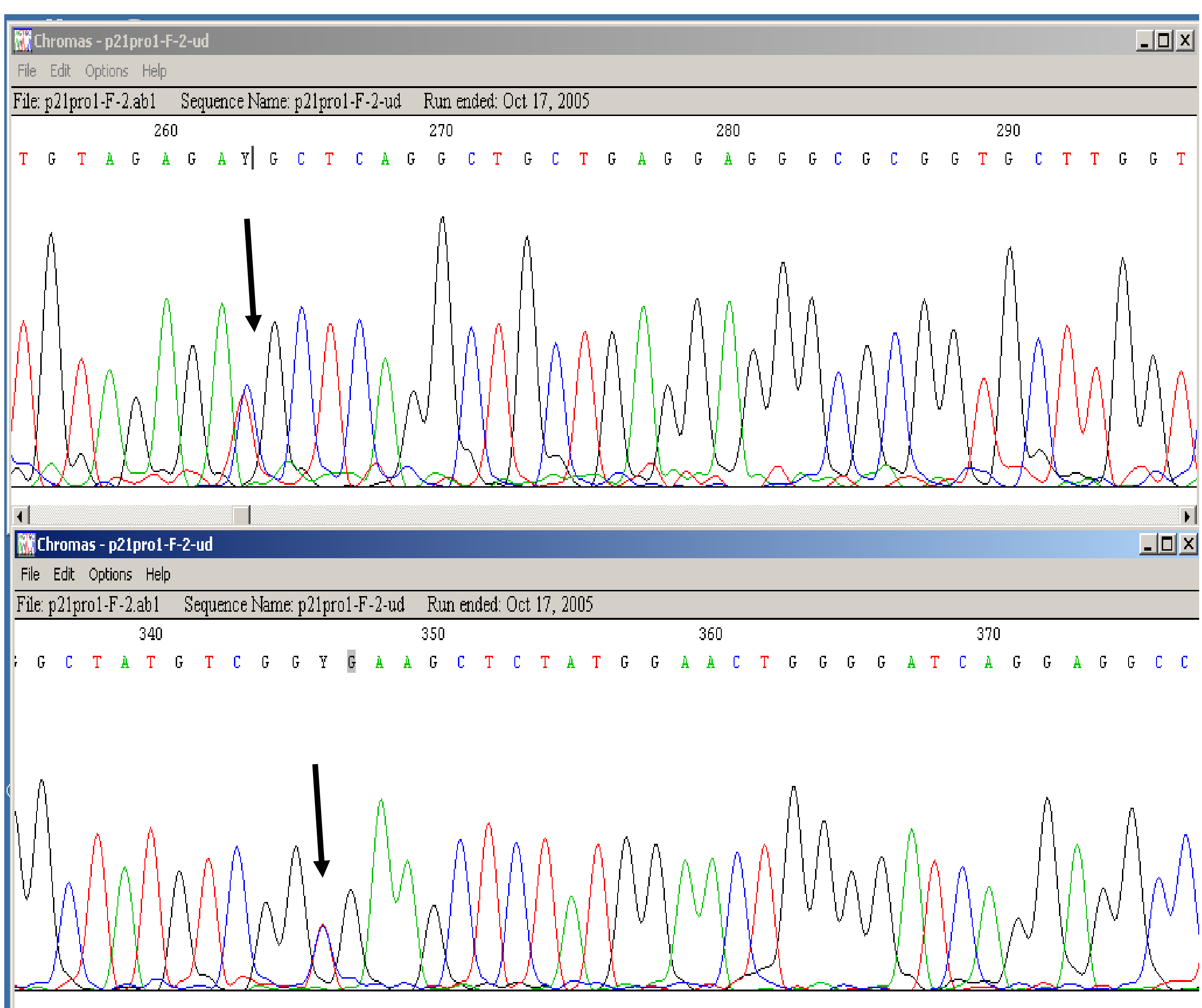


Figure 3: : Examples of polymorphic variants identified in the p21 promoter of centenarians:

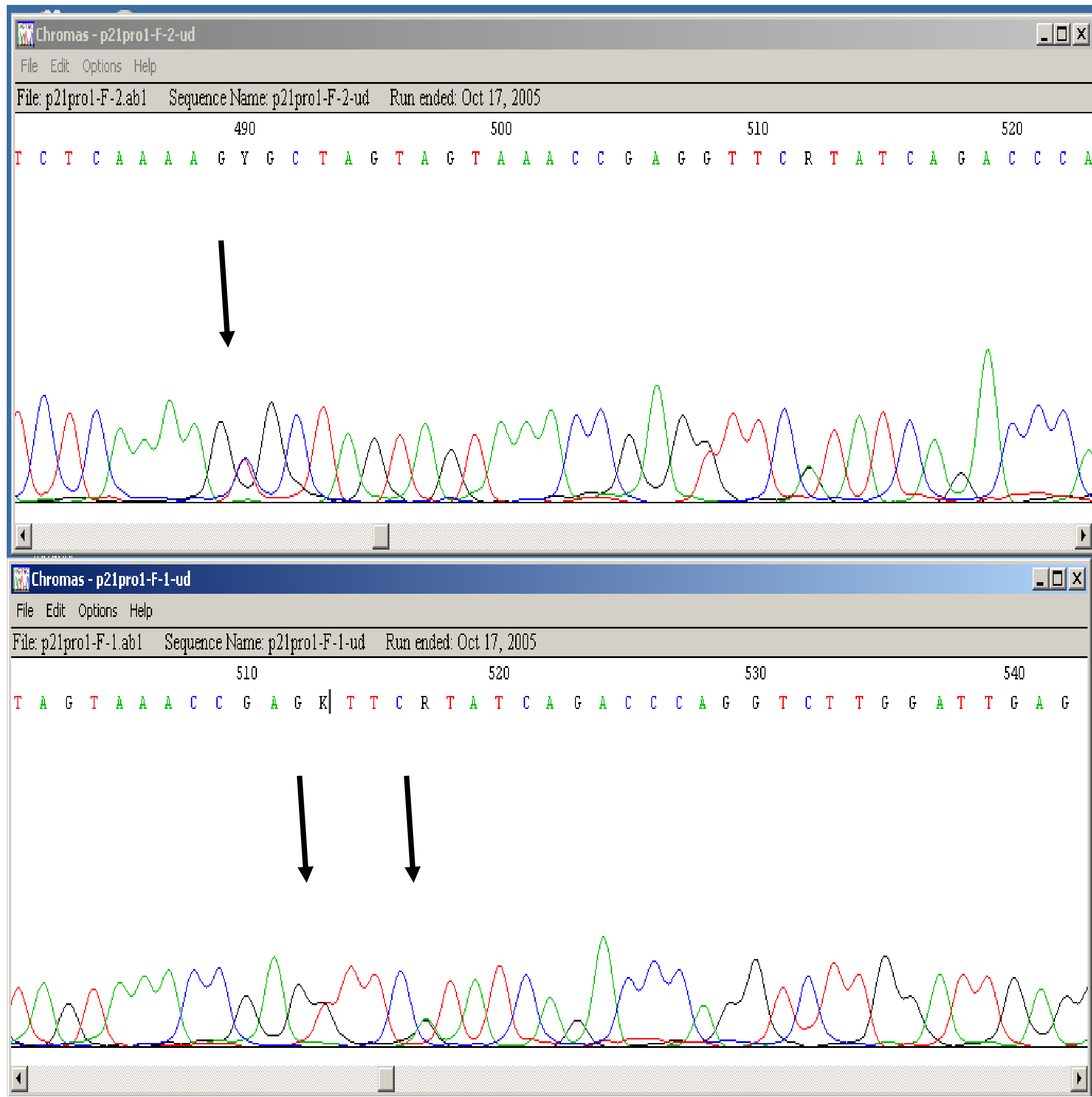


Figure 4 : Examples of polymorphic variants identified in the p21 promoter of centenarians:

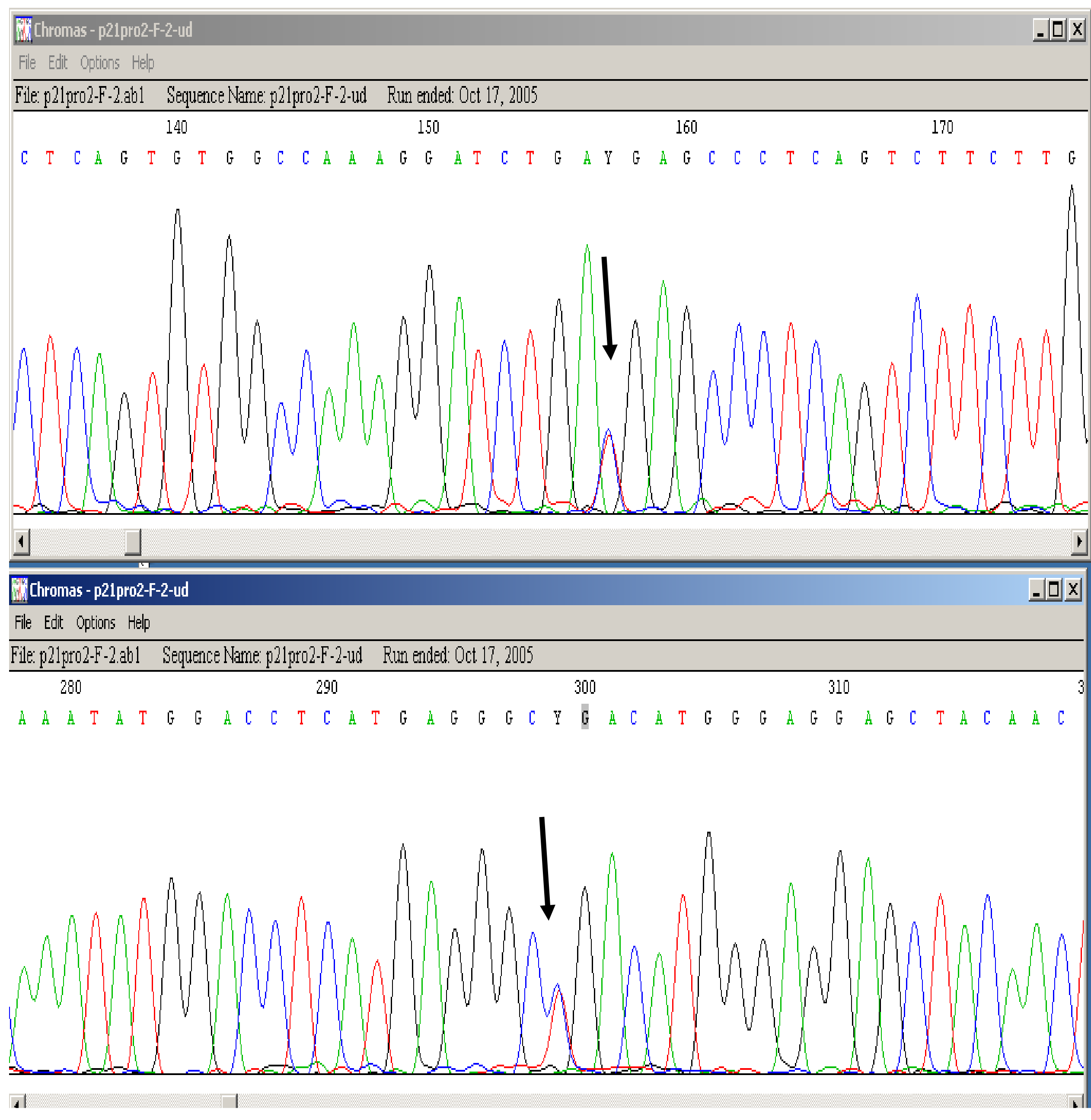


Figure 5 : Examples of polymorphic variants identified in the p21 promoter of

Figure 6 Examples of polymorphic variants identified in the p21 promoter of centenarians:

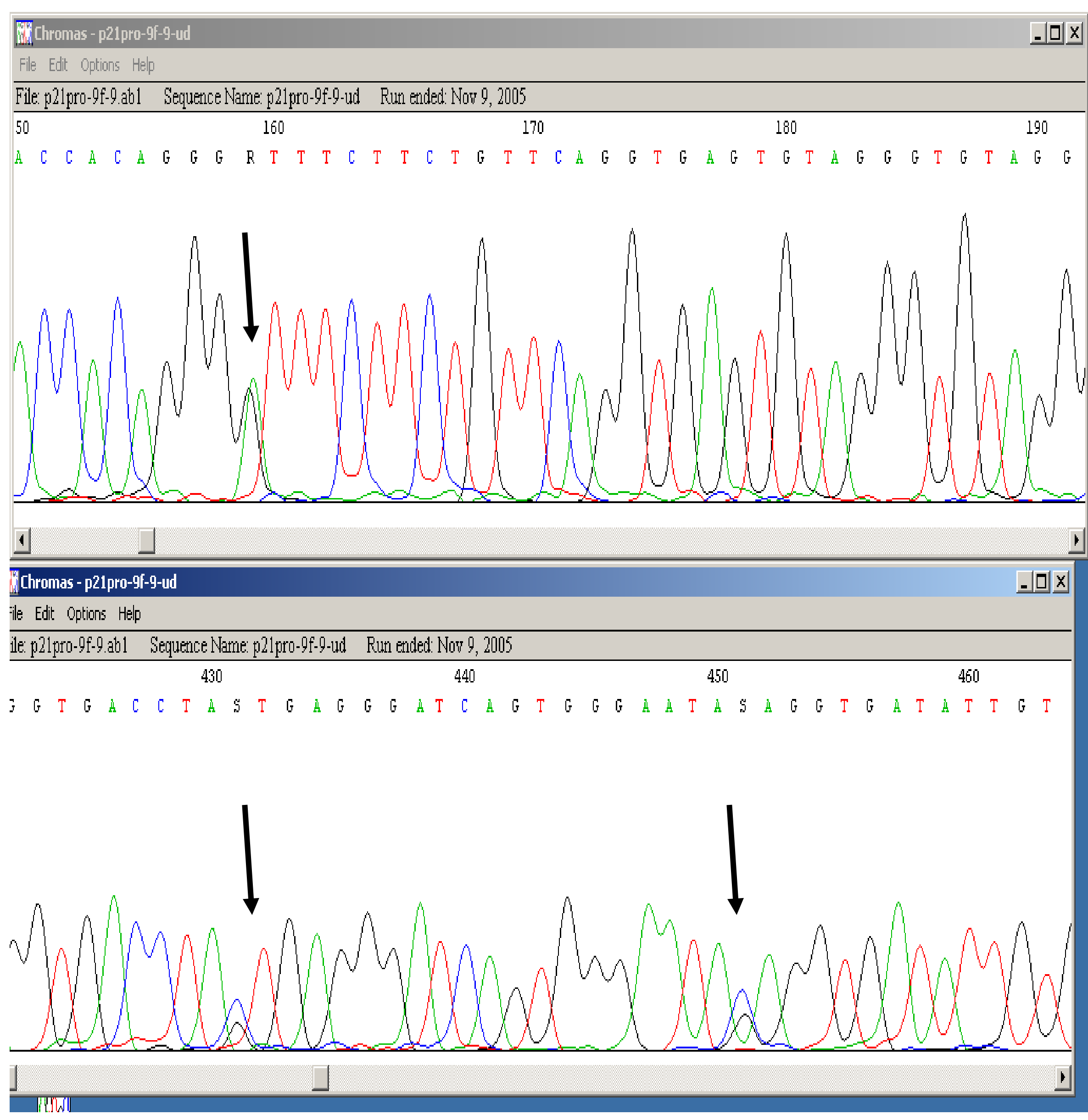
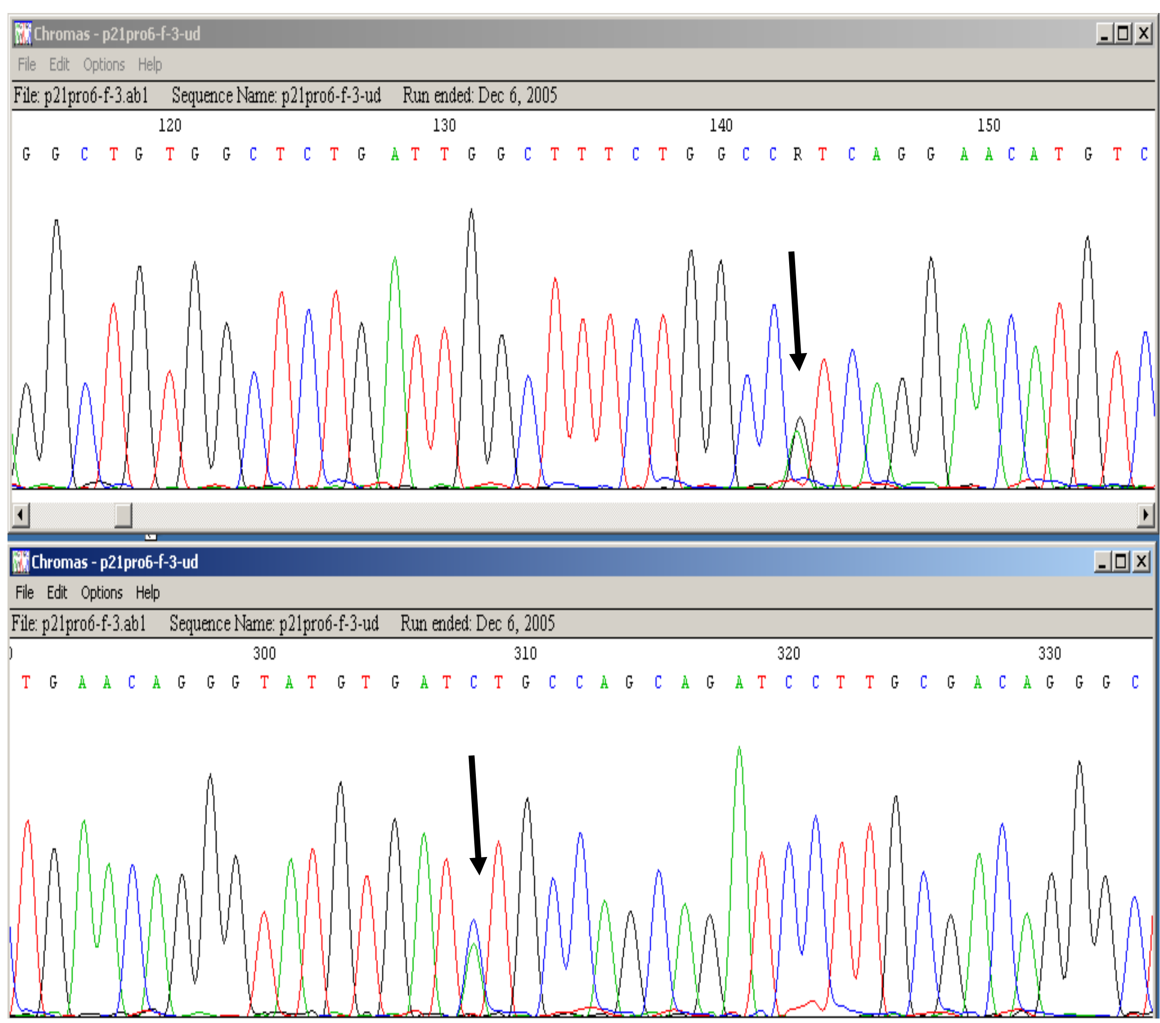


Figure 7 Examples of polymorphic variants identified in the p21 promoter of centenarians:



Analisys on NCBI:

Known RS and frequencies (reported on NCBI) othe the 29 polimorphic variants .
As up to 29 polimorphic variants:
-6 polymorphic variants were uncaractherized
-23 previously charachterized
up ot the 23 already charactherized only 6 had known frequencies

SNP#	rs
1	rs=4711458
2	rs=4711459
3	rs=4711461
4	rs=4714002
5	rs=471146
6	rs4714003
7	N.A.
8	rs=10947623
9	rs=12192827
10	rs=12192877
11	N.A.
12	N.A.
13	N.A.
14	N.A.
15	rs=4135234
16	rs=3829963
17	rs=3829964
18	rs =3829965
19	rs=4135235
20	rs=4135237
21	rs=3829966
22	rs=3829967
23	rs=3829968
24	rs=733590
25	rs=2395655
26	rs=2395655
27	rs=4151702
28	rs=4135239
29	N.A.

Table 1 List on SNPs and corrispondent Rs (when known)

7.2 Comparison of the frequencies of the polymorphic variants : Centenarians versus General Population

SNP#	GENERAL POPULATION		CENTENARIANS		Location
1	C :0.874	T:0.115	C;0,75	T; 0,25	-4568
2	T : O,876,	C: 0,115	T: 0,75	C:0,25	-4485
3	C: 0,9	T: 0.098	C:0,75	T: 0,25	-4341
4	T: 0,885	G: 0,105	T: 0,75	G: 0,25	-4322
5	A; 0,904	G; 0,095	A;0,75	G; 0,25	-4318
6	C; 0,85	T; 0,1185	C; 0,725	T; 0,25	-4248
7	NA		C; 0,75	T; 0,25	-4106
8	G; O,85	A; 0,1185	G; 0,77	A; 0,22	-3673
9	C; O,87	T;0,122	C; 0,71	T; 0,27	-3538
10	C; 0,91	T ; 0,08	C; 0,75	A; 0,25	-3510
11	A; 0,92	G; 0,07	A; 0,9	G; 0,1	-3431
12	NA		A; 0,75	G; 0,25	
13	G;1		G;0,88	A;0,11	-3285 -3009
14	NA		C;0,88	T;0,11	-2752
15	G;0,92	A;0,07	G;0,875	A;0.125	-2286

Table.2 The table belove shows the frequencies of the firs 15 polymorphic variants obtained by sequencing the DNA from centenarian subject and trough Sequenom analysis of DNA from general population subjects.
In addition on the left side, are reported the locations of each SNPs and the CIS ACTING elements close to each one (accordingly to Gartel et al., 1999 review). The comparison of the frequencies highlight a potential significant difference in the first 10 SNPs of the list which belong an HAPLOYPE where for Haplotype is meant a combination of alleles at multiple linked loci that are transmitted together.

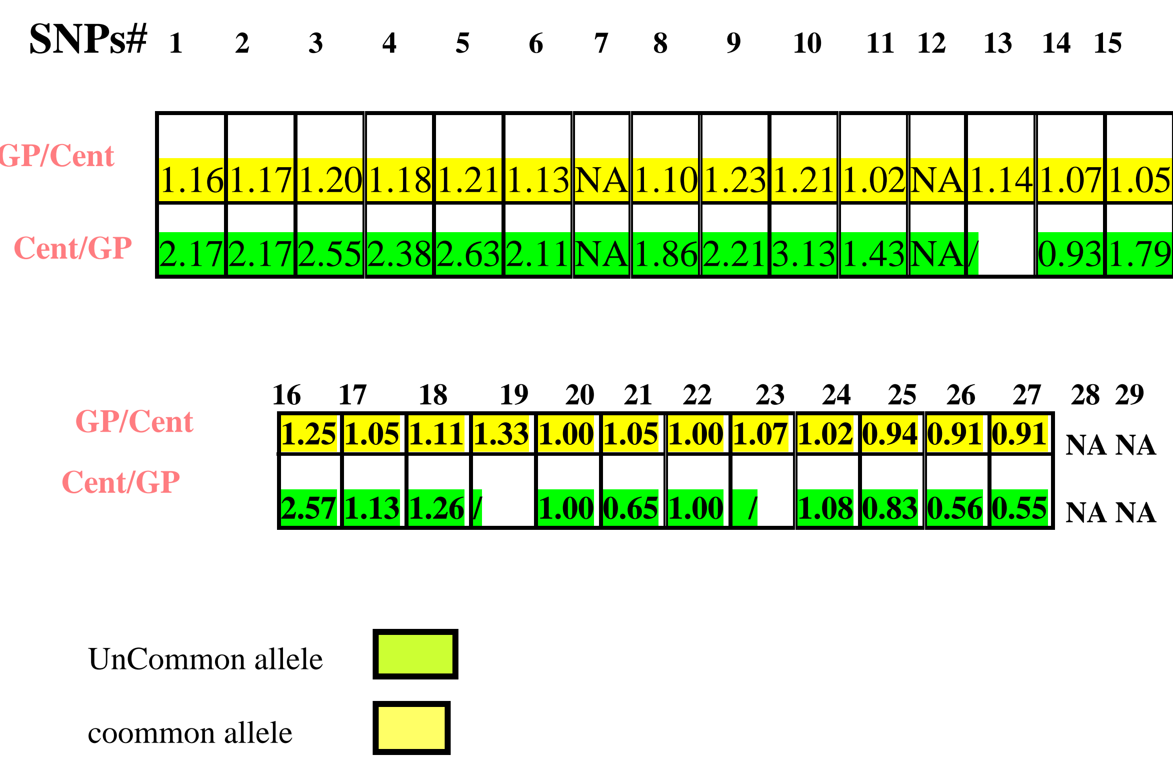
SNP#	GENERAL POPULATION		CENTENARIANS		SNP POSITION
16	C; 0,88	A; 0,105	C; O,71	A0,27	-2122
17	T:0,50	C:0,40	T; 0,55	C;0,45	-2009
18	A;0,785	G; 0,215	A; 0,71	G;0,27	-1997
19	A 1		A;0,75	T; 0,25	-1935
20	G;0,92	T;0,07	G; 0,92	T ; 0,07	-1613
21	C;0,96	T; 0,1	T;0,92	C; 0,07	-1552
22	T; 0,92	C;0,07	T;0,92	C; 0,07	-1545
23	A;1		A; 0,93	G;0,06	-1482
24	T; 0,7	C;0,29	T;0,68	C;0,31	-1309
25	A;0,665	G; 0,325	A;0,71	G;0,27	-807
26	G;0,805	C;0,195	G;0,88	C;0,11	-535
27	G;0,798	C;0,2	G;0,88	C;0,11	-515
28	NA		G;0,875	C;0,125	-150
29	NA		+cc 1		-75

KNOWN CIS ACTING ELEMENTS

Position	Element
-1924	C/EBP
-1394	P53,P73
-1263	C/EBP
-1203	RAR
-771	vitamin d
-592	stat1,2,3,
	SP1,SP3

Table.3 The table belove shows the frequencies of the last 29 polymorphic variants obtained by sequencing the DNA from centenarian subject and trough Sequenom analysis of DNA from general population subjects.In addition on the left side, are reported the locations of each SNPs and the CIS ACTING elements close to each one (accordingly to Gartel et al., 1999 review).

Ratio of the frequencies of the identified polymorphic variants



SNPs#	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
GP/Cent	1.16	1.17	1.20	1.18	1.21	1.13	NA	1.10	1.23	1.21	1.02	NA	1.14	1.07	1.05
Cent/GP	2.17	2.17	2.55	2.38	2.63	2.11	NA	1.86	2.21	3.13	1.43	NA	/	0.93	1.79

SNPs#	16	17	18	19	20	21	22	23	24	25	26	27	28	29
GP/Cent	1.25	1.05	1.11	1.33	1.00	1.05	1.00	1.07	1.02	0.94	0.91	0.91	NA	NA
Cent/GP	2.57	1.13	1.26	/	1.00	0.65	1.00	/	1.08	0.83	0.56	0.55	NA	NA

Table 4 The table belove shows the ratio between General population and Centenarian and Centenarian to General population for the respective frequencies of each allele. The ratio highlights a potential significative difference in the SNPs belonging the Haplotype (SNPs#1 to SNP#10). The SNP#16 may be potentially interesting.

HAPLOTYPE and frequencies

The existence of the haplotype has been validated trough bioinformatic analisys (see Materials and Metods- SNP alleles and genotypes statistic)

The frequencies of the haplotype carriers (almost all in etherozigoses) are:

5 among 10 centenarians (50%)

21 among 92 people in general population (23%)

P<0.158 (t-test)

7.3 **Sequenom analysis on adjuntive DNA samples**

The siginificance of haplotype representation is being verified by using Sequenom to determine the frequency of polymorphisms in ~200 centenarians and additional ~200 individuals in the general population. a $P < 0.158$ (t-test) is indicative of a trend.

7.4 Functional studies on the p21 promoter haplotype

7.4.1 Cloning of the promoter region containing the haplotype

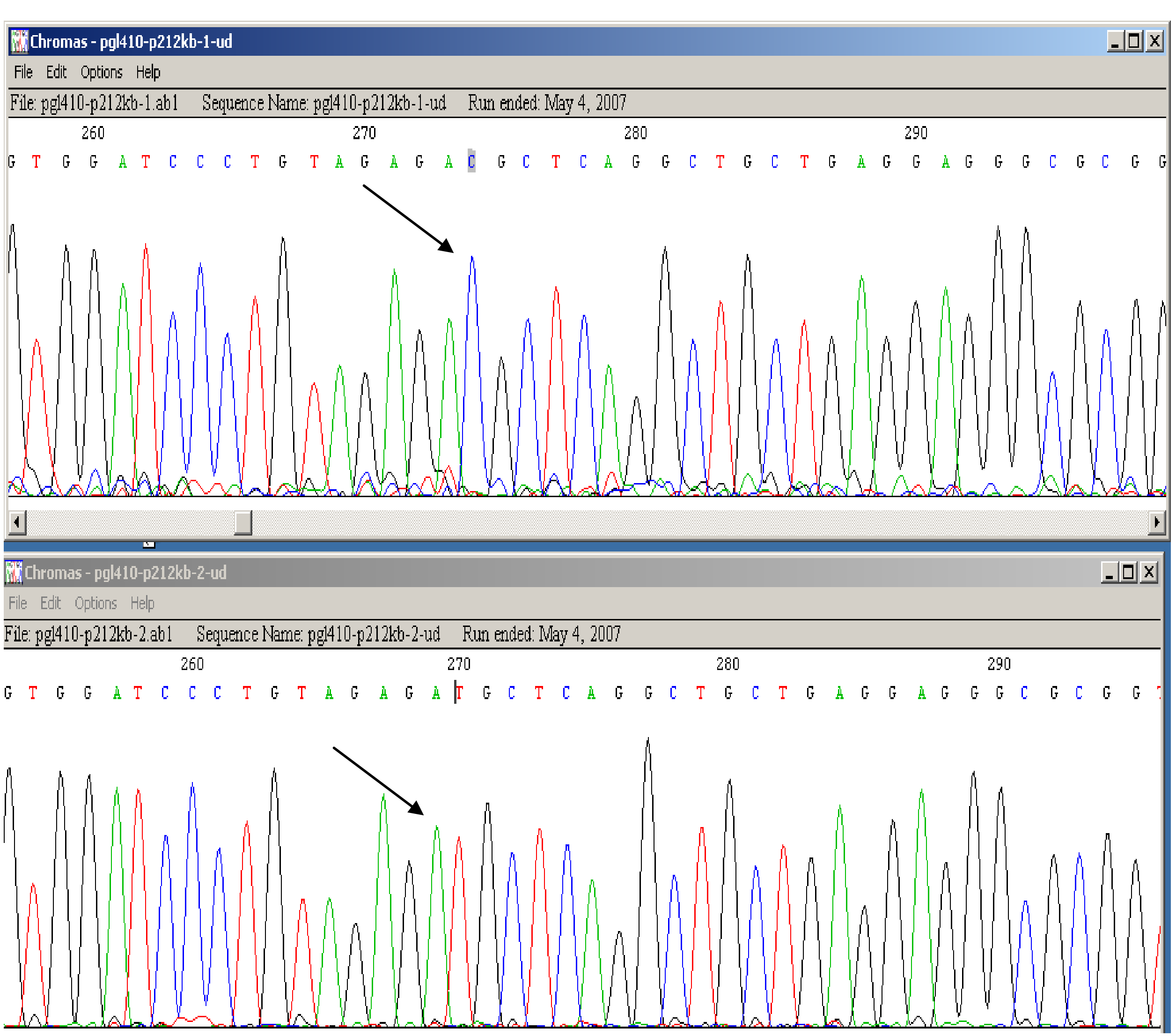


Figure 8: Sequence of the two cloned plasmid: Sequence of a region of the of the haplotype showing the two different allelels variants, the one more common in the Normal population (p21-Norm) (bottom) and the one more common in the centenarians one (p21-Cent)

7.4.2 Luciferase assays carried out on cells HCT116 (p53 WT and p53 null) transfected with p21-N and p21-C

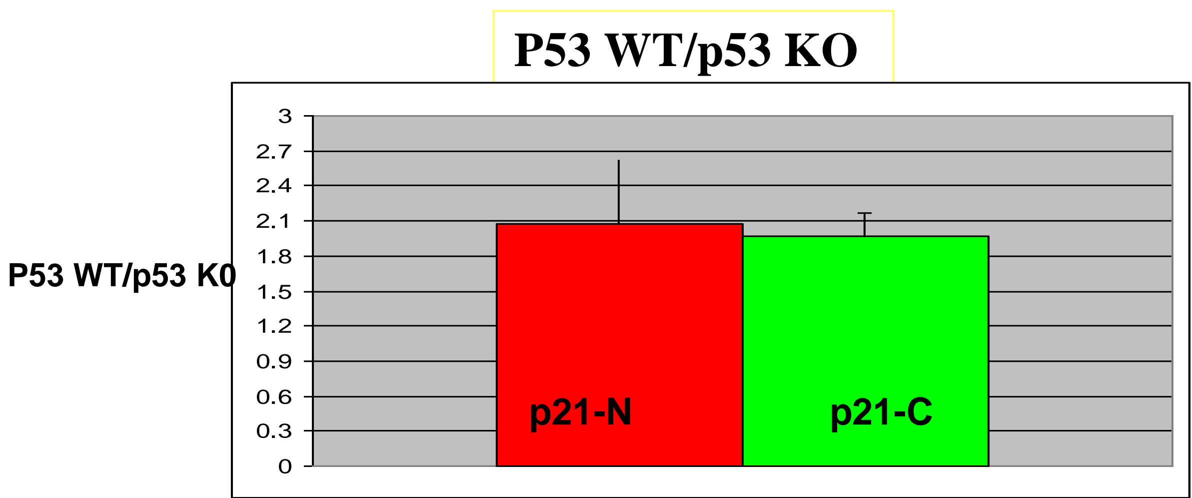


Figure 9: Luciferase assays were carried out on cells HCT116 (p53 WT and p53 null) transiently transfected with p21-Luc plasmids containing the 2 haplotype variants, p21 N (common population haplotype) and p21-C (centenarian population haplotype) . Initial assays carried out with a single preparation of the common (p21N) and centenarian (p21C) promoter haplotypes, shows that both promoter alleles were 2-fold more active in the presence of p53: no difference in p53 response

Luciferase Assays carried out on HCT116 cells (p53 WT or KO) transiently transfected with p21-N and p21-C

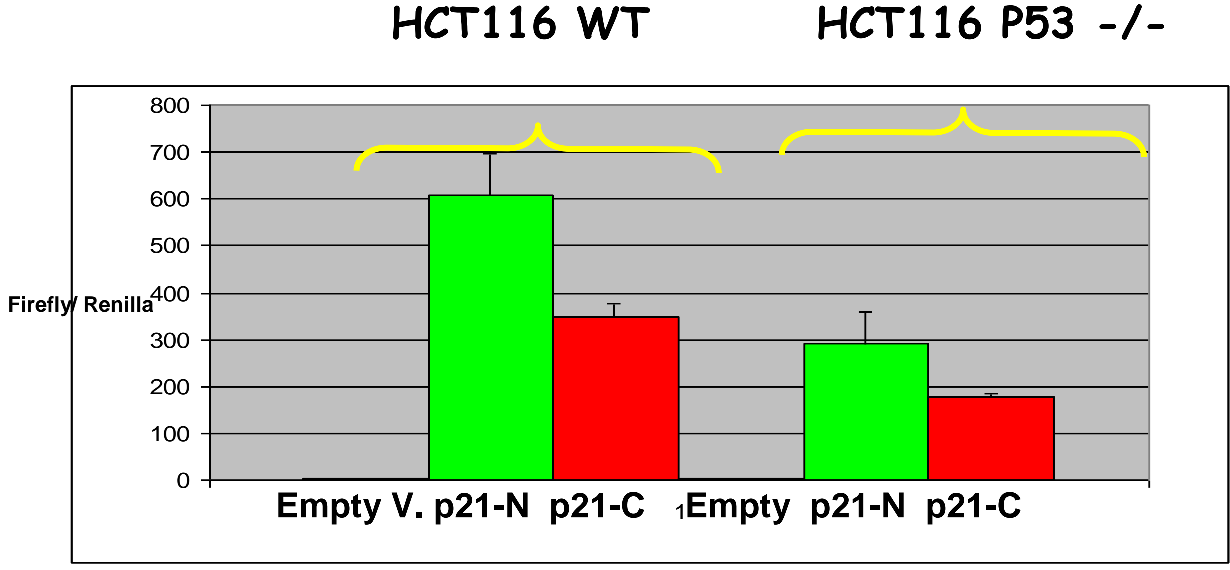


Figure 10: Luciferase assays carried out on cells HCT116 (p53 WT and p53 null) transiently transfected with p21-Luc plasmids containing the 2 haplotype variants, p21 N (common population haplotype) and p21-C (centenarian population haplotype) . The initial assay suggested ~2-fold decrease in the basal promoter activity in the centenarian haplotype

Quality of plasmid used for the transfection

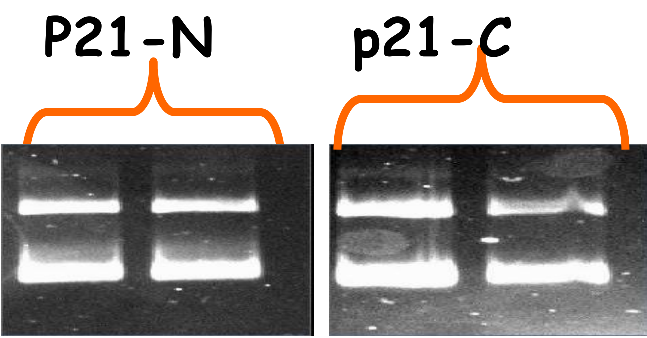


Figure 11: Analysis of the quality of the two plamid preparations run on an electroforesis gel showed that the two plasmids have similar quality

Luciferase Assays carried out on HCT116 cells (p53 WT or KO) transiently transfected with p21-N and p21-C in three indipendent DNA preparations

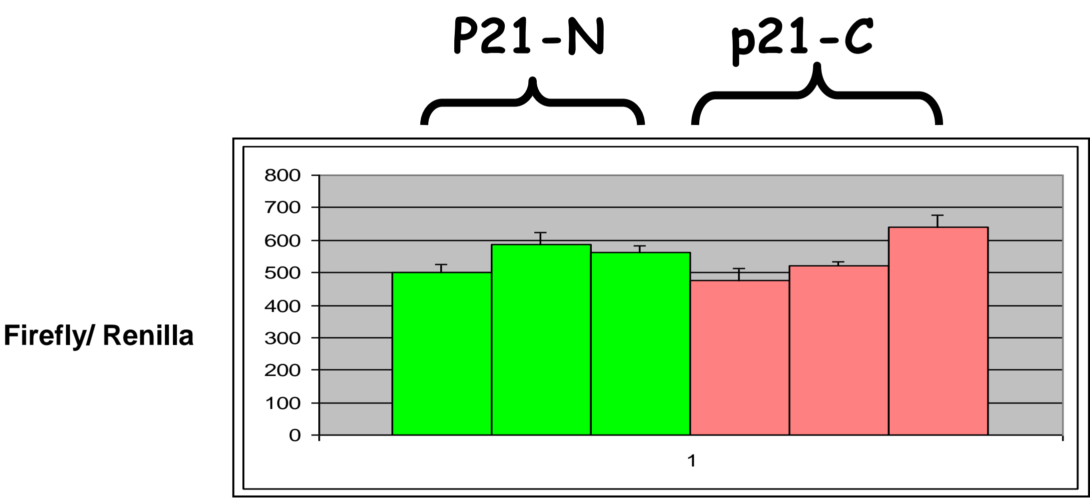


Figure 12: Luciferase assays carried out on cells HCT116 (p53 WT and p53 null) transiently transfected with three indipendent DNA preparation of p21-Luc plasmids containing the 2 haplotype variants, p21 N (common population haplotype) and p21-C (centenarian population haplotype) .
The three independent DNA preparations did not confirm the originally observed difference in basal promoter levels.

CHAPTER 8: RESULTS PROJECT #2

8.1.1 Microarrays analysis on changes in gene expression in aging: elaboration of a Centenarian-gene signature

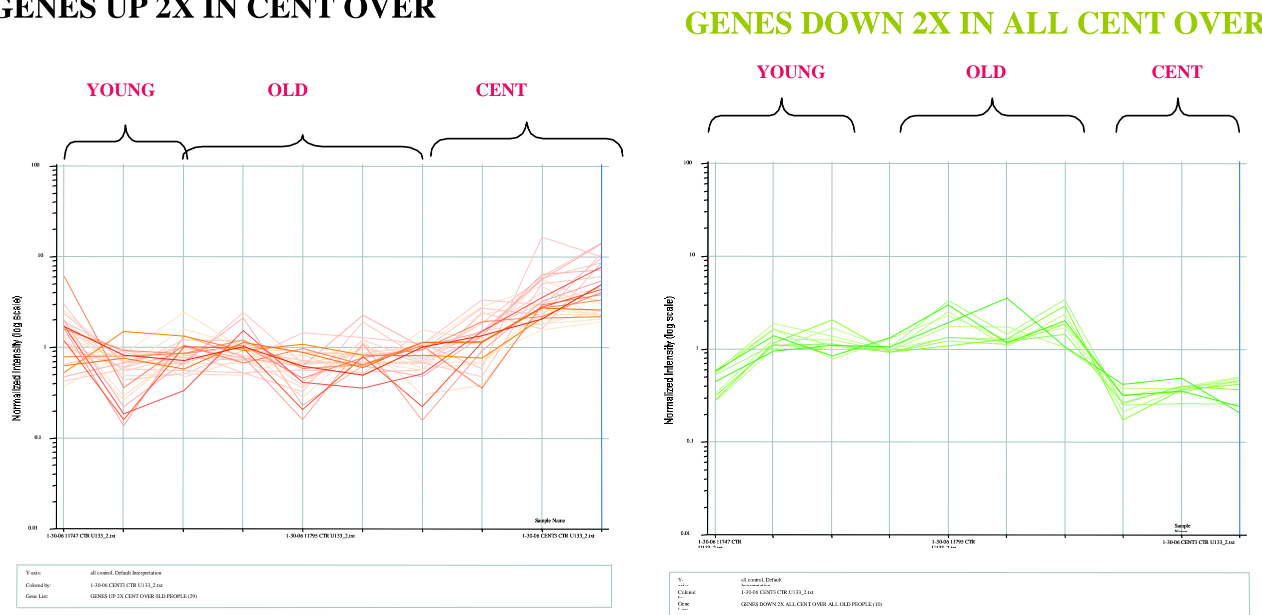


Figure 13: Identification of genes showing age correlations performed using the program GeneSpring.

The Microarray analysis of gene expression in 10 cultures from individuals of different ages, yields a tentative "centenarian signature": Identified 29 genes that show higher expression (>2X) in centenarians relative to old people and 10 genes that show lower expression (<2X) in centenarians versus old.

8.1.2 List of the centenarian signature-genes

- **Genes up regulated in the centenarians versus old people**

Systematic	Common	Gene Symbol	UniGene
233011_at	ANXA1		Hs.494173
209840_s_at	LRRN3		Hs.3781
204014_at	DUSP4; TYP; HVH2; MKP2; MKP-2	DUSP4	Hs.417962
209841_s_at	DKFZp761K2424; NLRR3; NLRR-3; FLJ11129; LRRN3	LRRN3	Hs.3781
201743_at	CD14	CD14	Hs.163867
230967_s_at	USP7		Hs.386939
214265_at	ITGA8		Hs.171311
220694_at	HSPC054		
232925_at			
222167_at	PVRL3		Hs.293917
230970_at	SSH2		Hs.335205
219352_at	HERC6;FLJ20637	HERC6	Hs.529317
1564378_a_at	EXT1		Hs.492618
228635_at	PCDH10		Hs.192859
1559249_at	ATXN1		Hs.434961
241970_at	PVRL3		Hs.293917
206197_at	NME5; NM23H5; NM23-H5	NME5	Hs.519602
237459_at	PCTK2		Hs.506415
215330_at	FLJ43663		Hs.150556
209911_x_at	HIST1H2BD; H2B/b; H2BFB; H2B.1B; HIRIP2; MGC90432; dJ221C16.6	HIST1H2BD	Hs.130853
202086_at	MX1; MxA; IFI78; IFI-78K	MX1	Hs.517307
230206_at	DOCK5		Hs.195403
236798_at			Hs.551751
215992_s_at	DKFZp586O1422; RA-GEF;	RAPGEF2	Hs.373588
39402_at	IL1B		Hs.126256
220898_at			
228275_at			Hs.551751
201272_at	AKR1B1; AR; ADR;	AKR1B1	Hs.521212
243788_at	PHF11		Hs.369039

- **Genes down regulated in the centenarians versus old people**

Systematic	Common	Gene Symbol	UniGene
213023_at	UTRN; DRP; DMDL; DRP1; FLJ23678	UTRN	Hs.133135
205018_s_at	MBNL2		Hs.372571
204841_s_at	EEA1; ZFYVE2	EEA1	Hs.567367
238420_at	TAOK1		Hs.399763
1552717_s_at	KAB		Hs.433891
204969_s_at	RDX	RDX	Hs.263671
225575_at	LIFR		Hs.133421
204864_s_at	IL6ST; CD130; GP130; IL6R-beta;	IL6ST	Hs.532082
229487_at	EBF		Hs.573143

8.2 Correlation of age-specific difference in gene expression with transcriptional effects of p21

8.2.1 The centenarian signature in the p21 (Wi38) model

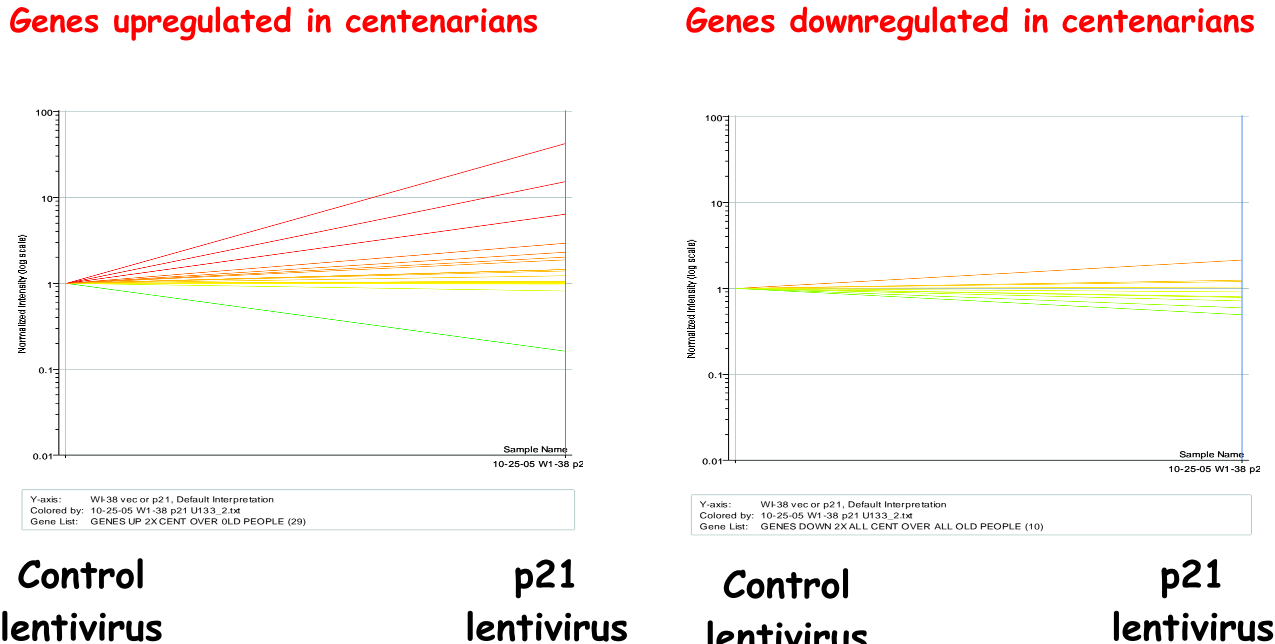


Figure 14: Microarrays analysis has been performed in cells WI38 where the ectopic expression of p21 has been obtained by transfecting those cells with a p21 lentivirus vector.
The image above show the behavior of the the genes comprising this signature in the p21 WI38 model : a subset of genes upregulated in centenarians is induced by p21 and a subset of genes downregulated in centenarians is inhibited by p21.

<u>a subset of genes that were up- or downregulated in centenarians showed the same response to ectopic expression of p21, yielding a putative “p21-centenarian” signature</u>

8.2.2 The p21-centenarian signature genes

down in cent over old and down in p21

WI-38 vec or p21 Sample Name ()*	W1-38 vec	W1-38 p21	
Systematic	Raw	Raw	Common
238420_at	283.1722	226.3846	TAOK1
204969_s_at	465.4081	228.6547	RDX
244187_at	971.4686	571.0795	SATL1
213023_at	171.0369	122.6468	UTRN; DRP; DMDL; DRP1; FLJ23678
205018_s_at	967.8409	872.7402	MBNL2
204841_s_at	277.5317	218.18	EEA1; ZFYVE2

up in cent over old and up in p21

WI-38 vec or p21 Sample Name ()*	W1-38 vec	W1-38 p21 U133_2.txt	
Systematic	Raw	Raw	Common
236798_at	316.8349	456.0996	
202086_at	62.55866	2620.27	MX1; MxA; IFI78; IFI-78K
209911_x_at	1570.459	3618.715	HIST1H2BD; H2B/b; H2BFB; H2B.1B; HIRIP2; MGC90432; dJ221C16.6
201272_at	5672.731	16654.81	AKR1B1; AR; ADR; ALDR1; MGC1804
228275_at	285.584	531.0973	
39402_at	28.33118	179.7332	IL1B; IL-1; IL1F2; IL1-BETA
209841_s_at	1618.85	2267.344	DKFZp761K2424; NLRR3; NLRR-3; FLJ11129; LRRN3
204014_at	23.40486	33.5471	DUSP4; TYP; HVH2; MKP2; MKP-2
209840_s_at	1062.826	1525.926	LRRN3
206197_at	14.67919	29.44429	NME5; NM23H5; NM23-H5
219352_at	30.15033	460.1755	HERC6; FLJ20637
232925_at	23.82106	29.10596	

The tables above list the p21-centenarian-signature genes. The raw data relative to the subset of genes that were up- or downregulated in centenarians show a concordant response to the ectopic expression of p21 (WI38 vec =control, WI38 p21= WI38 cells transfected with p21 lentivirus vector)

8.3 DNA damage response of the p21-centenarian signature genes showing a correlation stress/aging tested by RT-QPCR in an additional set of young and old samples (15) treated with p21-inducing drug doxorubicin

8.3.1 DNA damage response of the p21-centenarian signature genes showing a correlation stress/aging in Young subjects

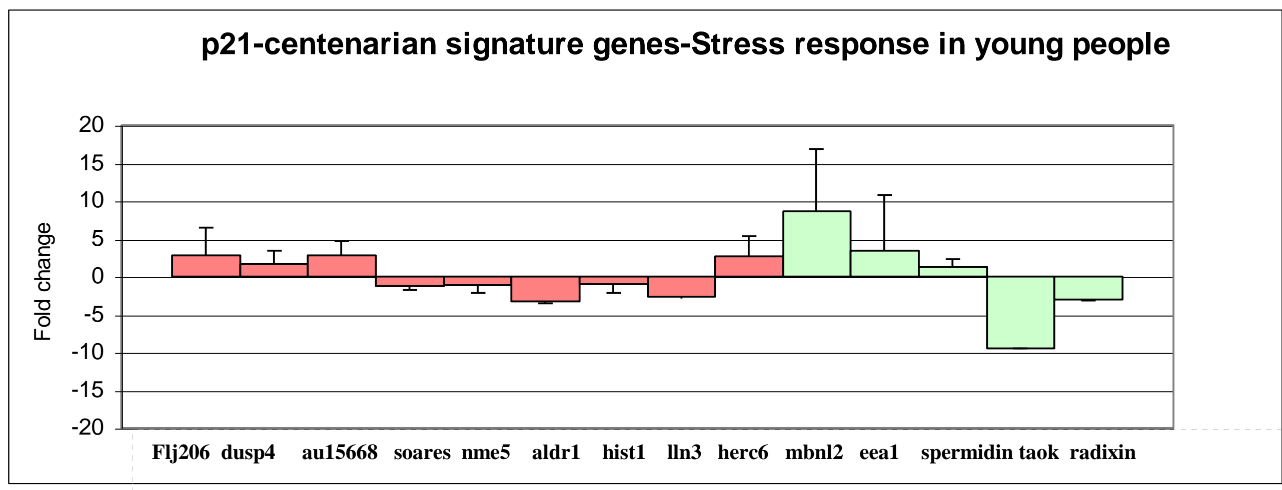


Figure 15

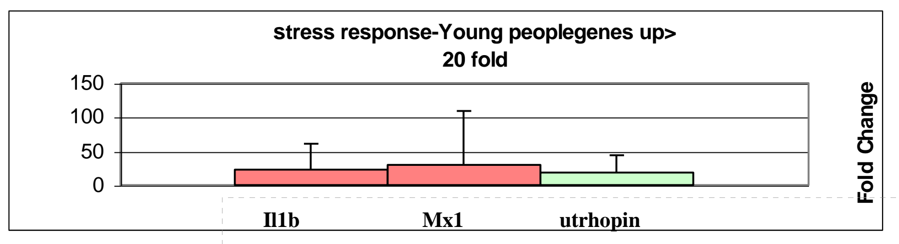


Figure 16

Figure 15-16: DNA damage response of the p21-centenarian signature genes showing a correlation stress/aging was tested by RT-QPCR in an additional set of young and old samples treated with p21-inducing drug doxorubicin .
Rapresented In red the induced genes of the p21-centenarian signature , in green the downregulaed ones.
The data above are relative to the response to stress in Young Subjects .
Some genes up regulated or downregulate in the p21-centenarian signature showed concordant response in the subset of young subjects samples. Notably Il1 Beta, MX1 and utrophin are strongly upregulated upon stress in Young subjects. (figure 16)

8.3.2 DNA damage response of the p21-centenarian signature genes showing a correlation stress/aging in old people

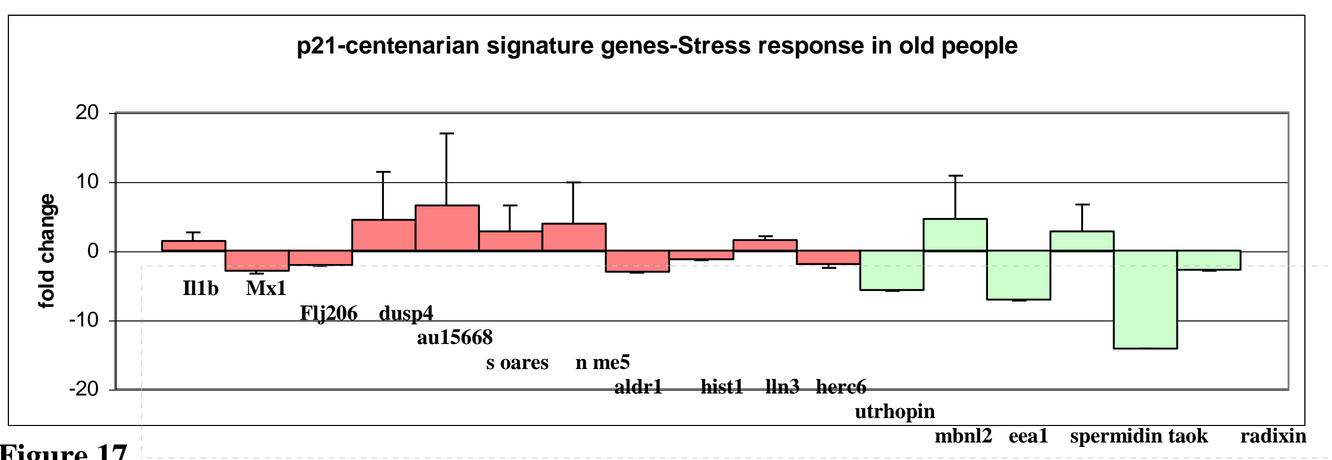


Figure 17

Figure 17: DNA damage response of the p21-centenarian signature genes showing a correlation stress/aging tested by RT-QPCR in an additional set old samples treated with p21-inducing drug doxorubicin.
Represented In red the induced genes of the p21-centenarian signature, in green the downregulaed ones.

Some genes up regulated or downregulate in the p21-centenarian signature showed concordant response in the subset of old subjects samples.

Interestingly, same genes showed different behavior in the old subjects compared to the young ones. Il1 beta is much strongly induced in the young subjects , like also MX1 . Flj206 is downregulated in old people upon stress while is upregulated in young subjects as well for HERC6.
Finally strongest differences are found in the Utrophin gene behaviour upon stress: in Young people it is strongly induced, in old the gene is downregulated.
More details are showed in the following figure.

8.3.3 Utrophin : an age-dependent response to DNA damage

UTROPHIN

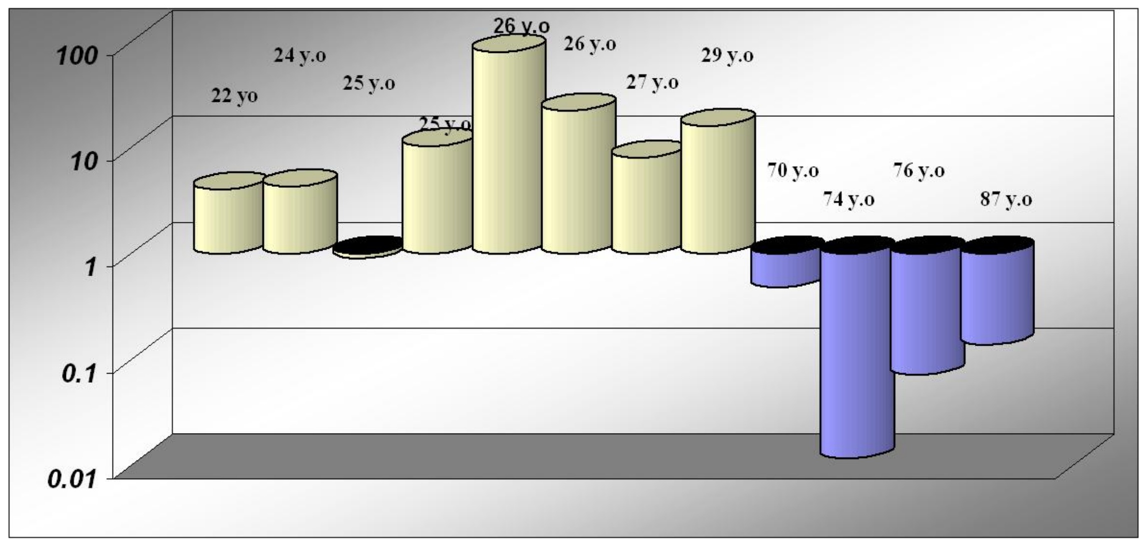


Figure 18

P<0. 03(t-test)

Figure 18: response for the gene Utrophin (gene comprising the p21-centenarian signature) to Doxorubicin treatments in dermal fibroblast from individuals of different ages (The age of each individual is indicated above)

In yellow values relative to young people, in blue the one relative to old ones.
Utrophin is one of the gene of the p21-centenarian signature showing the strongest differences in the response to the DNA damaging agent doxorubicin.
Notably the gene is generelly upregulated in young sujbects upon and downregulated in old subjects upond treatment with Doxorubicin.

8.3.4 GENERALITIES ABOUT UTROPHIN

Utrophin is a protein of the cytoskeleton. Utrophin was found during research into Duchenne's muscular dystrophy. The name is a contraction for *ubiquitous dystrophin*. The 900 kb gene for utrophin is found on the long arm of human chromosome 6. Utrophin was discovered due to its homology with dystrophin. It was found by screening a peptide containing the C-terminal domain of dystrophin against cDNA-libraries. The homology varies over its full length from less than 30% in regions of the central rod structural domain, to 85% (identity 73%) for the actin binding domain.

The tertiary structure of utrophin contains a C-terminus that consists of protein-protein interaction motifs that interact with dystroglycan, a central rod region consisting of a triple coiled-coil repeat, and an actin-binding N-terminus.

In normal muscle cells, utrophin is located at the neuromuscular synapse and myotendinous junctions. It is necessary for normal membrane maintenance, and for the clustering of the acetylcholine receptor. In adult humans, utrophin RNA is found ubiquitously, as the name implies, being abundant in the brain, kidney, liver, lung, muscle, spleen and stomach. In the human fetus during muscle differentiation, utrophin is found at the sarcolemma. It disappears when the fetus begins to express dystrophin.

Utrophin expression is dramatically increased in patients with Duchenne's muscular dystrophy (and female carriers), both in those muscle fibers lacking dystrophin and in rare, revertant fibers that express dystrophin.

No reports have yet associated mutation in the utrophin gene with disease, but it does not seem to play a critical role in development, since mice without utrophin develop normally.

8.4 Basal levels of the p21-centenarian signature genes showing a correlation stress/aging tested by RT-QPCR in an additional set of young and old samples (15)

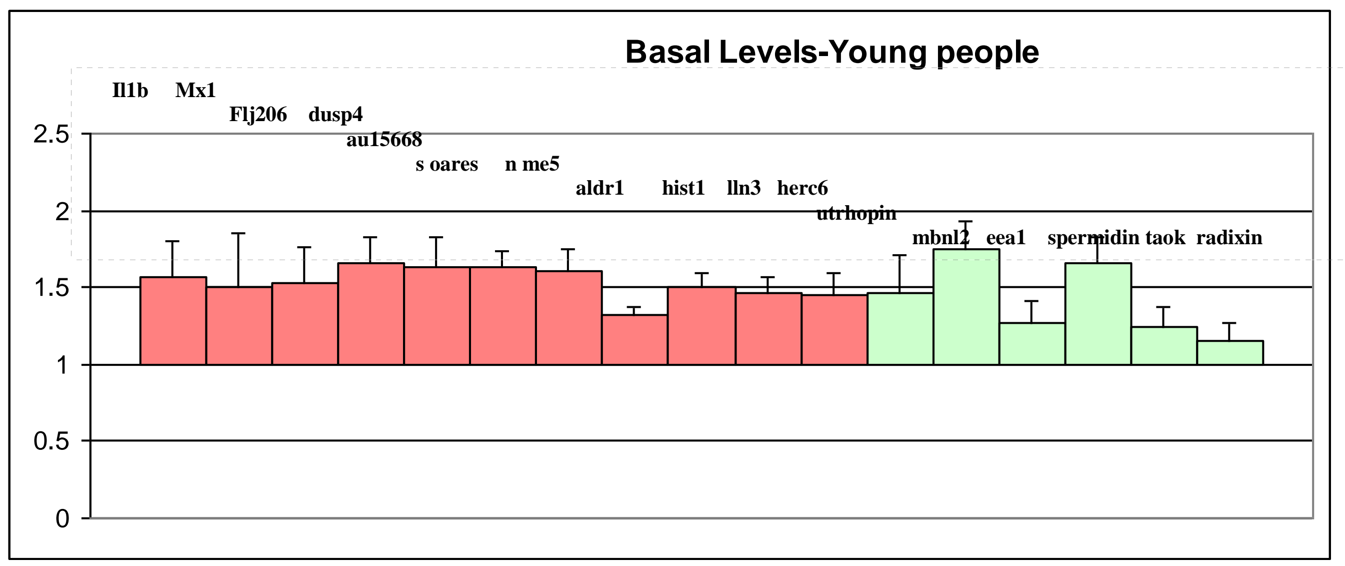


figure 19: basal levels-Young subjects

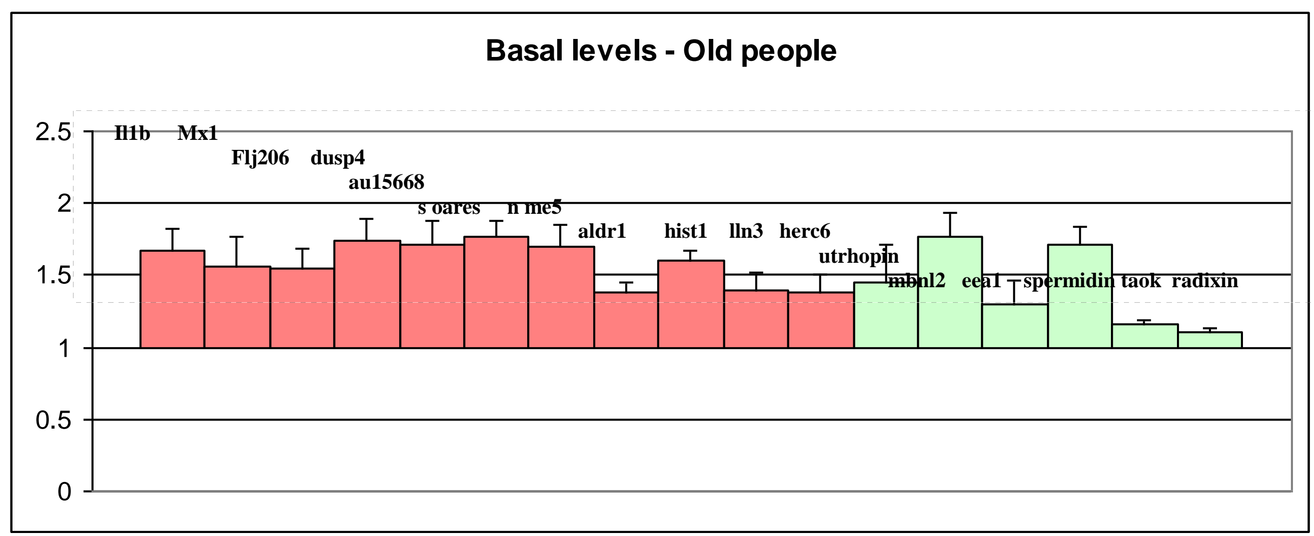


figure 20: basal levels –old subjects

figure 19-20
Basal levels of the p21-centenarian segnature genes were tested by RQ_PCR in the an additional set of young and old samples (15)
The basal levels of the p21-centenarian genes are , as showed in the microarrays analysis , are similar among young and old people.

8.5 Microarrays on an additional set of samples

We have extended the study to a set of fibroblast samples 15 (5 young, 4 old, 5 >90 y.o subjects) before and upon treatment with the DNA damaging agent Doxorubicin in order to evaluate whether the the data obtained are confirmed.

We recently got the program Genespring update in order to read the results from exon.1 chips. The analysis is in progress.

DISCUSSION

CHAPTER 9: Project #1

Identification of polymorphic variants in the p21 gene and their possible involvement in longevity

Induction of cyclin-dependent kinase inhibitor p21(Waf1/Cip1/Sdi1) triggers cell growth arrest associated with senescence and damage response.
In 2000 a paper from Roninson' lab came out showing that p21 also up-regulates multiple genes that have been associated with senescence or implicated in age-related diseases.

The main goal of this study consisted in testing the hypothesis that there are polymorphisms in the p21 gene that characterize the centenarian population and may affect the induction of the p21 regulated genes involved in age-related disease and eventually implicated in longevity and healthy aging.
Centenarians exhibiting not only an extreme longevity but having also have delayed, survived or escaped diseases associated with the aging process (such as cardiovascular disease, Alzheimer's disease, and some types of cancer) are an ideal group of people for the study of specific genes that are either directly associated with longevity or enable people to escape or delay the effects of age-related diseases.

The study was carried out in different steps by first sequencing the p21 gene and promoter using DNA from 10 centenarians in order to identify polymorphic variants and their frequencies in the centenarian population. Then we used the Sequenom strategy to determine the frequencies of the polymorphisms in about 100 individuals in the general population in order to identify polymorphisms that are present at a lower frequencies in the general population.

By sequencing of the 3 exons of the gene p21 we found an already characterized polymorphic variant which leads to a transition A transition A->C at codon codon 31 which leads to Arg-> Ser.
Accordingly to the data published on NCBI, this polymorphic variant is present in the general population at a similar frequencies found in the DNA of centenarian sequenced Sequencing the whole promoter lead us to find out that the promoter is strongly characterized by polymorphic variants.
Infact, sequencing of the whole promoter lead us to the identificaty 29 polimorphic variants.
Even tough just 2.5 kb of the promoter have been so far extensively studied most of the polymorphic variants have been already found by others so reported on NCBI but only few frequencies were known in the general population.

To have a better understanding of the frequencies of the whole polymorphic variants in the general population we used the Sequenom Strategy on about ~100 individual in the general population.
The analysis trough haplomap highlighted the presence of an Haplotype , a combination of alleles at multiple linked loci that are transmitted together. The Haplotype characterized by 10 polymorphic variants, is located in ~-4/-5 kb and located in the region where a novel p53 binding site has been recently found (Saramaki et al.,2006).
The frequencies of the haplotype carriers (almost all in etherozigoses) are (50%) centenarians and (23%) for the general population with a P< 0.158 (t-test) , indicative of a trend.

As far the other polymorphic variants we found another potential interesting one, a SNP (#16) located in proximity of the other p53 binding site (-2.300) as well p73 binding site.
The majority of the polymorphic variants, however, showed similar frequencies in the centenarian and general population. This, indirectly, is an indication of the validity of the sequencing results.

The siginificance of haplotype representation (as well the frequencies of the other polymorphic variants) is being verified by using Sequenom to determine the frequency of polymorphisms in ~200 centenarians and additional ~200 individuals in the general population.

In the meantime, I carry out functional studies (luciferase assays) for those polymorphic variants centenarian-specific in order whether the putative-centenarian - haplotype affects the p21 promoter activity.
In order to perform this study, it has been necessary to clone the whole promoter in plasmid contain the Luciferase reporter gene.
Thus I obtained the two plasmid representative of the centenarian-haplotype promoter and of the general population haplotype one.

The Luciferase assays were carried out on cells HCT116 (p53 WT and p53 null) transiently transfected with p21-Luc plasmids containing the 2 haplotype variants, p21 N (common population haplotype) and p21-C (centenarian population haplotype) .
The assays carried out with a single preparation of the common (p21N) and centenarian (p21C) promoter haplotypes, showed that both promoter alleles were 2-fold more active in the presence of p53: so in conclusion there is no difference in p53 response.
The initial assay suggested also ~2-fold decrease in the basal promoter activity in the centenarian haplotype.
In order to confirm this data the assays has been repeated in three independent DNA preparations which did not confirm the originally observed difference in basal promoter levels, despite the first two plasmids preparation showed similar quality.

Anyway, despite similar basal promoter activities and p53 response, as discussed in the introduction, there are many potential physiological situations p53 indepentent where the response of the two alleles may be different .
Interestingly the p21 promoter is induced by some signals involved in stress response and inflammation like TGF beta, INF Gamma, IL6 that, as discussed above, plays an important role in longevity (see inflammaging).

In conclusion the p21 promoter appears to represent a unique sensor that is able to integrate many extracellular stress signals into cellular decisions . the p21 promoter, regulated not only by p53 but also by a variety of very different extracellular signals , may generate different response depending on environmental factors and haplotype variants affected.

CONCLUSIONS #1

We tested the hypothesis that p21 is involved in longevity:

- We sequenced the gene and its promoter in 10 centenarians and found the total of 31 SNPs
- Out of 29 promoter SNPs 6 were previously unreported and 23 had no known frequencies
- We compared the frequencies of the SNPs in 10 centenarians and 92 members of general population

We found

- HAPLOTYPE located in -5kb region of the p21 promoter whose frequency is ~ 2 fold higher in centenarians than in the general population
- Large-scale analysis of haplotype frequencies is currently in progress
- The “centenarian” haplotype doesn’t affect the basal p21 promoter activity or its response to p53
- There are many other possible physiological conditions in which the centenarian allele of the p21 promoter may potentially show a different response (IL6, IFN,progesterone, vitamin E, Vitamin D etc)

CHAPTER 10: Project #2

Evaluation the differences in gene expression between centenarians, elderly, young in dermal fibroblast cultures and their response to p21 and DNA damage.

Aging is characterized by a deleterious and progressive decline in physiological effieciency.

It has been proposed the view that aging is a remodeling process where the body of survivors progressively adapts to internal and external damaging agents they are exposed to during several decades and their lifetime. Since multiple factors are involved in the aging process, it is still difficult to collectively understand the global network involved.

cDNA microarrays becomes a growing technology which can be used to better understand the global networking of individual genes. Thus in this study , we used cDNA micorarrays to investigate the relationship between DNA damage response and age related changes in gene expression.

Microarrays analysis were performed on actively dividely dermal fibroblasts cultures from subject young, elderly and centenarians treated and untreated with the DNA damaging agent doxorubicin which in turn acts inducing oxidative stress (thus cell senescence) and p21.
We used the unique materials from centenarians who can be considered as the best model of healthy aging and at the same time, intriguitly enough, they are also by definition extremely old people and show all the signs and the characteristics of a prolonged aging process.

Here first, by performing Microarrays analysis (using the program Genespring) analysis of gene expression in 10 cultures from individuals of different ages we identified genes showing age correlations (pilot study) thus elaborating a signature of genes that are upregulated or downreguated in centenarians compared to old people. (see table and list)
Interestingly we found major differences comparing old and centenarians than comparing young and centenarian subjects.
The centenarian "signature" was then tested in other different models particularly, we wanted to correlate age-specific difference in gene expression with transcriptional effects of p21.
To carry out this step we compared the data obtained from the Microarray analysis of gene expression from individuals of different ages, with the Microarrays data relative to fibroblast WI38 transfected with a lentivirus which induces an ectopic expression of p21. (see figure)
Interestingly, we found that a subset of genes that were up- or downregulated in centenarians showed the same response to ectopic expression of p21, yielding a putative "p21-centenarian" signature.

Some of those genes are involved in stress response and inflammation such as IL1beta, MX1, other in signal transduction such as TAOK but all are interestingly downstream of p21 , so stress responsive genes.

We then wanted to determine the determine the effects of DNA damage (which induces p21) on the expression of identified genes (trough RQ-PCR) in independent samples treated with the agent Doxorubicin which induces oxidative stress and p21.

Carrying out RQ-PCR assays on a subset of 14 samples for the main genes of the signature we found out that some genes differently respond to stress in young and old subjects such as Utrophin, flj206, Il1beta, MX1 .

Strikingly, upon stress, utrophin is generally downregulated in old people while it is upregulated in young subjects. ($p<0.03$)

Utrophin is is a protein of the cytoskeleton that was found during research into Duchenne's muscular dystrophy . Its functions is not really known : in case the behaviour of this gene were confirmed by our microarrays results (analysis in progress), it would be interesting to evaluate its functions.

The studies also highilighted that Il1 beta and MX1 are strongly upregulated in young people while weakly upregulated in old subjects (Il1beta) or downregulated (MX1).

The gene fjl206 and Herc6 are still generally upregulated in young people and downregulated in old people.

We also found genes such as TaoK and Radixin that show similar behaviour upon stress in the two groups, being both downregulated upon stress in young and old people.

Taok and Radixin are also the downregulated ones in the p21-centenarian signature (i.e. genes downregulated in centenarians compared to old people and downregulated by p21 induction).

The basal levels of those genes of the p21-centenarian signature genes are, as expected, expressed at similar levels in young and old people confirming the data obtained trough microarrays analysis.

Summarizing , the data obtained trough microarrays and RQ-PCR lead us to identified genes showing a strong relationship between p21-mediated damage response and aging

Testing the DNA damage response of the p21-centenarian signature genes showing a correlation stress/aging by RT-QPCR in an additional set of young and old samples (15) treated with p21-inducing drug doxorubicin, we found out that there is a subset of genes regulated by p21 that responde to stress in an age-specific manner.

We are postulating that there are changes in gene expression due to the interaction with enviromental factors might be gradually “fixed” in certain highly stress responsive genes thus resulting in a “Memory of stress” and that p21 may have a role in this process.

we extended the study to a set of fibroblast samples 15 (5 young, 4 old, 5 >90 y.o subjects) before and upon treatment with the DNA damaging agent Doxorubicin performing other Microarrays on those 15 subjects (under analysis).

CONCLUSIONS #2

- Microarray analysis of gene expression in dermal fibroblast cultures from ten individuals of different ages yielded a tentative "centenarian signature"

- A subset of genes that were up- or downregulated in centenarians showed the same response to ectopic expression of p21, yielding a putative "p21-centenarian" signature

- by RT-PCR in an independent set of 14 fibroblast cultures from subject of different ages treated with the p21-inducing DNA-damaging drug doxorubicin We found that some genes of the putative p21-centenarian signature showed a response aging-specificto stress
- -the gene utrophin is an example of gene showing the corresponding response to p21-inducing DNA-damaging drug doxorubicin

- These findings suggested the "memory of stress" hypothesis that postulates that some of the p21-mediated changes in gene expression become "fixed" over time in centenarians

- The "Memory of stress" hypothesis is being tested by microarray analysis of doxorubicin response in cultures from 14 indivuduals of different ages

GENERAL CONCLUSION

This work of this PhD thesis has been focused on elucidating the relationship between stress response mechanisms and aging (and healthy aging) carrying out two project that investigated the same problem from different angles.

One of the most fascinating theory of aging postulates that aging is a remodeling process where the body of survivors progressively adapts to internal and external damaging agents they are exposed to during several decades.
In this sense , stress response and adaptation mechanisms play a fundamental role in the aging process where the capability of adaptating effects, certainly, also is related the lifespan of each individual.
A key gene linking aging to stress response is indeed p21, an induction of cyclin-dependent kinase inhibitor which triggers cell growth arrest associated with senescence and damage response and notably is involved in the up-regulation of multiple genes that have been associated with senescence or implicated in age-related .

For this reason p21 is a interesting candidate gene linking stress response mechanisms, aging and potentially longevity.

Trough my the first part of this PhD project we tested the hypothesis that there are polymorphic variants in the p21 gene possibly involved in longevity.
By sequencing the whole gene and the promoter in the DNA from centenarians and general population we identified an haplotype potentially involved in longevity located in -4 kb of the promoter and in proximity of a novel p53 binding site.
Trough functional studies we also highlighted that the haplotype doesn't effect the response to p53 and the the basal promoter activity is similar to the variant more common in the general population. However, as discussed above , there are many different physiological situation in which the centenarian-haplotype and the general-population one might responde differently.

In the second study we investigate the relationship between stress response and aging from a "gene expression" point of view using Microarrays analysis on actively dividely dermal fibroblasts cultures from subject young, elderly and centenarians treated and untreated with the DNA damaging agent doxorubicin which in turn acts inducing oxidative stress (thus cell senescence) and p21.
we elaborate a gene expression signature of longevity and found that a subset of genes that were up- or downregulated in centenarians showed the same response to ectopic expression of p21, yielding a putative "p21-centenarian" signature.

We tested the genes of the signature trough RT-PCR in an independent set of 14 fibroblast cultures from subject of different ages treated with the p21-inducing DNA-damaging drug doxorubicin .
We highlighted that some genes of the putative p21-centenarian signature responde to stress in a aging-specific manner .

These findings suggested the “memory of stress” hypothesis that postulates that some of the p21-mediated changes in gene expression become “fixed” over time in centenarians. We are currently thesting this hypothesis by microarray analysis of doxorubicin response in cultures from 14 indivuduals of different ages .

We are also extending the study to other models (c.elegans) thanks to a collaboration set up with Dr. Dillin at Salk Institute where they are evaluating the role of some of the p21-centenarian signature genes in *c.elegans* .

The Dillin Lab is going to perform RNAi on long lived mutant worms to evaluated whether the long lived phenotype may become suppressed.

The concept of “fixation” of stress, memory of stress hypothesis that we are proposing has some interesting overlaps with some findings just recently pubblished by Lu et al., investigating gene regulation and DNA damage in the ageing human brain. (see Background)

Lu et al., examined the promoters of 30 different genes and they observed that DNA damage targeted the promoter of genes that are repressed with age but did not affected or that increase expression with age.

They suggested that selective DNA damage gene promoter sequences is a potential mechanism whereby the expression of specific genes could register the passage of time.

Some of the mechanisms of “registration” of passage of time could be epigenetics, infact just recently another paper came out suggesting that DNA methylation in the human cerebral cortex is dynamically regulated throughout the life span (Siegemund et al.,).

The concept of “registering” the passage of time thus the idea of a memory of “stress” are hopefully opening new scenario to the examination of aging from novel prospective where pilot genes involved in stress such as p21 might have a key role.

BIOGRAPHY

Beckman KB and Ames BN. The free radical theory of aging matures. *Physiol Rev* 78: 547-581, 1998.

Bey-Dih Chang, Keiko Watanabe , Eugenia V. Broude, Jing Fang, Jason C. Poole, Tatiana V. Kalinichenko, and Igor B. Roninson' PNAS | April 11, 2000 | vol. 97 | no. 8 | 4291-4296 Effects of p21$^{Waf1/Cip1/Sdi1}$ on cellular gene expression: Implications for carcinogenesis, senescence, and age-related diseases

Becker K.G., Owen A.B., and S.K. 2006. Transcriptional profiling of *aging* in human muscle reveals a common *aging* signature. *PLoS Genet.* 2: e115.

Blalock E.M., Geddes J.W., Chen K.C., Porter N.M., Markesbery W.R., and Landfield P.W. 2004. Incipient Alzheimer's disease: Microarray correlation analyses reveal major transcriptional and tumor suppressor responses. *Proc. Natl. Acad. Sci.* 101: 2173–2178.

Blalock E.M., Chen K.C., Sharrow K., Herman J.P., Porter N.M., Foster T.C., and Landfield P.W. 2003. Gene microarrays in hippocampal *aging*: Statistical profiling identifies novel processes correlated with cognitive impairment. *J. Neurosci.* 23: 3807–3819.

Capri M, Salvioli S, Monti D, Caruso C, Candore G, Vasto S, Olivieri F, Marchegiani F, Sansoni P, Baggio G, Mari D, Passarino G, De Benedictis G, Franceschi C. Human longevity within an evolutionary perspective: The peculiar paradigm of a post-reproductive genetics.Exp Gerontol. 2008 Feb;43(2):53-60.

Capri M, Salvioli S, Sevini F, Valensin S, Celani L, Monti D, Pawelec G, De Benedictis G, Gonos ES, Franceschi C.The genetics of human longevity.
Ann N Y Acad Sci. 2006 May;1067:252-63.

Campisi J. Cellular senescence and cell death. In: *Physiological Basis of Aging and Geriatrics* (3rd ed.), edited by Timiras PS. Boca Raton, FL: CRC, 2003, p. 47-59.

Cao S.X., Dhahbi J.M., Mote P.L., and Spindler S.R. 2001. Genomic profiling of short- and long-term caloric restriction effects in the liver of aging mice. *Proc. Natl. Acad. Sci.* 98: 10630–10635.

Cerinic, M. M., Generini, S., Partsch, G., Pignone, A., Dini, G., Konttinen, Y. T. & Del Rosso, M. Synoviocytes from osteoarthritis and rheumatoid arthritis produce plasminogen activators and plasminogen activator inhibitor-1 and display u-PA receptors on their surface. (1998) *Life Sci.* 63, 441-453

Chang E and Harley CB. Telomere length and replicative aging in human vascular tissues. *Proc Natl Acad Sci USA* 92: 11190-11194, 1995

Comfort, A. (1964). *Ageing: The Biology of Senescence*. Routledge & Kegan Paul, London.

De Martinis M, Franceschi C, Monti D, Ginaldi L.
Inflamm-ageing and lifelong antigenic load as major determinants of ageing rate and longevity.FEBS Lett. 2005 Apr 11;579(10):2035-9.

De Martinis M, Franceschi C, Monti D, Ginaldi L: Inflamm-ageing and lifelong antigenic load as major determinants of ageing rate and longevity. *FEBS Lett* 2005, 11;579(10):2035-9

Dimri GP, Lee X, Basile G, Acosta M, Scott G, Roskelley C, Medrano EE, Linskens M, Rubelj I, Pereira-Smith O, et al. A biomarker that identifies senescent human cells in culture and in aging skin in vivo. *Proc Natl Acad Sci USA* 92: 9363-9367, 1995

Dotto GP.
p21(WAF1/Cip1): more than a break to the cell cycle?Biochim Biophys Acta. 2000 Jul 31;1471(1):M43-56.
Dudek, S. M. & Johnson, G. V. (1994) *Brain Res.* 651, 129-133 Transglutaminase facilitates the formation of polymers of the β-amyloid peptide

Evert J, Lawler E, Bogan H, Perls T.
Morbidity profiles of centenarians: survivors, delayers, and escapers.
J Gerontol A Biol Sci Med Sci. 2003 Mar;58(3):232-7.

Franceschi C, Valensin S, Bonafè M, Paolisso G, Yashin AI, Monti D, De Benedictis G. The network and the remodeling theories of aging: historical background and new perspectives Exp Gerontol.2000 Sep;35(6-7):879-96

Franceschi C, Bonafe M, Valensin S, Olivieri F, De Luca M, Ottaviani E, De Benedictis G: Inflammaging. An evolutionary perspective on immunosenescence. *Ann N Y Acad Sci* 2000, 908:244-54

Franceschi C, Monti D, Sansoni P, Cossarizza A: The immunology of exceptional individuals:the lesson of centenarians. *Immunol Today* 1995, 16:12-16

Fraser H.B., Khaitovich P., Plotkin J.B., Paabo S., and Eisen M.B. 2005. *aging* and gene
expression in the primate brain. *PLoS Biol.* 3: e274.

Fenton M, Barker S, Kurz DJ, and Erusalimsky JD. Cellular senescence after single and repeated balloon catheter denudations of rabbit carotid arteries. *Arterioscler Thromb Vasc Biol* 21: 220-226, 2001.

Finkel T and Holbrook NJ. Oxidants, oxidative stress and the biology of ageing. *Nature* 408: 239-247, 2000.

Giresi P.G., Stevenson E.J., Theilhaber J., Koncarevic A., Parkington J., Fielding R.A., and Kandarian S.C. 2005. Identification of a molecular signature of sarcopenia. *Physiol. Genomics* 21: 253–263.

Gartel AL, Tyner AL.
Transcriptional regulation of the p21((WAF1/CIP1)) gene.
Exp Cell Res. 1999 Feb 1;246(2):280-9.

Gartel AL, Serfas MS, Tyner AL.
p21--negative regulator of the cell cycle.
Proc Soc Exp Biol Med. 1996 Nov;213(2):138-49.

Gartel AL, Tyner AL.
The growth-regulatory role of p21 (WAF1/CIP1).
Prog Mol Subcell Biol. 1998;20:43-71.

Hayflick L. The limited in vitro lifetime of human diploid cell strains. *Exp Cell Res* 37: 614-636, 1965

Hawse J.R., Hejtmancik J.F., Horwitz J., and Kantorow M. 2004. Identification and functional clustering of global gene expression differences between age-related cataract and
clear human lenses and aged human lenses. *Exp. Eye Res.* 79: 935–940.

Howie, A. J., Burnett, D. & Crocker, J. (1985) *J. Pathol.* 145, 307-314The distribution of cathepsin B in human tissues.

Hurteau GJ, Spivack SD.
mRNA-specific reverse transcription-polymerase chain reaction from human tissue extracts.Anal Biochem. 2002 Aug 15;307(2):304-15.

Jensen, L. E. & Whitehead, A. S. (1998) *Biochem. J.* 334, 489-503.

Kanungo MS. A model for ageing. *J Theor Biol* 53: 253-261, 1975

Kim, S.K. Genome-wide Views of Aging Gene Networks, Cold Spring Harbor ,2007

Kyng K.J., May A., Kolvraa S., and Bohr V.A. 2003. Gene expression profiling in Werner syndrome closely resembles that of normal aging . *Proc. Natl. Acad. Sci.* 100: 12259–12264.

Kirkwood T.B. and Austad S.N. 2000. Why do we age? *Nature* 408: 233–238.

Krtolica A and Campisi J. Cancer and aging: a model for the cancer promoting effects of the aging stroma. *Int J Biochem Cell Biol* 34: 1401-1414, 2002

Lee C.K., Weindruch R., and Prolla T.A. 2000. Gene-expression profile of the ageing brain in mice. *Nat. Genet.* 25: 294–297.

Lee C.K., Klopp R.G., Weindruch R., and Prolla T.A. 1999. Gene expression profile of aging and its retardation by caloric restriction. *Science* 285: 1390–1393.

Lee C.K., Allison D.B., Brand J., Weindruch R., and Prolla T.A. 2002. Transcriptional profiles associated with aging and middle age-onset caloric restriction in mouse hearts. *Proc. Natl. Acad. Sci.* 99: 14988–14993.

Lee C.K., Pugh T.D., Klopp R.G., Edwards J., Allison D.B., Weindruch R., and Prolla T.A.2004. The impact of alpha-lipoic acid, coenzyme Q10 and caloric restriction on life span and gene expression patterns in mice. *Free Radic. Biol. Med.* 36: 1043–1057.

Lee H.M., Greeley G.H., Jr., and Englander E.W. 2001. Age-associated changes in gene expression patterns in the duodenum and colon of rats. *Mech. Ageing Dev.* 122: 355–371.

Lener T., Moll P.R., Rinnerthaler M., Bauer J., Aberger F., and Richter K. 2006. Expression
profiling of *aging* in the human skin. *Exp. Gerontol.* 41: 387–397.

Liang W.S., Dunckley T., Beach T.G., Grover A., Mastroeni D., Walker D.G., Caselli R.J.,Kukull W.A., McKeel D., Morris J.C., et al. 2006. Gene expression profiles in anatomically and functionally distinct regions of the normal aged human brain. *Physiol. Genomics*

Lu T., Pan Y., Kao S.Y., Li C., Kohane I., Chan J., and Yankner B.A. 2004. Gene regulation and DNA damage in the ageing human brain. *Nature* 429: 883–891.

Ly D.H., Lockhart D.J., Lerner R.A., and Schultz P.G. 2000. Mitotic misregulation and human aging *Science* 287: 2486–2492.

Macleod KF, Sherry N, Hannon G, Beach D, Tokino T, Kinzler K, Vogelstein B, Jacks T.
p53-dependent and independent expression of p21 during cell growth, differentiation, and DNA damage.Genes Dev. 1995 Apr 15;9(8):935-44.

Mandavilli BS, Santos JH, and Van Houten B. Mitochondrial DNA repair and aging. *Mutat Res* 509: 127-151, 2002

Martin G.M. 2002. Gene action in the aging brain: An evolutionary biological perspective. *Neurobiol. aging* 23: 647–654.

McCarroll S.A., Murphy C.T., Zou S., Pletcher S.D., Chin C.S., Jan Y.N., Kenyon C., Bargmann C.I., and Li H. 2004. Comparing genomic expression patterns across species identifies shared transcriptional profile in *aging Nat. Genet.* 36: 197–204.

Medvedev, Z. A. (1990). "An attempt at a rational classification of theories of ageing." *Biol Rev Camb Philos Soc* 65(3):375-398

Melov S. and Hubbard A. 2004. Microarrays as a tool to investigate the biology of *aging*
A retrospective and a look to the future. *Sci. aging Knowledge Environ.* 2004

Melk A., Mansfield E.S., Hsieh S.C., Hernandez-Boussard T., Grimm P., Rayner D.C., Halloran P.F., and Sarwal M.M. 2005. Transcriptional analysis of the molecular basis of human kidney *aging*using cDNA microarray profiling. *Kidney Int.* 68: 2667–2679.

Oemar, B. S., Werner, A., Garnier, J. M., Do, D. D., Godoy, N., Nauck, M., Marz, W., Rapp, J., Pech, M. & Luscher, T. F. (1997) *Circulation* Human connective tissue growth factor is expressed in advanced atherosclerotic lesions.

Partridge, L., and Mangel, M. (1999). "Messages from mortality: the evolution of death rates in the old." *Trends in Ecology and Evolution* 14(11):438-442

Park, S. C., Yeo, E. J., Han, J. A., Hwang, Y. C., Choi, J. Y., Park, J. S., Park, Y. H., Kim, K. O., Kim, I. G., Seong, S. C., *et al.* Aging process is accompanied by increase of transglutaminase C.(1999) *J. Gerontol. A Biol. Sci.*

Ricciarelli R., d'Abramo C., Massone S., Marinari U., Pronzato M., and Tabaton M. 2004.
Microarray analysis in Alzheimer's disease and normal *aging*. *IUBMB Life* 56: 349–354.
Rodwell G.E., Sonu R., Zahn J.M., Lund J., W

Rodwell G.E., Sonu R., Zahn J.M., Lund J., Wilhelmy J., Wang L., Xiao W., Mindrinos M.,Crane E., Segal E., et al. 2004. A transcriptional profile of *aging* in the human kidney.
PLoS Biol. 2: e427

Saramäki A, Banwell CM, Campbell MJ, Carlberg C.

Regulation of the human p21(waf1/cip1) gene promoter via multiple binding sites for p53 and the vitamin D3 receptor.
Nucleic Acids Res. 2006 Jan 24;34(2):543-54. Print 2006.

Seki, T., Selby, J., Haupl, T. & Winchester, R. (1998) *Arthritis Rheum.* 41, 1356-1364Use of differential subtraction method to identify genes that characterize the phenotype of cultured rheumatoid arthritis synoviocytes

Segev F., Mor O., Segev A., Belkin M., and Assia E.I. 2005. Downregulation of gene expres-
sion in the ageing lens: A possible contributory factor in senile cataract. *Eye* 19: 80–85.

Shringarpure R and Davies KJ. Protein turnover by the proteasome in aging and disease. *Free Radic Biol Med* 32: 1084-1089, 2002.

Siegmund KD, Connor CM, Campan M, Long TI, Weisenberger DJ, Biniszkiewicz D, Jaenisch R, Laird PW, Akbarian S.
DNA methylation in the human cerebral cortex is dynamically regulated throughout the life span and involves differentiated neurons.
PLoS ONE. 2007 Sep 19;2(9):e895.

Sims, B., Powers, R. E., Sabina, R. L. & Theibert, A. B. (1998) Elevated adenosine monophosphate deaminase activity in Alzheimer's disease brain. *Neurobiol. Aging* 19, 385-391.

Statistics. 2006. *Health, United States, 2006.* National Center for Health Statistics. Hyattsville, Maryland.

Tan Q., Christensen K., Christiansen L., Frederiksen H., Bathum L., Dahlgaard J., and Kruse T.A. 2005. Genetic dissection of gene expression observed in whole blood samples of elderly Danish twins. *Hum. Genet.* 117: 267–274.

Thomas R.P., Guigneaux M., Wood T., and Evers B.M. 2002. Age-associated changes in
gene expression patterns in the liver. *J. Gastrointest. Surg.* 6: 445–454.

Vasto S, Malavolta M, Pawelec G.Age and immunity.Immun Ageing. 2006 Feb 24;3:2.

Vasto S, Candore G, Balestrieri CR, Caruso M, Colonna-Romano G, Grimaldi MP, Listi F, Nuzzo D, Lio D, Caruso C: Inflammatory networks in ageing, age-related diseases and longevity. *Mech Aging Dev* 2006

Van Den Biggelaar AHJ, De Craen AJM, Gussekloo J, Huizinga J, Heijmans BT, Frölich M, Kirkwood TBL, Westendorp RGJ: Inflammation underlying cardiovascular mortality is a late consequence of evolutionary programming. *The FASEB Journal* 2004, 18:1022-1024

Van Den Biggelaar AH, De Craen AJ, Gussekloo J, Huizinga TW, Heijmans BT, Frölich M, Kirkwood TB, Westendorp RG.
Inflammation underlying cardiovascular mortality is a late consequence of evolutionary programming.FASEB J. 2004 Jun;18(9):1022-4.

Visala Rao D., Boyle G.M., Parsons P.G., Watson K., and Jones G.L. 2003. Influence of
ageing, heat shock treatment and in vivo total antioxidant status on gene-expression profile and protein synthesis in human peripheral lymphocytes. *Mech. Ageing Dev.* 124: 55–69.

Vousden KH, Lu X.
Live or let die: the cell's response to p53.
Nat Rev Cancer. 2002 Aug;2(8):594-604.

Welle S., Brooks A., and Thornton C.A. 2001. Senescence-related changes in gene expres-
sion in muscle: Similarities and differences between mice and men. *Physiol. Genomics* 5: 67–73.

Welle S., Brooks A.I., Delehanty J.M., Needler N., and Thornton C.A. 2003. Gene expres-
sion profile of *aging*in human muscle. *Physiol. Genomics* 14: 149–159.

Welle S., Brooks A.I., Delehanty J.M., Needler N., Bhatt K., Shah B., and Thornton C.A.
2004. Skeletal muscle gene expression profiles in 20-29 year old and 65-71 year old women. *Exp. Gerontol.* 39: 369–377.

Weinert BT, Timiras PS.Invited review: Theories of aging.
J Appl Physiol. 2003 Oct;95(4):1706-16.

Yoshida S., Yashar B.M., Hiriyanna S., and Swaroop A. 2002. Microarray analysis of gene expression in the *aging* human retina. *Invest. Ophthalmol. Vis. Sci.* 43: 2554–2560

Zahn J.M., Sonu R., Vogel H., Crane E., Mazan-Mamczarz K., Rabkin R., Davis R.W., Becker K.G., Owen A.B. and Kim S.K 2006. Trascriptional profiling of aging in human muscle revelas a commona ging signature PLOS GENet 2: e115