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The role of mitochondria in the regulation of gamma rays induced mTOR-dependent senescence

TESI PRESENTATA DA:
Dott.ssa Elisa Mariani
(matricola 376182)

RELATORE:
Chiar.mo Prof. Giovanni Romeo
CORRELATORE
Dott. Giuseppe Gasparre
COORDINATORE:
Chiar.mo Prof. Giorgio Lenaz

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INDEX

1. INTRODUCTION

1.1 CELLULAR SENESCENCE

Senescence was formally described in 1965 by Hayflick, as a process that limits the proliferation of human fibroblast in culture [1]. Hayflick experiments showed that human fibroblast, over many cell doublings, underwent a decline in cell proliferation. Eventually, all cells in the culture lost the ability of divide [2]. A phenotype similar to senescence described by Hayflick was observed on overexpression of an oncogenic version of *H-Ras* (*H-Ras^{V}12*) in fibroblast *in vitro*. Normal cells forced to express high levels of the oncogene, rather than proliferate, stopped dividing and suffered morphological and molecular changes similar to those observed by Hayflick [3]. Based on these observations, cellular senescence refers to the essentially irreversible growth arrest that occurs in cells subjected to several kinds of stress in order to limit the proliferation of damaged cells or of cells reaching the "Hayflick limit".

Cellular senescence limits the proliferation of cells, therefore it can be induced only in mitotic cells (epithelial, stromal and vascular cells that comprise the major renewable tissues and organs such as skin, intestines, liver and kidney).

Recently it has been demonstrated the presence of senescent cells even in some kinds of tumors, such as pre-malignant tumors and tumoral cells at early stage of tumorigenesis [4, 5, 2, 6].

Senescent growth arrest is essentially irreversible, in contrast to what observed in quiescent cells; in fact it was verified that senescent cells cannot be stimulated to proliferate even in the presence of growth factors [2].

Only after inactivation of pathways that are responsible to regulate and maintain senescence phenotype (for example p53-p21 and p16^{INK4A}-pRb pathways) can be reverted the proliferative arrest that characterized these cells. For example, inactivation of the p53 pathway permits senescence reversal [7, 8], likewise inactivation of some interleukins also abrogates the proliferative arrest [9, 10].

Another feature of senescent cells, which distinguishes them from quiescent cells, is that senescent cells are metabolically active even if not proliferate.

Senescent cells become resistant to cell-death signals, such as apoptotic signals, and they acquire widespread morphological changes, as well as changes in gene

expression, distinguishing them from proliferating cells. Taken together, all these changes define what is called "senescent phenotype" [2].

Senescent can be induced by many stimuli (Fig.1)

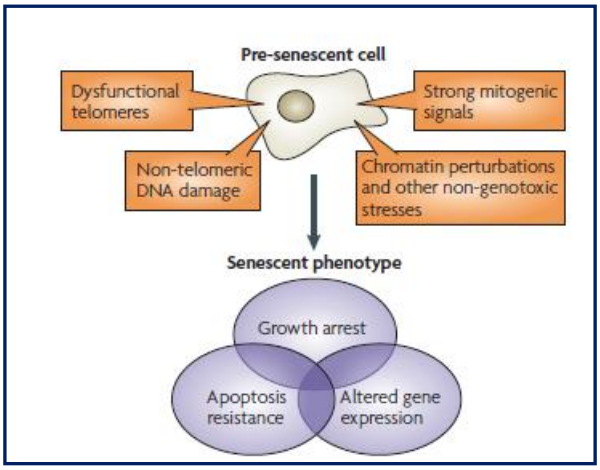


Fig.1: Mitotically competent cells respond to various stressors by undergoing cellular senescence [2].

It is possible to distinguish several type of senescence, based on the type of stimuli that induce it:

1) ***Replicative Senescence*:** this type of senescence is caused by telomere erosion, due to the gradual loss of DNA at the ends of chromosomes during each S phase. It is this type of senescence that Hayflick described in your experiment [1].

2) ***Premature cellular senescence:*** senescence can be induced in the absence of telomere loss or dysfunction. This type of senescence has been termed premature, since it arises before replicative senescence. Several kinds of stress can determine the activation of this type of senescence, hence it is possible to distinguish between:

- *Stress-induced senescence:* *in vitro* premature senescence can result from inadequate culturing conditions [11, 12], oxidative stress [13, 5, 14] and exposure to agents that cause DNA damage such as chemotherapeutic drugs and ionizing radiation [2].
- *Oncogene-induced Senescence (OIS):* normal cells respond to oncogenes over-expression or loss of tumor suppressor genes by undergoing senescence [3].

- PTEN loss-induced senescence (PICS): this type of senescence is rapidly induced as a consequence of loss of *PTEN* tumor supppressor gene [6].

Cell senescence is generally accompanied by morphological changes, that facilitate the identification both *in vivo* and *in vitro*.

To date, an exclusive marker that can be used to identify the activation of cellular senescence as not yet been defined, and on the other hand it is clear that not all senescent cells express the same markers [2, 15].

The marker that is commonly used to identify senescent cells is Senescence-Associated β-galactosidase (SA-βgal) [26]. It has been demonstrated that senescence cells are characterized by an increase in SA-β-GAL activity. Its increase activity in senescent cells derives from lisosoma β-D-galactosidase, which is encoded by the *GLB1* gene [27]. Acid β-D-galactosidase i san eukaryotic hydrolas localized in the lysosome. It clave β-linked terminal galactosyl residues froma wide range of substrates (gangliosides, glycoproteins and glycosaminoglycans). The increase in SA-β-GAL activity in senescent cells is likely due to an expansion of the lysosomal compartment, as a result of the accumulation of damaged macromolecules in lysosomes [28]. The increase expression of β-D-galactosidase and the expansion of the lysosomal compartment, cause an increase in β-galactosidase activity that can be measured also at suboptimal pH6 [27]. Senescent cells can be easily identified with respect to proliferating cells using a cytochemical assay that evaluate the activity of β-galactosidase.

The hallmark of cellular senescence is an inability to progress through the cell cycle. Senescent cells arrest growth, usually with a DNA content that is typical of G1 phase [16]. The feature and stringency of the senescent growth arrest vary depending on the species and the genetic background of the cell. For example, it has been shown that mouse fibroblast senesce with a G2/M DNA content as a result of a defect in the stress-signaling kinase MKK7 [17].

Likewise, oncogenes overexpression cause a fraction of cells to senesce with a DNA content that is typical of G2 phases [18, 19].

Cell senescence is generally accompanied by morphological changes, which allow distinguishing them from proliferating and terminally differentiated cells.

In general, senescence cells increase in size, in some cases a two-fold increase compared to non-senescence cells it has been observed. Furthermore, cells can become large, flat and multinucleated.

Beside those that are considered common alterations of senescent cells, there are morphological changes that are peculiar to a particular type of senescence.

For example, a flat cell phenotype is commonly seen in cells undergoing *H-Ras^{V}12*-induced senescence [3, 20], stress-induced senescence [21] or DNA-damage induced [22]. Cell senescing due to *BRAF^{E600}* expression or the silencing of p400, however, acquires a more spindle-shaped morphology [23, 24]. Melanocytes undergoing *H-Ras^{V12}*-induced senescence display extensive vacuolization as a result of endoplasmic reticulum stress caused by the unfold protein response (UPR) [20]. Furthermore, vacuolization it has been demonstrated in fibroblast underwent *H-Ras*-induced senescence, where it has been demonstrated the involvement of mitochondria in the regulation of cellular senescence [25].

Cellular senescence can be associated with an altered chromatin structure, at least *in vitro*. It has been demonstrated that cycling or quiescent cells, labeled with DNA dyes such as DAPI (4’,6-diamidino-2-phenylindole) display overall homogenous staining patterns, instead senescent cells often show strikingly different punctuate pattern [29]. These nuclear foci which characterized senescent cells are called SAHFs (Senescence-Associated Heterochromatic Foci), and correspond to highly compact heterochromatic regions. These SAHFs are specifically enriched in proteins such as histone H3 methylated on Lys 9 (H3K9me) and HP1 (heterochromatin protein 1). It was verified that SAHFs are formed in proximity of the promoters of several E2F target genes, which are then silenced [2, 29].

In this way, SAHFs silence the expression of genes that are needed for cell cycle progression, thereby contributing to the block of cell proliferation. The p16^{INK4A}-pRB regulates the SAHFs formation [29].

Cells undergoing senescence exhibit profound changes in their transcriptomes. As a result senescent cells secrete numerous factors, such as chemokines, growth factors and proteases [5, 10, 30]. It was verified that these factors have autocrine activities on the cells which they were secreted and a paracrine action on surrounding cells. These factors can affect surrounding cells by activating signal

transduction pathways that may lead to multiple pathologies including cancer [30]. Factors secreted by senescent cells constitue the SASP (Senescence associated secretory phenotype).

SASP factors can be divided into the following categories:

1) soluble signaling factors (interleukins, chemokines and growth factors);
2) secreted proteases;
3) secreted insoluble proteins and extracellular matrix components (ECM).

A recent study indicates that, for the induction of several SASP factors, persistent DNA damage is required. Because DNA damage is typical of some but not all kind of senescence, the SASP is produced only when senescence is accompanied by the activation of DDR [31].

1.1.1 Replicative Senescence

Whit the expression "Replicative senescence" we refer to senescence induced in normal cells which are reaching the "Hayflick Limit" [1]. The proliferative arrest is caused, as previously discussed, by telomere erosion, an event that in mammalian cells occurs at the end of each replicative cycle. Telomeres are stretches of repetitive DNA (5'-TTAGGG-3') and associated proteins that cap the ends of linear chromosomes and protect them from degradation or fusion by DNA-repair processes [2]. Telomeres length is maintained by specific enzyme called *telomerase*. Most normal cells do not express telomerase, or express it at levels that are too low to prevent telomere shortening [2]. Moreover, DNA polymerase cannot completely replicate DNA ends, a phenomenon called the end-replication problem. As a consequence, cells lose 50-200 bp of telomeric DNA during each S-phase. When telomeres reach a critical minimal length the replication is blocked and this phenomenon triggers the activation of DNA damage response (DDR). The DDR enables cells to sense damaged DNA, particularly double strand breaks (DSBs), and to respond by arresting cell-cycle progression and repairing the damage if possible [2, 10]. The DDR is associated with the appearance of SAHFs, at the level of which are located multiple proteins. Multiple proteins participate in the DDR, including protein kinases (ATM (ataxia telangiectasia mutated) ATR(ataxia telangiectasia and Rad3 related) CHK1 e CHK2 (checkpoint kinase 1 and 2), adaptor proteins (53BP1 e MDC1 (mediator

of DNA damage checkpoint protein-1)) and chromatin modifiers, for example γ-H2AX.

The kinases CHK1 and CHK2 are responsible to propagate the damage signal to effector molecules such as the phosphatase CDC25 and p53. The effector molecules halt the cell cycle progression. If DNA damage exceeds a threshold, cells are destined to undergo either apoptosis or senescence; the severity and the type of the damage are important factor that can determine the activation of senescence instead apoptosis [32].

In addiction to p53, it was demonstrated that $p16^{INK4A}$-pRB pathway is essential for induction of senescence [33,34]. The relative contribution of these pathways to senescence depend on cell type (Fig. 2).

Fig.2: Replicative senescence is driven by telomere erosion, and can results in activation of p16 and/or p53 [6]

The dependence of replicative senescence on telomere shortening is evident from its bypass by the ectopic expression of the catalytic subunit of the telomerase holoenzyme (hTERT). This enzyme is responsible to elongates telomeres and then to block the induction of senescence [2]. The limited life span of most primary cells is explained by the fact that, in contrast to stem cells, telomerase is not express or is express at low level in human somatic cells [35]. Likewise, tumor cells often express telomerase [36] or elongate their telomeres through a mechanism termed alternative lengthening of telomeres (ALT) [37].

1.1.2 Oncogene-induced Senescence (OIS)

Normal cells respond to oncogenes by undergoing senescence. This phenomenon was observed for the first time, in normal human fibroblast expressed an oncogenic form of *Ras* (*H-Ras*^{V12}) oncogene [3].

This type of senescence, that is induced prior of replicative senescence, is call Oncogene-Induced Senescence (OIS). To date, it was shown that other members of the Ras signalling pathway, such as RAF, MEK, MOS and BRAF, as well as

pro-proliferative nuclear proteins (E2F-1), cause senescence when overexpressed or expressed as oncogenic versions [3].

Recently, it has been shown that even loss of tumor suppressor genes can trigger senescence in mouse and human cells. This is the case of INK4A (Inhibitor of cyclin-dependent kinase 4A), NFR or ARF(alternative reading frame).

It has been established that cells underwent OIS, respond to oncogenic signalling trigger a DDR, which is associated with hyperproliferation and DNA hyper-replication [6, 18].

Although the mechanism that induced DDR in OIS is different from that of replicative senescence, effectors and primary pathways activated are the same [32]. In fact, cells underwent OIS are characterized by SAHFs formation, indeed OIS fails in cells lack ATM activity or when cells cannot sense DNA damage or transduce DDR signals to p53 [18]. Several experimental data have demonstrated that p53 and $p16^{INK4A}$-pRB pathways are involved in OIS activation. The relative contribution of these pathways to OIS depends on cell type [6]. For example, in murine cells, inactivation of p53 or its upstream regulator, $p19^{ARF}$, is sufficient to bypass *H-Ras* ^{V12} induced senescence [38, 3]. Instead, in human cells p16 ^{INK4A} it seems to play a more prominent role than p53 in regulating OIS activation, in fact it has been shown that some cells depend solely on this pathway for OIS regulation[34] (Fig. 3).

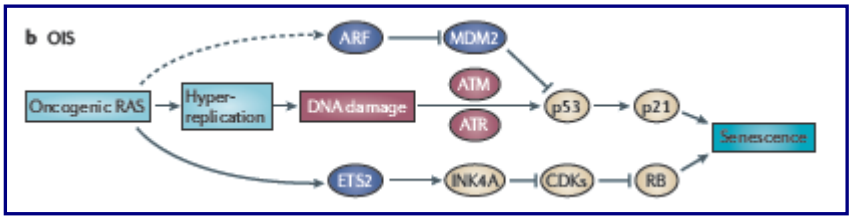


Fig.3: In OIS, the activation of p53 is driven by stabilization through phosphorylation by DDR and by ARF-mediated stabilization [6]

Although most of extant knowledge about OIS refers to *in vitro* experiments, some mouse models provide physiological evidence for OIS *in vivo* [6]. Taken together, these studies demonstrate that senescence is a primary response that is elicited to limit tumour progression, at least in tumour cells at early stage of tumorigenesis [6]. In 2005, four groups simultaneously reported evidences on the protective physiological role of OIS *in vivo*. This was shown for murine lung

adenomas, T-cell lymphomas, prostate tumors, as well as human benign melanocytic nevi [39-42] that are characterized by a low proliferative index and elevation of SA-β-Gal activity, as well as by the induction of some senescence markers. For example, nevi are benign melanocytic tumors that generally lack proliferative activity. They are commonly characterized by mutation in *BRAF* gene (generally $BRAF^{V600E}$) [43], Michaloglou and collaborators showed that the cell cycle arrest of human nevi has hallmarks of OIS: nevi undergo long-term cell cycle arrest, express elevated level of p16 and display increased SA-β-Gal activity. These results indicate that OIS in nevi acts as a barrier to melanoma development [44, 45]

1.1.3 PTEN-Loss Induced Cellular Senescence (PICS)

A senescence response, recently characterized is PICS (Pten-loss Induced Senescence). PICS is rapidly induced after loss of PTEN, it is distinct from OIS on several dimension. In fact PICS is induced even in absence of DDR and does not follow an initial phase of DNA hyper-replication [46].

PICS can occur in cells that are treated with aphidicolin, a molecule that block S-phase entry and prevents DNA replication [46]. Instead, in $H\text{-}RAS^{V12}$ –induced senescence, aphidicolin treatment is responsible to abrogate senescence [18].

Furthermore, the SAHFs formation is not observed in PICS, in fact cells do not respond to loss of PTEN activating a DDR; and inhibition of ATM has not effect on PICS induction [46].

Similar to OIS, p53 have a primary role in PICS, but it was shown that p53 up-regulation is caused by mTOR-mediated translation [46,47]. Several experiments have demonstrated that ARF is not involved in p53 regulation in PICS, differently to what observed in OIS [46, 47, 48,49].

PTEN regulate PICS activation even due to its nuclear function. It has been shown that nuclear PTEN acts up-regulating INK4 through the regulation of ETS2 [50].

PTEN loss driver senescence from two perspectives:

1) through p53 up-regulation as result of mTOR hyper-activation;

2) through INK4A up-regulation as result of disassembly of the CDH1-containing anaphase-promoting complex (APC/CDH1) and accumulation of ETS2

As in the case of OIS, even PICS can have an important role in limiting the transformation of early lesions to malignant cancer, almost in prostate cancer [3]. (Fig.4)

Fig.4: Model of PICS; p53 regulation is mainly mediated by mTORC1. In addiction, the ETS2-INK4A pathway is also required for senescence induction [6]

1.1.4 Stress-induced Senescence

Different type of stress can induce senescence. For example, *in vitro* senescence can result from inadequate culture conditions, such as abnormal concentration of nutrients and growth factors or the presence of ambient of O_2 levels [10, 2].

Severe DNA damage, especially damage that creates DSBs, cause many cells to undergo senescence [51, 52]. Oxidative stress, citotoxic agent, chemioterapeutic drugs and ionizing radiation are responsible to induce senescence as a consequence of activation of DDR [52, 53]. Many chemotherapeutic drugs cause severe DNA damage, and then induce senescence in normal cells but also in tumor cells *in vitro* and *in vivo* [53].

Mouse models demonstrated that tumor cells with wild type p53 are more likely to senesce in response to chemotherapy than tumor cells with mutant p53, at least in cell culture and cancer-prone mouse models [54,55,56]. At the same time, exposure to ionizing radiation can induce senescence in normal cells and tumoral cells. Similarly, the exposure to ionixing radiation is able to activate a senescence process in normal cells as well in tumor [52, 53]. An important therapeutic implication that can derived by these observation is that DNA damage therapies, such as radiotherapy and chemotherapy, are more likely efficacious in tumours that can maintain the capacity to senescence, for example in tumor cells that not present mutations in p53 e $p16^{INK4A}$-pRB pathways [53,54, 55, 56].

1.1.5 The regulators of cellular senescence: p53-p21 and $p16^{INK4A}$-pRb pathways

Independently from the stimuli that induced senescence, p53-p21 and $p16^{INK4A}$-pRB pathways are directly involved in establish the senescence growth arrest and

to regulate the appearance of the morphological modification typical of senescence cells [2].

These pathways interact but can independently halt cell-cycle progression. In fact, different stimuli can lead to activation of one or the other pathway. In addition the propensity with which cells engage p53-p21 or $p16^{INK4A}$-pRB pathways is cell type and species-specific [2].

Finally, although most cells senesce owing engagement of p53-p21 or $p16^{INK4A}$-pRB pathways, or both, there are examples of senescence that is independent of these pathways [24,57].

In general, stimuli that generate DNA damage or that activate DDR, induce senescence primarily through the p53 pathway.

p53 is regulated at multiple point, for example by MDM2, an E3-ubiquitin protein ligase which facilitates p53 degradation via proteasoma. Another p53 regulator is ARF, which inhibits MDM2 activity [58]. When activated, p53 induced the growth arrest through p21 expression. p21 is a cyclin-dependent kinase inhibitor which acts on cyclinE-A/Cdk2 complexes (Fig.4)

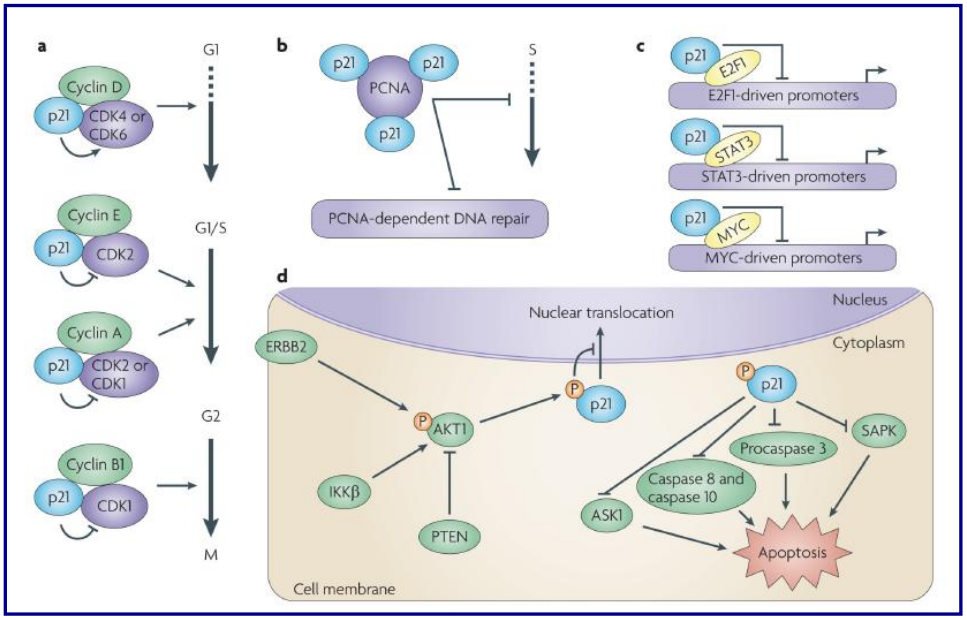


Fig.4: Nuclear and cytoplasmatic activity of p21

Stimuli that produce a DDR can also engage the $p16^{INK4A}$-pRB pathway, but this usually occurs secondary to engagement of the p53 pathway [59,60].

Nonetheless, some senescence-inducing stimuli act primarily through the $p16^{INK4A}$-pRB pathway. This is the case of *H-Ras* induced senescence, in which

oncogenic Ras induces p16 expression by activating ETS transcription factors [62].

The $p16^{INK4A}$-pRB pathway is crucial for generating SAHFs, which silence the genes that are needed for cell proliferation [61]. SAHFs require several days to develop, during which time there are transient interactions among chromatin-modifying proteins such as HIRA (HIstone Repressor A), ASF1a (Anti-Silencing Function 1a) and HP1. Once established, SAHFs no require p16 or pRb for maintenance. Although SAHFs are not present in all senescence cells, the $p16^{INK4A}$-pRB pathway might establish chromatin states that are functionally equivalent to SAHF [2].

Even p53-p21 pathway can be induced without the involvement of DDR, as demonstrated in PICS where p53 increase expression depends on mTOR-hyper-activation [46].

p16 and p21 are both cyclin-dependent kinase inhibitor (CDKIs) and are responsible to halt cell-cycle progression. p21 acts primarily as "universal" inhibitor of cyclin-Cdks complexes, instead p16 acts on cyclin D-Cdk4-6 complexes. p16 and p21 can keep pRb in an active, hypophosphorylated form. In proliferating cells, pRb is maintained in a hyperphosphorylated inactive form by cyclin-Cdk complexes, thereby pRb cannot inhibit the transcriptional factor E2F. The final effect of pRb inactivation is the transcription of genes which control cell proliferation.

1.1.6 Senescence in vivo

Much of the current knowledge on the causes and consequences of senescence derives from *in vitro* studies. Only during the past decade cellular senescence has been shown to occur *in vivo* [2]. In rodents, primates and humans, senescence cells are found in many renewable tissues, such as vasculature, haematopoietic system, many epithelial organs and the stroma [2,33, 63]

Cells that express senescence markers are relatively rare in young organisms, but their numbers increase with age. Senescence observed in age tissues is caused mainly by telomere dysfunction (replicative senescence).

Cells that express the typical markers of a senescent cells are found at sites of chronic age-related pathology, such as osteoarthritis and atherosclerosis [64,65].

In addition, senescent cells are associated with benign dysplastic or preneoplastic lesions [24,66,67,68] and benign prostatic hyperplasia [69] but not with malignant tumours. As previously discuss, senescent cells can be found in normal ad tumour tissues following DNA damaging chemiotherapy [53,54,55,56]. Taken together these results support the hypothesis that senescence suppress or limiting the development of cancer.

1.1.7 Senescence and tumorigenesis

Although initially the hypothesis that senescence could be correlated with tumorigenesis originated a lively debate, nowadays the idea that senescence is a powerful physiological mechanism, able to block tumour proliferation is quite well established. Several independent studies performed in human as well as in animal models, have supported the idea that senescence, as well as apoptosis, is an efficient mechanism that cells can used to suppress tumour proliferation [70]. The presence of senescent cells had been identified in animal models and in human, in close association with pre-malignant stages of tumorigenesis.

The original identification of senescent tumour cells was obtained from lung adenomas, pancreatic intraductal neoplasias and melanocytic nevi associated with the presence of oncogenic $BRAF^{V600E}$, which are all pre-malignant tumours [15, 4]. Senescent cells have been found in hyperplastic lesions such as benign prostatic hyperplasia [69], and also in a familial cancer syndrome known as neurofibromatosis type 1, caused by loss-of-function mutations in *NF1* tumour suppressor gene [71].

Taken together these data emphasize a close association between senescence and tumour, but it is important to underline that senescence is associated with tumours at pre-malignant stage of tumorigenesis, but not with malignant tumours [4]. As previously discuss, senescent cells are identified initially in pre-malignant tumours such as lung adenomas, pancreatic intraductal neoplasias and nevi melanocytic [68,66,39]. By contrast senescence was absent in their corresponding malignant stages, which are respectively lung adenocarcinomas, pancreatic ductal adenocarcinomas and melanomas [68,66,72]. The different behaviour observed in pre-malignant lesions compared to aggressive tumor is due to the fact that in most cases the differentiation towards a malignant phenotype is associated to an inhibition of p53 and p16 pathways.

It would however be incorrect to conclude that tumoral cells have lost their ability to senesce. It is possible to induce senescence also in malignant tumoral cells if correctly stimulated. Interestingly, in animal models, the induction of such a process in tumoral cell line can be effective from a therapeutic point of view.

Initial studies conducted on humans have shown that senescence can be induce after treatment with chemotherapeutic drugs, and the result is regression of the malignant phenotype [2].

In the past few years, the concept of pro-senescence therapy has emerged as a novel theraputic approach to treat cancer [6]. To date, are in developing therapeutic strategies to activate senescence in tumour cells are under development.

These drugs can be subdivided in several categories [6]:

1) drugs that can enhance p53 activity and function;

2) drugs with the ability to modulate the cell cycle machinery, for example drugs which act on p27 and pRb;

3) drugs that target oncogene or tumour suppressor genes;

4) telomerase inhibitors for the induction of replicative senescence .

Each of these strategies is developed in order to be used alone for senescence induction and cancer treatment. However, the idea of using pro-senescence strategies in combination with traditional treatment protocols (for example chemotherapy and radiotherapy) is emerging, with the aim of combining the positive effect of single treatment and then to improve the effectiveness of treatment. Such an idea emerged from experiment conducted in mouse model affected by lymphomas.

Mouse model of chemotherapy have shown that MYC-initiated lymphomas respond to ciclophosphamide by inducing tumour cell senescence, but the most interesting data is that, activation of senescence correlated with a better prognosis following chemiotehrapy [55]. These experimental evidences suggest that senescence can improve the effectiveness of chemotherapeutic treatment [2]. Of note, two reports analysing senescence markers in biopsies from patients with lung or breast cancer after neoadjuvant chemotherapy have observed chemotherapy-induced senescence and its association with treatment success

[54,56]. More recently, the analysis of biopsy material from patients with prostate cancer has shown that chemotherapy induces marker of senescence [30].

Although are actually in developing several strategies to induce the activation of senescence, it must bear in mind the potential problems that might arise from senescence-induced therapies. One of the most important problems is that, cancer cells in a senescence-like state might remain as 'dormant' tumour cells and therefore represent a dangerous potential for tumour relapses [2].

For this reason, the therapeutical protocols based on the use of molecules that induce senescence might induce at the same times the activation of processes which are responsible to eliminate senescence cells.

Senescent cells can secrete molecules and factors, such as IL-6 and IL-8, which are responsible to recruit immune cells (such as neutrophils, macrophages and natural killer cells) to sites of senescence and to clearance it [73]. Thus, these data suggest to promoting immune cell function, while also engaging a pro-senescence response, may prove to be beneficial for the clearance of senescence cells and may result in tumour regression. In virtù del ruolo svolto dalle cellule del sistema immunitario, è chiaro che potenziarne l'azione è sicuramente di fondamentale importanza nel potenziare il fenomeno della regressione tumorale.

SASP component, such as interleukin, might then have an important role in sustaining senescence activation and tumour clearance, but it is important to bear in mind that some SASP molecules might also stimulate the malignant phenotype of nearby tumour cells [30].

Before introducing the use of induced senescence for therapeutic purposes, it will be therefore necessary to complete studies considering also the side effects that are generated by such a process.

1.2. mTOR

mTOR (mammalian target of rapamycin) is a serine/threonine kinase which regulates protein synthesis, cell growth and proliferation in response to pleiotropic inputs including growth factors, nutrients, stress and energy [74].

It was identified in the yeast *Saccharomyces Cerevisiae* during a screen for resistance to the immunosuppressant drug rapamycin [75, 76].

mTOR is a member of the phosphoinositide 3-kinase (PIKK) family, whose catalytic domain has omology to that of PI3Ks [77].

Unlike PI3K family member which phosphorylate lipids, the PIKK (ATM; ATR, DNA-PKcs, mTOR, SMG1 e TRRAP) act as serine/threonine kinases [78].

mTOR is a central signalling molecule, than it is implicated in various syndrome and hamartoma syndromes [79]. There are several hamartoma syndromes which are caused by mutations or deregulation of mTOR pathway, such as TSC1-TSC2 (Tuberous Sclerosis Complex)[81, 82], NF1 (Neurofibromatosis Type I) [82], LKB1 (Peutz-Jeghers Syndrome) [84], PTEN (Cowden disease) [84,85] e FLCN (Birt-Hogg-Dubé sindrome) [86-88].

mTOR is a high molecular weight proteins (280Kda) that contains several structural domains. The N-terminus posses 20 HEAT repeats that are implicated in protein-protein interactions. The C-terminus contains the kinase domain, which has sequence similarity with PI3K. Upstream to the catalytic domain are present the FRB domain responsible to the interaction with rapamycin. Furthermore, upstream to FRB domain there is the FAT domain, typical of PIKK kinases. Finally in the C-terminus region is present the FATC domain, that is necessary for mTOR activity, in fact deletion or even single base mutations abrogates the activity of mTOR [89].

In mamamlial mTOR formed two distinct complexes, called respectively mTORC1 and mTORC2 (Fig. 5).

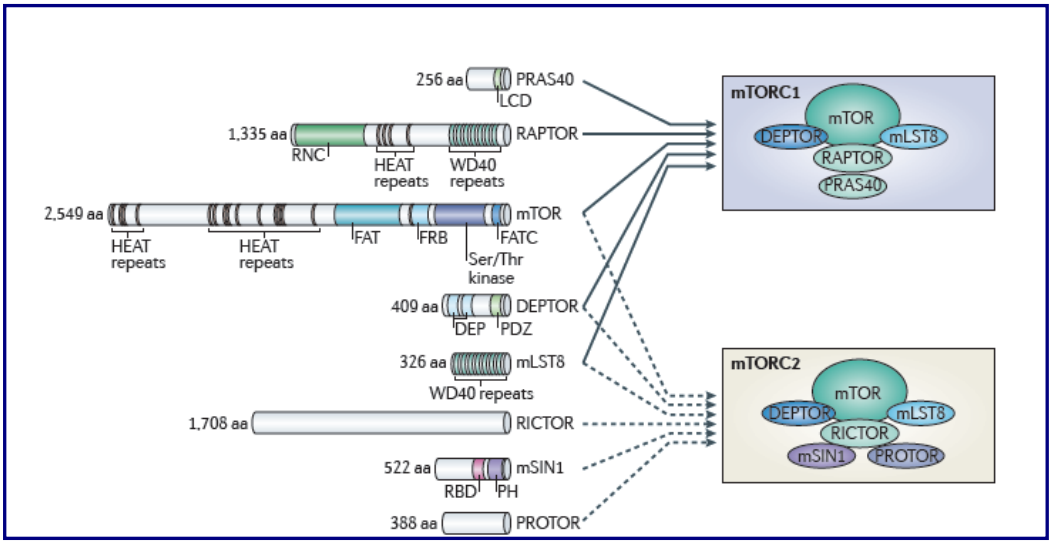


Fig.5: Domain organization of mTOR and mTORc proteins [90]

mTORC1 is a rapamycin-sensible complex. Rapamycin was originally isolated from the bacterium *Streptomyces Hygroscopicus*, inhibits proliferation of mammalial cells and possess immunosuppressive properties [74]. Rapamycin in

cells binds the protein FKBP12 (FK506-binfìding protein), then the complex rapamycin-FKBP12 binds and inhibits raptor-bind to mTOR, instead do not have effect on rictor [91]. Prolonged treatment with rapamycin can inhibits mTORC2 in some tissues and cell lines. This effect may be due to the sequestration of the pool of mTOR in the complex rapamycin-FKBP12 [92].

The subunits that composed mTORC1 are: mTOR, RAPTOR (Regulatory associated protein of mTOR), PRAS40, GβLe DEPTOR. RAPTOR acts as an adaptator subunit and is important to recruit substrates to mTOR, PRAS40 e DEPTOR are negative regulators of mTORC1 complex, instead GβL is a positive regulator of the complex [74,90].

mTORC2 complex is composed by the following subunits, some of which are common to mTORC1: mTOR, RICTOR, PROTOR, DEPTOR, GβL e mSIN1. RICTOR, as well as RAPTOR, acts as a scaffold; PROTOR regulates complex assembly, instead mSIN1 target mTORC2 to membranes [90].

mTORC1 and mTORC2 are responsible to regulate different processes: mTORC1 is responsible to regulate protein synthesis, proliferation and cellular growth. mTORC2 regulates proliferation and actin reorganization [90].

mTORC1 promotes cell growth through phosphorylation of various regulators of translation, including the well-characterized ribosomal S6K1, which activates the S6 ribosomal protein (S6), and the eukaryotic translation initiation factor 4E-binding protein1 (4EBP1), which leads to its uncoupling from the elongation initiation factorE (eiF4e) [74].

S6K1 and 4EBP1 associate with mRNAs and regulate mRNA translation initiation and progression, thus controlling the rate of protein synthesis.

Unphosphorylated 4E-BP1 suppresses mRNA translation: however when phosphorylated by mTORC1, 4E-BP1 dissociates from eIF4E allowing eIF4E to recruit the translation initiation factor eIF4G to the 5’ end of most mRNAs [90].

When phosphorylated by mTORC1 S6K1 promotes mRNA translation by phosphorylating or binding multiple proteins, including eukaryotic elongation factor 2 kinase (eIF2K), S6K1 Aly/REF-like target (SKAR), 80kDa nuclear cap-binding protein (CBP80) and eIF4B, which collectively affect translation initiation and elongation [90]. eIF4B is responsible to enhances the activity of eIF4A, an RNA helicase that unwind the structured 5’UTR of many mRNAs [90].

Moreover, studies in yeast *S.Cerevisae* demonstrated that mTORC1 suppress autophagy [93,94].

Recently, several experimental data have demonstrated that mTORC1 is involved in the regulation of cellular senescence. In a recent report, the persistent expression of Wnt proteins in mouse epiderms led to hyperproliferation of epithelial stem cells, ultimately causing them to undergo senescence. These action seemed to occur through Wnt-mediated activation of mTOR pathway [95].

The involvement of mTOR in the regulation of cellular senescence it has been shown even in PICS; where it was shown that p53 up-regulation is caused by mTOR-mediated translation consequent to the loss of PTEN [46,47].

Recently it has been shown in fibroblast that PI3K/AKT pathway activation in response to PTEN knockdown, mutant PI3K or activated AKT expression, induces cellular senescence. It was demonstrated that AKT-induced senescence senescence is p53-dependent, and that mTORC1 have a pivotal role in regulation p53 translation and stabilization of p53 protein following nucleolar localisation and inactivation of MDM2 [96].

mTORC1 acts as a signal integrator for four major regulatory inputs: growth factors, nutrients, energy and stress [90].

mTOR responds to growth factors via the PI3K/AKT pathway [74, 90]. Binding of insuline to its receptor activates the PI3K pathway, which converts PIP2 (phosphatidiylinositol-4,5-phosphate) in PIP3. PIP3 accumulation is antagonized by the tumor suppressor gene PTEN. PIP3 recruits PDK1 and AKT to the membrane, resulting in the phosphorylation and activation of AKT by PDK1.

When AKT is activated, phosphorylates and inactivates TSC1 and TSC2 and, consequently, activates mTORC1 [97-99]. In fact, TSC1 and TSC2 form a complex which have been shown to negatively regulate mTORC1 [105]. TSC2 was shown to have a GTPase activating protein (GAP) activity towards the Rheb (ras-homolog enriched in brain) GTPase; this event stimulates the intrinsic GTP hydrolysis activity of Rheb to promote its transition from an active GTP-bound form to an inactive GDP-bound form [100].

Conversely inactivation of TSC1/TSC2 by AKT phosphorylation lead to GTP binding and Rheb activation which ultimately promotes the activation of mTORC1.

AKT also phosphorylates PRAS40 a component of mTORC1, which causes it to bind to 14-3-3 proteins and prevents it from inhibiting mTORC1 [101].

mTORC1 senses also cellular energy [90]. The mTORC1 pathway indirectly senses low ATP by a mechanism that involved the AMPK kinase. Both AMP and ATP are allosteric regulators of AMPK, when the AMP:ATP ratio increases, AMPK phosphorylates TSC2, therefore the GAP activity of TSC1-TSC2 towards Rheb is stimulating, and the mTORC1 signaling is inhibited [90]. Moreover AMPK phosphorylates Raptor, causing it to bind to 14-3-3 proteins, which lead to mTORC1 inhibition [101]. Other stressor that not primarly impinge on cellular energy can inhibit mTORC1 through the involvement of AMPK. For example, DNA damage result in inhibition of mTORC1 activity through the p53-dependent upregulation of AMPK [102, 103]. Sestrin1 and 2 are two transcriptional targets of p53 that are impliocated in the DNA damage response, and it was recently shown that sestrin potently activate AMPK, thus mediating the p53-dependent suppression of mTOR activity upon DNA damage [104]

1.3 MITOCHONDRIA

Mitochondria are cytoplasmic organell present in every eukaryotic cells, animal or vegetal with an aerobic metabolism. Mitochondria are the main producers of energy in a cell [106, 107], but they are involved in other metabolic processes, such as Krebs cycle, pyruvate and fatty acid oxidation, nitrogen metabolism and heme metabolism [108, 109]. Moreover, mitochondria are involved in calcium homeostasis maintenance, production and regulation of reactive oxygen species (ROS) and in the regulation of apoptosis [106, 107, 108]. Mitochondria are endowed with a two-membrane system in which the outer and the inner membrane differ in lipoprotein content and permeability and thus create two separate sub-compartments; the intermembrane space and the inner matrix, each hosting different metabolic reactions and molecular processes. The outer mitochondrial membrane is more permeable, mainly due to the voltage dependent anion channel (VDAC) known as porin [110], while the inner membrane is almost completely impermeable, particularly for protons, which allows the formation of the proton gradient crucial for the mitochondrial energy production. The low permeability of inner membrane is attributed to the absence of porin and to the

elevate presence of cardiolipin, a phospholipid which modulates the activities of some respiratory chain enzyme [111].

The surface of the inner membrane folds into tubular formations called *christae* (Fig.6) which protrude in the matrix and increasing the surface of energy production. In the *christae* are located almost all the protein that constitute the electron transport chain.

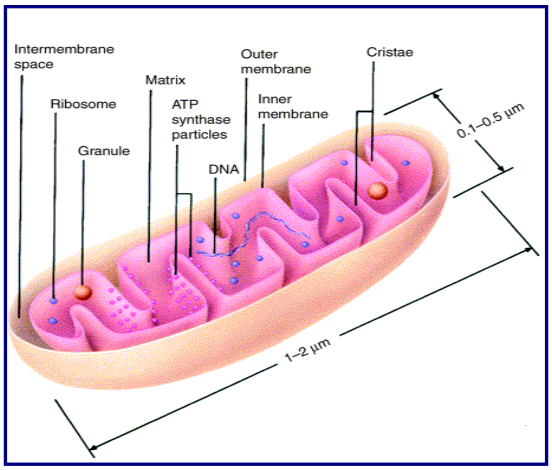


Fig.6: Mitochondrial structure [112]

Mitochondria are the main producers of energy in a cell; through the process called *oxidative phosphorylation*, protein complexes of the electron transport chain (CI, CII, CIII and CIV) and ATP synthase (CV), situated in the inner mitochondrial membrane, utilize hydrogen to reduce oxygen into water and generate ATP (Fig. 7).

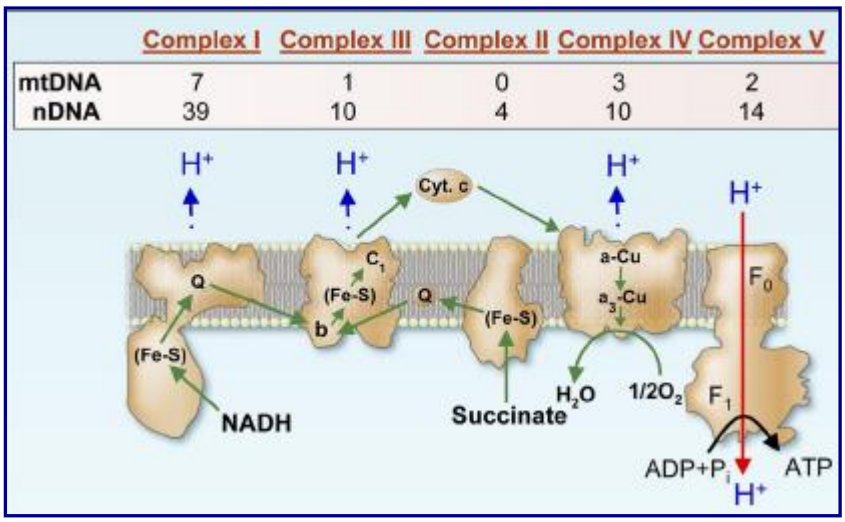


Fig.7: Protein subunits of the five respiratory complexes encoded by nuclear and mitochondrial genes. [109]

1.3.1 Mitochondrial genome

Mitochondria are unique among eukaryotic organelles in that they contain their own genetic system. Only a small number of genes necessary to account for the molecular architecture and biological function of the organelle are present in mtDNA. Because of this limited coding capacity, mitochondrial are genetically semi-autonumous, in fact they rely on the expression of nuclear genes for all of their biological function [109]. For example, the majority of subunits that comprise the complexes of electron transport chain, and the enzyme involved in replication and transcription are nucleus encoded.

Human mtDNA is a double-stranded molecules that consists of 16569 bp 113,114,115] is located in mitochondrial matrix and carries genes encoding for 13 subunits of electron transport chain complexes, as well as 2 rRNAs and 22 tRNAs (Fig. 8).

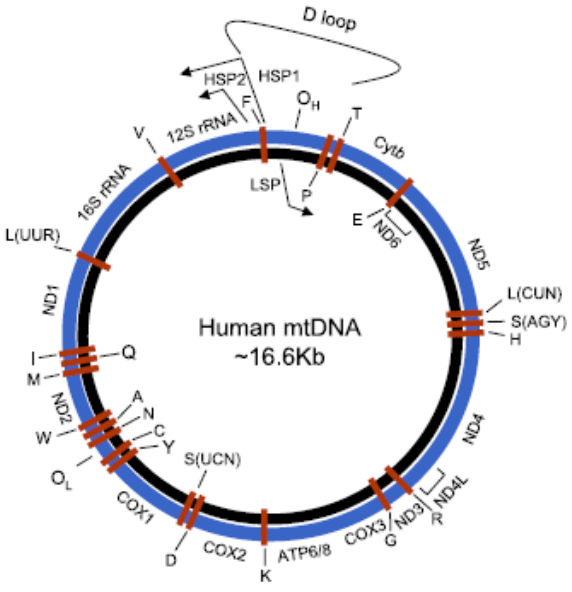


Fig.8: Schematic representation of the human mitochondrial genome

mtDNA has some characterists that are different from those of the nuclear genome (nDNA). The genetic information of mtDNA is extremely compacted, mitochondrial genes lack introns and there are little or no intergenic regions. Some respiratory protein genes overlap and finally, another characteristic of mtDNA is the use of mitochondria specific codons, which differ from the universal genetic code [116].

Although mitochondria do not contain introns, recent discoveries showed that mtDNA is actually protein-coated and packed in aggregates called mitochondrial *nucleoids*, together with the machinery necessary for its replication and transcription [117, 118] such as POLG (mtDNA polymerase gamma) or TFAM (mitochondrial transcription factor) to which a role in mtDNA packaging and organization has recently been assigned [118-120].

The only non coding regions in mtDNA is the D-loop (displacement loop), a control region which contains replication and transcription factor binding sites.

Mitochondrial genome is inherited maternally, in fact only mitochondria from the oocyte contribute to gamete development, while sperm mtDNA tends to be eliminated via proteosoma after fertilization through their ubiquitination in spermatogonia [121-123].

Mammalial cell generally have many mitochondria, each of them carries several (2-10) mtDNA molecules [124], that are not always genetically uniform. The coexistence of both mutated and wild type mtDNA in a cell is a condition called heteroplasmia. On the other hand, homoplasmy is a condition typical of a cell that present only one mtDNA genotype [124]. A direct consequence of polyploidia and heteroplasmy is the threshold effect. The phenotypic effect of a mtDNA mutation depends on the percentage of its mutant load. The portion of mutated molecules needs to be reached before the functional consequences of a mtDNA mutation begin to arise, depending on the type of the mutation and on the tissue in which it occurred. In fact, tissue that have an elevated oxidative metabolism, such as brain, skeletale muscle and heart, have a low threshold with respect to other tissue.

1.3.2 Mitochondrial biogenesis

The energetic demands can vary substantially between different cell types and in different physiological situations requiring an adaptation in mitochondrial

biogenesis, which is a complex phenomenon that needs the participation and coordination of nuclear and mitochondrial genomes [109]. Many activators and coactivators are involved in the coordinated upregulation of nuclear and mitochondrial genes that are necessary for an efficient mitochondrial biogenesis (for example nuclear genes encoding respiratory subunits, mitochondrial transcription and replication factors, heme biosynthetic enzymes, protein import machinery).

Physiological signals may activate specific transcription factors through different pathways and this can result in an activation of genes required for mitochondrial biogenesis and respiratory function. The nuclear transcriptional factors involved in regulating mitochondrial biogenesis are: Nuclear Respiratory Factors 1 and 2 (NRF-1 and NRF-2), YY1 and CREB. They act on the majority of nuclear genes encoding subunits of the respiratory chain complexes [109], and are also involved in the expression of mitochondrial transcription and replication factors (TFAM, TFB1M and TFB2M), heme biosynthetic enzymes and other proteins required for respiratory function [125, 127].

In addition to these transcription factors, mitochondrial biogenesis is controlled by transcriptional coactivators, such as PGC-1α (PPARγ coactivator-1), PGC-1β and PRC, which interact with other transcription factors and coactivators [126].

PGC-1α is the main regulator of mitochondrial biogenesis in mammalial cells. It was identified in brown fat tissue (BAT), through its interaction with PPARγ (*Peroxisome Proliferators-Activated Receptor γ)*, a regulator of adipocyte differentiation [109]. It is expressed at high levels in tissues where mitochondria are abundant, such as brown adipose tissue (BAT), heart and skeletal muscle. Instead, the expression level is low in tissues such as liver and white adipose tissue [131].

PGC-1α can induce mitochondrial biogenesis by interaction with several transcription factors, such as NRF-1, NRF-2, ERRα and YY1 [109].

Recently it was shown that AMPK kinase, a key regulator of cellular energy homeostasis, is the mainly regulator of mitochondrial biogenesis upon energy depletion through inhibition of PGC-1α activity[129, 130]. In condition of energy depletion, AMPK is activated and is responsible to phosphorylates PGC-1α on specific serine and threonine residues. This results in increased mitochondrial

gene expression and activation of ATP production; instead all the molecular pathways that use ATP are inhibited.

More recently, it has been demonstrated that *PGC-1α* gene expression is regulated by mTORC1 [132]. In skeletal muscle it has been demonstrated that pharmacological inhibition of mTORC1 is responsible to decreased PGC-1α expression and of some of its interactors such as NRF-1, NRF-2 and ERR-α. The result is a decrease in mitochondrial biogenesis, even as proteins expression than in terms of oxygen consumption [132]. It has also been demonstrated that mTORC1 and PGC-1α interact with YY1 (Ying-Yang 1), which seems directly bind mitochondrial gene promoters. mTORC1 were shown to directly interact with YY1 and PGC-1α, and mTORC1 was demonstrated to regulate the transcriptional function of YY1-PGC-1α by altering their physical interaction [132].

In another study it has been demonstrated that mTOR and raptor can be purified in mitochondrial fraction; moreover pharmacological inhibition of mTORC1 through rapamycin, resulted in a marked alteration of mitochondrial transcriptoma [133].

1.3.3 Oncocytic tumors: a model to study mitochondrial dysfunction

Oncocytic tumors are an interesting model to study the effect of mitochondrial dysfunction on cancer development. Oncocytic tumors may arise in different types of tissues, most often in those of epithelial origin such as thyroid, kidney or pituitary gland [134].

They are characterized by an aberrant mitochondrial hyperplasia, that is responsible for their "swollen" appearance [135]. This feature can be appreciated through ultrastructural analysis, which displays cells packed with enlarged globular or ovate mitochondria with a stack of lamelliform, tubular or flat cristae and occupying up to 60% of the cytoplasm [134, 136, 137].

The majority of oncocytic neoplasms is considered benign and display low-proliferating, non-invasive behaviour [134, 135, 138-144]. Moreover, a strong association between complex I disruption and HIF-1 α (Hypoxia inducible factor 1) destabilization has been shown. The mitochondrial mutation lead to an increase of α-chetoglutarate/succinate ratio, which may be responsible for a higher affinity/activity of the prolyl hydroxylase (PHD) responsible to mediate HIF-1α

degradation. Such metabolic switch was suggested to explain why onocytic tumors might excape malignancy [144].

The mitochondrial hyperplasia is caused by a compensatory effect as a consequence of a respiratory dysfunction triggered in presence of a retrograde signalling from the organelles to the nucleus [135]. This stress response can be activated in response to several kinds of stimuli, such as loss of mitochondrial function caused by collapse of electrochemical potential , impaired respiratory chain activity or by the accumulation of unfolded proteins in the organelle [135].

Several studies have investigated the increase in mitochondrial biogenesis in oncocytic tumors as well as in the only existing oncocytic model, the XTC.UC1 cell line [135]. The group of Prof. Romeo fully characterized the bioenergetic competence of XTC.UC1. It has been shown that the energetic impairment is due to a decrease in both complex I and complex III activity due to the presence of mtDNA mutations respectively in ND1 and cytocrome b [142]. A similar decrease in complex I subunits content was reported in a case of a rare nasopharynx oncocytoma, along with a mtDNA copy number ncrease [140]. Instead, decrease in activity of complex I and IV was reported in vivo in a peculiar case of Warthin tumor [144].

All these data point at a dysfunction at least in respiratory complex I as the main trigger for the subsequent increase in other mitochondrial proteins and, hence, for the compensatory effect in oncocytic cells [135].

However how mtDNA mutations contributes to oncocytic development is not so clear. It is worth noting that the mtDNA mutations underlying the oncocytic phenotype may not be the primary hit in tumorigenesis as previously discuss [141]. The hypothesis of the active contribution of the mtDNA mutation to the oncocytic transformation fits well with the model of the compensatory effect. In fact, based on this model, the mtDNA mutation may cause the respiratory dysfunction which triggers a retrograde signalking to the nucleus resulting in mitochondrial biogenesis [135].

1.4 COWDEN SYNDROME AND PTEN

Cowden Syndrome (CS) belongs to the family of the *PTEN* hamartoma tumor syndromes (PHTS) that also includes Bannayan-Riley-Ruvalcaba syndrome (BRRS), Proteus syndrome (PS) and Proteus like syndrome [145].

CS is a highly variable, autosomal-dominant hereditary cancer susceptibility syndrome. The patients affected by CS developed multiple hamartomas, unusual skin and facial findings, abnormal CNS findings and increased risk of malignancies.

Mutations in the tumor suppressor gene *PTEN* (Phosphatase and Tensin homologue on chromosome 10) are the cause of CS [146].

The majority of mutations occur in exon 5, but mutations in the other exons, except for the first, have also been reported. CS patients are characterized by germ-line mutations of *PTEN* including nonsense, frameshift, splice site, missense mutations, as well as deletion/insertion of one or more exons all leading to monoallelic loss of function.

PTEN is a "haplo-insufficient" tumor suppressor gene, which is particularly sensitive to dosage. Generally, tumor suppressor genes must be fully inactivated to participate in tumor initiation and progression, as explained by Knudson's "two-hit Hypothesis". It has been shown in some cases, that many deleted regions in a tumor or in patients are not accompanied by mutations or deletion of the other allele. This suggests that these deletions could be passenger events, not implied in tumorigenesis. The alternative explanation is that the deleted region includes tumor suppressor genes for which the loss of one single copy has a role in tumorigenesis. Another hypothesis is that the single mutation acts as a dominant negative towards the other allele resulting in loss of both copies function, mimicking the effect of homozygous or, third hypothesis, both copies of the gene are necessary to maintain its function. In this case a single-copy loss of function mutation may be sufficient to cause the phenotype.

PTEN dosage-dependence was first demonstrated in mice [183] in which loss of one allele of *PTEN* has been shown to promote the progression of a lethal polyclonal autoimmune disorder with high penetrance. These findings suggested that a residual wild type allele is not enough to sustain normal function. Similarly *PTEN* heterozygosity appeared to promote epithelial cancers, such as prostate cancer, in mouse model of PTEN loss [184].

1.4.1 PTEN: Structure and functions.

The *PTEN* (Phosphatase and tensin homologue deleted in chromosome 10) tumor-suppressor gene is located on chromosome 10q23.3, a genomic region that suffers

loss of heterozygosisty (LOH) in many human cancers [147]. Somatic deletion or mutations of this gene have been identified in a large fraction of tumors, including glioblastomas, endometrial and advanced prostate cancer, thus placing *PTEN* among the most commonly mutated genes in human cancer [148].

PTEN encodes for a dual protein/ lipid phosphatase. There are two major domains of PTEN, the N-terminal phosphatase domain and the C-terminal domain. Tumor suppressor function requires both the phosphatase domain and the C2 or lipid membrane binding domain. The PZD domain is responsible to the interaction with other proteins, instead the PEST domain may contribute to protein stability (Fig. 9).

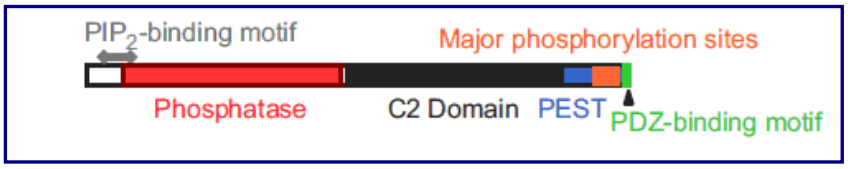


Fig. 9: PTEN domains structure

PTEN primary target is the phosphatydil-inositol 3,4,5 triphosphate (PPI3) which is the direct product of the phosphatidylinositol 3-kinase (PI3K). Loss of PTEN function, either in murine embryonic stem cells or in human cancer cell lines, results in accumulation of PIP3 mimicking the effect of PI3K activation and triggering the activation of its downstream effectors, PDK1, AKT/PKB and Rac1/cdc42. Activated PDK1 phosphorylates AKT at Thr308 activating its serine-threonine kinase activity.

Once phosphorylated in Thr308, further activation occurs by PDK2 (mTORC2 or DNA-PK) by phosphorylation at Ser473. AKT activation stimulates cell cycle progression, survival, metabolism and migration through phosphorylation of many physiological substrates [150].

PTEN therefore negatively regulates the AKT pathway leading to decreased phosphorylation of AKT substrates such as TSC2 and PRAS40 that control mTOR activity, p27, p21, glycone synthase kinase 3 (GSK3A), BCL-2-associated agonist cell death (BAD), apoptosis signal regulating kinase 1 (MPK3K5), WT1 regulator PAWR (also known as PAR 4) and CHK1, as well as members of the fork-head transcription factor family (for example FOXO1, FOXO3 and FOXO4) and others.

Changes in phosphorylation alter the activity and/or localization of these proteins, which in turn affects processes such as cell cycle progression, metabolism, migration, apoptosis, transcription and translation (Fig.10).

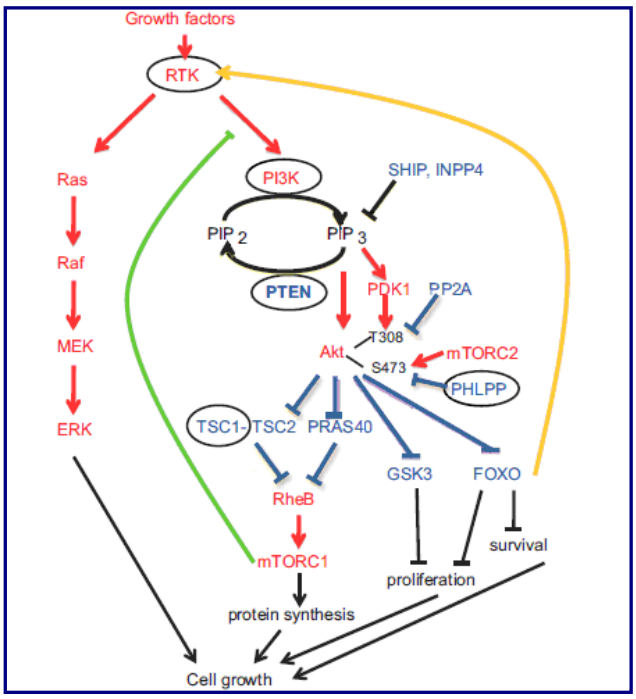


Fig.10: The PTEN-PI3K Pathway.

PTEN phosphatase also targets different proteins, including proteins that regulate cell migration and invasion, such as p130 and FAK, and pathways that control proliferation and differentiation, such as MAPK.

It has recently demonstrated that PTEN have a nuclear function [151,152]. It is important to notice that PTEN does not contain typical nuclear localization signals (NLS) or nuclear export motifs (NES). Several mechanisms for the nucleus-cytoplasmic shuttling of PTEN have been postulated, including simple diffusion; export dependent on a putative cytoplasmic localization signal, active shuttling by

the RAN-GTPase or major vault protein (MVP), phosphorylation dependent shuttling and monoubiquitylation-dependent import [151].

It has been shown that nuclear PTEN acts to maintain chromosomal stability [152]. The binding of PTEN to centromere protein C1 (CENP-C1) is required for centromere stability, and its nuclear localization is required for DNA double-strand breaks (DBS) repair that is mediated by DNA repair protein RAD51. PTEN also regulates the tumor suppressor function of anaphase-promoting complex (APC) and of its regulator E-cadherin, in the nucleus, independently of its lipid phosphatase activity. Altered APC-CDH1 activity has been implicated in multiple tumor types.

Furthermore *in vitro* studies demonstrated that nuclear PTEN induces G0-G1 arrest since cells expressing wild type PTEN protein exhibit a slower growth rate and higher G1:S ratio than cells expressing the nuclear-localization-defective mutant. This increase in the number of cells in G0-G1 is probably a direct result of down regulation of cyclin D1 by nuclear PTEN. It might lead to decreased tumor growth, explaining another aspect of PTEN tumor-suppressor function [50].

1.5 BIRT-HOGG-DUBE' SYNDROME AND FLCN

Birt-Hogg-Dube syndrome (BHD) is an autosomal dominant condition, caused by germline mutations in the *FLCN* (folliculin) gene. Patients affected by this syndrome are characterised by skin fibrofolliculomas, multiple lung cysts, spontaneous pneumothorax, and renal cancer [153].

1.5.1 FLCN: structure and functions

FLCN is located on chromosome 17p11.2; and it consists of 14 exons [153].

The majority of mutations, identified in *FLCN* sequence, were predicted to introduce a premature stop codon into *FLCN* and therefore to result in protein truncation [154]. This includes the "hot spot" for insertions/deletions in exon 11.

It is unclear whether the truncated FLCN is targeted for degradation or remains in the cell with an altered function

Somatic mutations in the remaining wild type allele of *FLCN* or loss of heterozygosity on chromosome 17p11.2 have been identified in BHD-associated renal tumors, supporting Knudson "two-hit" hypothesis and a tumor suppressor

role for *FLCN* [80]. However there is evidence that FLCN does not behave as typical tumor suppressor protein.

In a study of five BHD patients [81] the authors found no evidence of somatic mutations and loss of heterozigosity in fibrofolliculomas suggesting that haploinsufficiency is enough to cause benign tumor growth in the skin.

FLCN consisting of a short hydrophobic N-terminal sequence, one N-glycosylation site, three myristoylation sites and a glutamic-acid rich coiled coil domain centrally located in the protein [154].

Two FLCN-interacting proteins have been described: FNIP1 and FNIP2. FLCN-binding protein 1 (FNIP1) was identified in 2006 [155] as an evolutionary conserved protein that interacts with and phosphorylates FLCN.

FNIP1 also binds AMPK, which is a negative regulator of mTOR and a key protein for energy sensing in cells [155,156] demonstrated that both FLCN and FNIP1 are phosphorylated by AMPK. This interaction between FNIP1 and FLCN was also shown to be modified by external influences, since treatment with an AMPK inhibitor (compound C), rapamycin or amino acid starvation affected the phosphorylation status of FLCN, further indicating a role for FLCN in energy sensing and the mTOR pathway.

FNIP2, is a second FLCN-binding protein and it is homologous to FNIP1 (49% identity, 74% similarity). As with FNIP1, it is conserved across species and binds AMPK [157,158] *in vitro* kinase assays also suggest that FNIP2 is phosphorylated by AMPK [158]. FNIP1 and FNIP2 are able to form homo- and heterodimers, as well as multimers [158] suggesting a functional association between these two proteins.

Further research has shown that Ser 62 is a phosphorylation site in FLCN, this work also suggests that ser62 phosphorylation is indirectly up-regulated by AMPK [159]. FLCN also appears to be phosphorylated at Ser302 by unknown kinases downstream of mTORC1 [160]. Since mTORC1 is known to be indirectly down-regulated by AMPK, this process could be associated with an unknown feedback mechanism that regulates mTOR signaling.

The molecular functions of FLCN are poorly understood, but it is known that FLCN and AMPK interaction, as mediated by FNIP1 and FNIP2, is involved in mTOR signaling [155].

Nevertheless the functional role of FLCN in mTOR signaling is undetermined, since several recent publications have reported contradictory effects on an indicator of mTOR activation (known as phosphorylated ribosomal protein S6 / p-S6R) when *FLCN* expression is reduced. Two studies recently reported that transient downregulation of *FLCN* by siRNA in human cell lines results in reduction of phosphorylation of p-S6R [158, 161]. Reduction of p-S6R was also observed in renal cysts developing in mice heterozygous for *FLCN* [162]. In contrast, kidney-specific homozygous knockout of *FLCN* resulted in an increase in phosphorylated p-S6R, which contributed to the development of polycystic kidneys [163].

Recent research has also indicated a role for FLCN in other signaling systems and cellular processes (Fig.11).

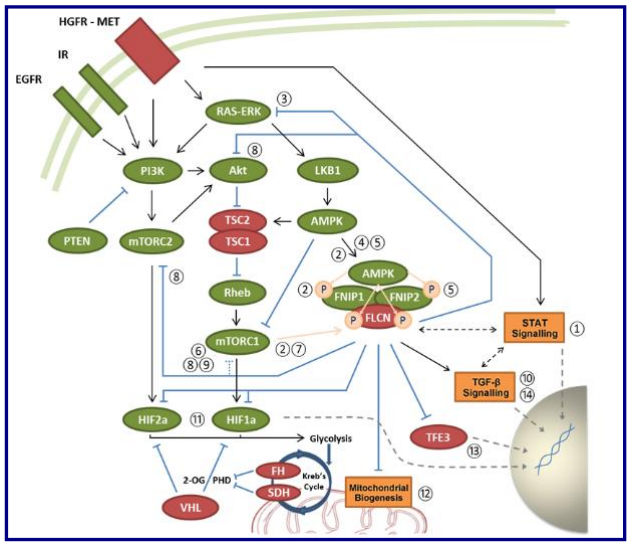


Fig.11: FLCN pathway and interactors.

Other cellular pathways seem to be controlled by FLCN function; using a RCC cell line derived from a BHD patient [164] demonstrated that FLCN also influences HIF-1α signaling. The authors suggested that a high level of HIF-mediated expression in these FLCN-null cells alters cell metabolism through elevated levels of metabolic enzymes. This altered metabolic state parallels a phenomenon known as the Warburg effect, which is commonly seen in cancerous cells [165] postulated that cancer should in fact be interpreted as a mitochondrial disease, and further work [166] suggests that the loss of FLCN in BHD syndrome results in mitochondrial dysfunction, as indicated by a high level of mitochondrial gene expression.

2.MATERIALS AND METHODS

Cell lines

The following cell line was utilized for the study:

- RPE1: ephitelial cell line derived from human retina with non-rearranged cariotype
- XTC.UC1 (*Thyroid Oncocytoma Cell Line*): cell line derived from an oncocytic thyroid tumor [Zielke A et al., 1998].
- HCT116: human cell line derived from a colon-rectal tumor
- HEK293: an immortalized cell line derived from human embryonic kidney
- HPS11: ci-hybrid cell line derived from the fusion of fibroblast enucleated from a health patient and, as cytoplasmic donors the *143B TK*$^-$ deprived of their mtDNA (Rho0) [G.Attardi e M.King, 1989]

RPE1 and XTC.UC1 were cultured in DF12 medium (Dulbecco's modified eagle's medium/nutrient mixture F-12 ham (*Sigma*)) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin G-streptomycin.
HCT116, HEK293 and HPS11 were cultured in DMEM (*Dulbecco's Modified Eagle Medium*) supplemented with 10% FBS, 1% penicillin G-streptomycina and 1% L-glutammine.
Cells were maintained in 5% $C0_2$ at 37°C

γ-rays treatment

For γ-rays treatment is followed a standard protocol, previously optimized in our laboratory. The biological irradiator IBL437C (89-294) is used for the treatment. Cell are growth in T25 flaks, and the treatment is started the day after the seeding. Each cell lines is irradiated at 4Gy for 3/4 days as indicated in Table 1.

	RPE1
T0	Untreated control
IR4	4 doses of 4 Gy

RL	4 doses of 4 Gy and 120 hours of recovery

	XTC.UC1
T0	Untreated control
IR4	4 doses of 4 Gy
RL	4 doses of 4 Gy and 120 hours of recovery

	HCT116
T0	Untreated control
IR3	3 doses of 4 Gy
RL	3 doses of 4 Gy and 120 hours of recovery

Rapamycin treatment

Cells are treated with rapamycin (*Cell Signaling*), a specific inhibitor of mTORC1 complex. Rapamycin is a bacterial macrolide with antifungal and immunosuppressive activity. Rapamycin forms a complex with the immuniphilin FKBP12 which then inhibits the activity of mTORC1. The treatment leads to the desphosphorylation and inactivation of p70S6K kinase.

Cells are seeded in T25 cm^2 flasks, the day after the culture medium was replaced with medium containing rapamycin 10nM (optimal dose that induces dephosphorylation of p70S6K).

For each cell lines a flask was subjected to a combined treatment rapamycin+radiation. The time and doses of irradiation are the same indicated in table 1. After the last dose of radiation, the medium is substituted with fresh medium containing rapamycin. After 120h of recovery the cells are utilized for the successive analysis.

SA-β-Gal assay

The marker that is commonly used to identify senescent cells is Senescence-Associated β-galactosidase activity (SA-βgal) [26]. In normal cells, β-galactosidase, a lysosomal enzyme, is active at pH 4; instead senescent cells are characterized by an increase activity of this enzyme. Consequently, the β-galactosidase activity can be measured also at suboptimal pH6.

For the assay, cells are seeded in T25 cm^2 flasks, washed twice with PBS and then incubated with a fixing solution for 7' at RT. This solution contains:

- Glutaraldeide 2%
- Formaldeid 0.2%
- PBS

Then the flasks are washed five times with PBS and then incubated with a staining solution. The staining solution contains:

- Citric acid+Sodium Phosphate Buffer 40mM at pH 6
- $K_4[Fe(CN)_6]x3H_2O$ 5mM
- $K_3[Fe(CN)_6]$ 5mM
- NaCl 150mM
- $MgCl_2$ 1mM
- X-Gal 1mg/ml
- H2O

The flasks are incubated at 37°C (without CO_2) and protected from light.

The day after the staining solution is eliminated and the flasks washed twice with PBS and then microphotographeted.

Flow cytometry

In order to confirm the proliferative arrest induced in a senescent cell, the distribution of cells in the different phases of cell cycle was analized.

The protocol derive from the original Nusse protocol (Nusse, M., et al "*Flow citometric analysis of G1- and G2/M phase subpopulations in mammalial cell nuclei using side scatter and DNA contenent measurements*" . Cytometry **11:** 813-821 (1990)).

Cells are seeded at low confluence and subjected to γ-rays treatment as indicated in table 1.

At the end of the treatment, cells are first pelletted at 1500rpm for 10', then are resuspended with Nusse 1 Solution at a final concentration of $2x10^6$ cells/mL. The Nusse 1 Solution contains:

- NaCl 584mg/L
- Trisodic Citrate 1139 mg/L
- RNAsi 10mg/L
- Nonidet P40 300 µg/L
- H_2O

Cells are incubated for 30'/1h in ice. Then is added Nusse 2 Solution (same volume of Nusse 1 Solution) to reach a final concentration of $1x10^6$cells/mL. The solution is vortexed and the sample is transferred in a cytoflurimetric tube and incubated with Propidium Iodide (50µg/mL) for 15' and then analysed with a flow cytometer (BD-FACSAria).

DCDFA

To evaluate the hydrogen peroxide production, cells are incubated with dichlorodihydrofluorescein diacetate (DCFDA) as indicated by manufacturer. Cells were seeded in dishes ($60mm^3$) and irradiated following the protocol optimized for each cell lines. After 120h of recovery in incubator, cells were washed twice with PBS, then is added DMEM without red-phenol and cells are incubated for 30' at 37°C with 2µM of DCDFA. At the end of the incubation, cells is washed twice with PBS, fresh DMEM without red-phenol is added and then the dishes is analyzed at immunofluorescent microscope.

Cloning

PTEN and FLCN complete cDNA were cloned in two different plasmid vectors, respectively pcDNA 3.1(-)PURO and pcDNA 3.1(+)ZEO.

The pcDNA 3.1(-)PURO plasmid derived from pcDNA3.1 NEO (*Invitrogen*), and carries a puromycin resistant casset, for selection in mammalial cells, which substitues the neomycin cassette of the original vector.

For PTEN cloning are used the following primers:

PTEN-EcoRI-FW CGGAATTCGCCACCTGACAGCCATCATC

PTEN-HindIII-RV GACAAGCTTTCAGATTTTGTAATTG

The reaction is prepared as indicated:

- cDNA 1 µl
- BUFFER 10X 2 µl
- dNTPs (2mM) 2 µl
- $MgCl_2$ 1,6 µl
- PRIMER FW+RV 2+2 µl
- Fast Taq (*Roche*) 0.2 µl
- H_2O 9,2 µl

And the theromcycler impostated as indicated:

Temperature	***Time***	***Cycle***
96°C	10 min	1
96°C	30 sec	30
60°C	30 sec	
72°C	30 sec	
72°C	7 min	1

The PCR product is then cloned in pGEM-T Easy vector system (*Promega*) for the amplification. First the polyA is added at the PCR product by the addiction of 0,2µl of Taq Gold (*Invitrogen*) and the mixture incubated 15min at 72°C. Then, the transformation reaction is prepared as following indicated:

- PCR 1,5µl
- pGEM 1 µl
- Buffer 2X 5 µl
- T4 DNA ligase 1 µl
- H_20 1,5 µl

O/N at 4°C.

The day after transformation reaction is performed: 4 µl of the ligation mix is added to 100 µl of DH5α competent cells, in a pre-chilled tube. The mix is incubated 30' in ice, then heated for 1 min at 42°C and then 5' in ice. Then 100 µl of LB medium is added to the mix, and the batteria is growth at 37°C for 1h.

Then the mixture is plated in a LB+AMP plate add with X-Gal (80µg/µl) and left growth O/N at 37°C.

The white colony are picked and used to performed a *PCR-colony reaction* in order to verify the presence of the insert:

- Buffer 10X 1,5µl
- $MgCl_2$ 1,5 µl
- dNTPs (2mM) 3 µl
- PRIMER FW+RV 0,6+0,6 µl
- Taq GOLD 0,1 µl
- H_2O 6,7 µl
- DNA 1 µl

Thermocycler program:

Temperature	***Time***	***Cycle***
96°C	7 min	1
96°C	30 sec	35
53°C	45 sec	
72°C	1 min	
72°C	7 min	1

The colony which showed to have the insert is inoculated in 2 ml di LB+AMP, to proceed to DNA extraction (mini prep).

The correct mini prep is restricted with the specific restriction enzyme as indicated:

- 14µl mini prep
- 1 µl EcoRI (Fermentas)
- 1 µl HindIII (Fermentas)
- 4 µl TANGO Buffer 2X

O/N at 37°C.

The restriction is verified on agarose gel 1%, the residual mix is then loaded on a crystal violet gel (60 ml of TBE and 30 µl of crystal violet) to extract the digest insert. The insert was then extract from the gel using a kit (*Quiagen*).

The vector is restricted and dephosphorylated by added 1µl of CIAP enzyme (fermnetas), the mix is incubated for 30' at 37°C, then the enzyme is inactivated at 65°C for 5' and then in ice. Finally, the dephosphorilated insert is purified by plate (Millipore) and is resuspended in 40µl of MilliQ water.

The ligation reaction is performed as indicated:

- 2 µl Buffer 10X
- 1 µl T4 DNA ligase (Fermentas)
- 12 µl PTEN (EcoRI/HindIII)
- 1,5 µl pcDNA 3.1 (-)PURO (EcoRI/HindIII)
- 3,5 µl H_2O

O/N at 16°C.

The ligation reaction is then transformed in DH5α, following the protocol described above, and finally plated in LB+AMP (37°C O/N). The colony is picked up, inoculated in 2 ml LB+AMP for DNA extraction (mini-prep).

DNA extracted is then sequenced to verify the presence of insert, its right orientation and the frame with vector sequence. Finally, the correct insert is inoculated in 50ml LB+AMP, for midi prep extraction (*Roche*).

FLCN cDNA is cloned in two different vectors pcDNA 3.1(+)ZEO and pcDNA 3.1(-)PURO. Before being inserted in pcDNA 3.1(-)PURO, FLCN is cloned in pcDNA3.1mycHis vector, in order to inserted the myc flag at 3'end.

The following primers are used for the cloning:

FLCN-EcoRV-FW TAGATATCCGCCACCATGAATGCCATCGTGGCT

FLCN-XhOI-RV ATCTCGAGCGTTCCGAGACTCCGAGGC

Then FLCN-myc is cloned in pcDNA3.1 (-)PURO, using the following primers:

FLCN-EcoRV-FW TAGATATCCGCCACCATGAATGCCATCGTGGCT

FLCN-BamHI-RV TGGATCCTCAATGGTGATGGTGATGATGACCGGT

The PCR mix is performed as indicated:

- 2,5 µl dNTPs
- 2,5 µl Buffer 10X
- 0,2 µl Taq EasyA
- 1 µl insert
- 17,3 µl H_2O

Theromcycler is programmed as following reported:

Temperature	***Time***	***Cycle***
95°C	2 min	1
95°C	40 sec	30
60°C	30 sec	
72°C	12 min	
72°C	10 min	1

The PCR product is purified by gel extraction (*SIGMA*) and then restricted with the specific restriction enzyme. Then is transformed in pGEM vector (*Promega*).

The double digestion is performed in two following step.

FLCN and pcDNA 3.1 (-) PURO is first digested with EcoRV (NEB):

-12 µl FLCN/ 10 µl pcDNA 3.1(-)PURO

- 1 µl EcoRV (NEB)

- 0,2 µl BSA 100X

- 2 µl Buffer 10X

- 4,8/6,8 µl H_2O

O/N at 37°C.

The restriction is verified on 1% gel agarose and then purified, by purification plate (Millipore), before proceeding to the second digestion.

Then all the purified PCR product is used for the digestion reaction with BamHI (Fermentas):

- 17µl insert/pcDNA 3.1(-)
- 2 µl Buffer 10X
- 1 µl BamHI(Fermentas)

37°C O/N.

The pcDNA 3.1(-)PURO is dephosphorylated with the use of CIAP enzyme, and then purified by plate (*Millipore*). FLCN is loaded a crystal violet gel and then extracted with a kit (Sigma). The ligation reaction is performed as indicated:

-2µl Buffer 10X

- 1 µl T4DNA ligase

- 9,9 µl FLCN

- 1,3 µl pcDNA 3.1(-)PURO

-5,8 µl H2O

16°C O/N

All the successive steps followed the same protocol used for PTEN cloning.

FLCN is finally cloned in pcDNA3.1(+)ZEO. FLCN in pGEM vector and pcDNA 3.1(+)ZEO is both digested with EcoRV and NotI (Roche). Then the ligation is performed following the protocol described before. All the successive steps followed the same protocol of the other cloning.

Transfection

Cells is seeded the day before in concentration that allow to reach the 70-90% of confluence in 24 h. The transfection reaction is performed using the X-treme GENE HP DNA Transfction Reagent (roche), following the manufacturer protocol.

Prepare in a tube 200 µl(for a 6 wells dishes) and 600 µl (for a T25 flask) of serum free medium and add 2 µg or 6 µg, respectively for a 6 wells and for a flask, of transfection reagent. Mix well and incubate for 15' at RT. Add directly the mix, containingthe plasmid and the transfection reagent, to the cell culture without changing the medium. Then , the cells are incubated at 37°C for 48h.

Cellular lysate

The cells are first pelleted at 1500g for 5', then the medium is removed and the pellet is washed twice with PBS buffer.

The pellet is resuspended in RIPA buffer (Tris-HCl pH7.4 50mM, NaCl 150mM, SDS 0,1%, Triton-X 1%, EDTA 1mM) with Phosphatases Inhibitor (10X-*Roche*)+ Protease Inhibitor (25X-*Roche*)+$NaOV_3$ (Tyrosine Phosphatase Inhibitor-1mM).

The suspenction is incubated for 15' in ice, then is passed, at least 4 time, through an insuline syringe.Then it is frozen and thawed twice and centrifuged 10' at 13.000rpm at 4°C. Finally the supernatant is transferred in a new tube.

Proteins lysates are quantified using the Lowry High Standard Curve method (Bio-Rad) following the manufacturer instruction.

SDS_Page and Western blot

40 µg of proteins are loaded on 10% poly-acrilammide gel. The protein is resuspended in Laemly Buffer (Tris-HCl pH 6.8 125mM, 4% SDS, glicerol 20%, bromo-phenol blue 0,000025% and β-mercaptoethanol 10%). The samples is denaturated at 99 °C for 5'.

Run is performed in Running Buffer 1X (Tris pH 8.3 25 mM, glycine 193 mM, SDS 1%) at 120 V for ~2 hours. At the end of the run the proteins are transfered on nitrocellulose membrane (Perkin-Elmer). The transfer is performed in pre-chilled Transfer Buffer solution 1 X (Tris pH 8.3 25mM glycine 193 mM, methanol 20%) at 300mA for 1 hour at 4°C.

Western blotting is performed following the *WesternBreeze® Chemiluminescent Water Blot Immunodetection Kit* (*Invitrogen*). After transfer the nitrocellulose membrane is incubated in *Blocking Solution* (Blocker/Diluent A: concentrated buffered saline solution containing detergent, Blocker/Diluent B: concentrated hammersten casein solution, e H_2O) for at least 30'.

Primary antibodies are diluited in Blocking Solution and incubated for 1 hour at RT.

For the experiments are utilized the following primary antibodies:

- Mouse anti-PTEN (Cascade Bioscience) 1:1000
- Rabbit anti-p16 (*Santa Cruz*) 1:500
- Rabbit anti-p21 (*Santa Cruz*) 1:1000
- Mouse anti-p53 (*Santa Cruz*) 1:1000
- Rabbit anti-p70S6K1 (Cell Signaling) 1:1000
- Rabbit anti-Phospho-p70S6K1 (Cell Signaling) 1:1000
- Mouse anti-Myc (Invitrogen) 1:1000
- Rabbit anti-NDUFA9 (Santa Cruz Biotecnology) 1:2000
- Rabbit anti-SDHA-SDHB (Mitosciences) 1:1000
- Rabbit-ATPase5B 1:1000
- Mouse anti-TFAM (Santa Cruz Biotecnology) 1:10000
- Mouse anti-Catalase (Bethyl laboratories) 1:1000

- Rabbit anti-Prx3 (LabFrontiers) 1:100000
- Rabbit anti-MnSOD (Invitrogen) 1:1000
- Mouse anti-Tubulin (*Sigma*) 1:10000
- Mouse anti-Actin (*Sigma*) 1:10000

Then the nitrocellulose membrane is washed 3 times for 5' in *Wash Solution* (concentrated buffered saline solution containing detergent), the membrane is incubated with secondary antibodies for 30'(anti-mouse or anti-rabbit).

Then the membrane is washed 3 times for 5' in wash solution and twice with water. Finally *Chemioluminescent Substrate* (Solution of CDP-star chemiluminescent substrate for alcaline phosphatase e Chemiluminescent Substrate Enancher) is added and incubated for 5' before proceeding to the developing.

RNA extraction

RNA extraction was performed using the Trizol reagent (*Invitrogen*) according to the manifacturer protocol.

Cells are first pelleted at 1500 rpm for 5', then the pellet are washed twice in PBS buffer.The pellet is re-suspended in 300 µL of Trizol Reagent and transfered in a new tube.Follows an incubation a RT for 5'.

60 µL of chloroform are added to separated the two phases. The tube is shaker 15" in hands and then incubated at RT for 2-3'. Then the tube is centrifugated at 12.000g for 15' at 4°C. The aqueous phase is removed avoid drawing any of the interphase or organic layer, and transferred to a new tube and the RNA isolation phase begins.

200 µL of isopropanol is added and the mix is incubated 10' at RT. Then is centrifugated at 1200g for 10' at 4°C. The supernatant is removed to leave only the pellet in the tube. The pellet is washed by adding 200µL of ethanol (70%) and centrifugated at 7500g at 5'. Finally the ethanol is removed, the pellet is air dried, but not completely to avoid that the pellet can loose solubility.

Then the pellet is resuspended in 20-40 µL of RNAase-free water and incubated for 10' at 55°C-60°C to help resuspension. The integrity of the RNA is verified on electrophoresis gel at 1%. For the quantification is utilized the Nanodrop (*Thermo Scientific*).

Reverse transcription (RT-PCR)

RT-PCR was performed using the GoScript™ Reverse Transcription System kit (*Promega*) according to the manufacturer protocol.

1µg of total RNA is transcripted

A mix is made combiningthe following reagents:

1. total RNA 1µg/reaction
2. 1µL Random Primers (0.5µg/reaction)
3. Nuclease Free water X µL

To the final volume of 5µL.

The mix is heated at 70°C in a heat block for 5' and then chilly in ice for 5'.

Then the transcription mix, containing the *Reverse Transcriptase* enzyme, is prepared as indicated (final volume 15 µL):

GoScript 5X Reaction Buffer	4 µL
$MgCl_2$ (1.5-5mM)	1.2-6.4 µL
PCR nucleotideMix (0.5mM each)	1 µL
GoScript ™ RT	1 µL
ddH_2O	6 µL

The reaction is then performed in the thermocycler (Applied Byosistem 2700 PCR System):

Temperature	***Time***	***Cycle***
25°C	5 minuti	1
42°C	1 ora	1
70°C	1 minuto	1

Semi-quantitative PCR

The semi-quantitative PCR was performed on the cDNA to verify the efficacy of retrotrascription. A house keeping gene (tubulin) was amplified being sure of its expression in all tissue.

cDNA has been used at different diluitions (1:1, 1:10, 1:100, 1:1000)

The mix is prepared ad indicated (15 µL):

1. cDNA 1µL
2. 2. Buffer 10X 2.5 µL
3. dNTPs 2.5 µL
4. Taq GOLD (*Invitrogen*) 0.2 µL
5. $MgCl_2$ (25mM) 1.5 µL
6. Primer FW (10 µmol) 1 µL
7. Primer Reverse (10 µmol) 1 µL
8. H_2O X µL

The reaction is then performed in the thermocycler

Temperature	*Time*	*Cycle*
96°C	5 minuti	1
94°C	30 secondi	30
61°C	30 secondi	
72°C	30 secondi	
72°C	7 minuti	1

Real Time quantitative PCR (qRT-PCR)

The quantitative real time PCR was performed using the reverse transcriptase mix GoTaq-qPCR Master Mix (*Promega).*

The reaction mix is performed as indicate (20 µL)

1. GoTaq Master Mix 10 µL
2. Primer Forward 1 µL
3. Primer Reverse 1 µL
4. cDNA 2µL
5. H_2O 6 µL

The reaction is performed in the 7500 Fast Real Time PCR System (*Applied Biosystem*), the machine program is:

Temperature	**Time**	**Cycle**
95°C	5 minutes	1
95°C	10 second	40
59°C	20 second	
60°C	30 second	

Analysis was performed using both the ΔCt and the ΔΔCt statistical metod (Kenneth J.Livak and Thomas D.Schmitthen. Methods 25 402-408 (2001).

Sequencing Human Mitochondrial DNA with the mitoSEQr™ kit (Applied Biosystem)

The mitoSEQr system (MitoAll) supplied by Applied Biosystem is an innovative kit designed for sequencing the whole mitochondrial genome from total DNA. A set of 46 primer pairs amplifies, partly overlapping, fragments covering the whole mitochondrial genome and permits to obtain the sequence of the entire 16.6Kb long mitochondrial chromosome. Another set of 9 primers is designed to sequence the D-loop region (about 1.1Kb).

All these primer pairs anneal at the same temperature allowing to perform all the 46 amplifications in one single step. Each primer is also tailed with a universal M13 sequence tag on the 5' end to permit sequencing with M13 primers which allow using only one sequencing reaction mix for all amplicons.

PCR amplification

Amplification is carried out in a final volume of 10 µl on a thermocycler (applied Biosystem *GeneAmp 9700* or *2700 PCR System*). Each amplification reaction contained.

Master mix ampliTaq Gold 2X	5 µL
Human genomic DNA(0.5ng/µl)	1 µL
PCR primer mix (0,6 pmol/µL)	2 µL
Glycerol 50%	1,6 µL
ddH_2O	0,4 µL

The reaction was performed in a thermocycler following the protocol below:

Temperatur	***Time***	***Cycle***
96°C	5 min	1
94°C	30 sec	45
60°C	45 sec	
72°C	45 sec	
72°C	10 min	1

The positive outcome of the PCR reaction is verified by loading a part of the PCR on a agarose gel.

Agarose Gel Electrophoresis

The positive outcome of the reaction is verified by loading a part of the PCR on an agarose gel.

2% and 1% agarose gel is made up with standard 1X TBE buffer (0.05M Tris, 0.05 M boric acid, 0.01 M EDTA) incorporating Ethidium Bromide at a concentration of 0.5 µg/ml (corresponding to 5 µl of EtBr (100mg/ml) in a 100 ml gel). 2µl of each PCR reaction was added to one volume of water plus loading dye (6X), 2µl (100ng/µl) of 1 Kb or 100 bp DNA Ladder (*Invitrogen*) were loaded into one well on each row of wells on the gel. The gel is run at 100V for 20', then the gel is visualized with a UV trans-illuminator at λ 254 nm.

PCR cleanup

PCR products diluited in sterile water to a final volume of 120µl is transferred to a filetr plate which is placed in a vacuum filtration manifold. A vacuum pressure of approximately 250-380mmHg is applied for 5 minutes or until the plate was dry. Plates is removed from filtration manifold and 50µl of MilliQ water is added to each well. Filter plate is then placed on a shaker and gently shaken to resuspend DNA. Finallly, PCR product is purified and can be transferred in a new plate.

Sequencing reaction

For the sequencing reaction are used the reagents supplied by Applied Biosystem. The reaction is prepared as indicated:

Big Dye Terminator v.3.1 RR mix	0,5 µL
Primer FW o RV (M13) (3,2 pMol)	1 µL
Buffer 5X	2 µL
PCR purificata	2 µL
ddH_2O	4,5 µL

The reaction was performed in a thermocycler (Applied Byosistem 2700 PCR System) following the protocol below

Temperature	**Time**	**Cycle**
96°C	1 min	1
96°C	10 sec	30
50°C	5 sec	
60°C	4 min	

For the sequences analysis is utilized the software SeqScape v.25.

Copy number assay UPL (Roche)

The copy number assay is performed to evaluate the occurrence of LOH in CS and BHD patients and XTC.UC1 cell line. The experiment is designed using the Universal Probe Library (Roche) technique. A set of primers are designed along both *FLCN* and *PTEN* gene and the corresponding probes are used to perform the assay:

FLCN

Exon 4

FW GGGATGGGAATGAGGACAG

RV CCGACTGTTCATCTGAATGG

Probe 35

Exon9

FW CTGTCCTTGTCATCTGTCTTGC

RV TCTCCTCCTCTTCAGCCTCA

Probe 33

Exon 14

FW ACAGGGATGGCTGTCCAC

RV GCTTCCTTCCAGCAGTTGAGA

Probe 19

PTEN

Exon 1

FW ACTTGACCTGTATCCATTTCTGC

RV TCCGTCTACTCCCACGTTCTA

Probe68

Exon 9

FW GCTGAGGCAGGCAGATTG

RV GTAGAAATGGGGTTCATGATGTT

Probe 64

The DNA is first quantified, an optimal concentration is established in 20 ng/µL or 40 ng/µL. Primer concentration is optimezed at 0.5 µM .The reaction mix comprises (10µl):

10 µl Master mix

1+1 µl Primer FW+RV

0.2 µl Probes

5.8 µl H_2O

Then 2 µl of DNA is added.

The reaction is performed in the LightCycler 480 Real Time PCR System (Roche Diagnostic). The machine program is:

95°Cx5'

95°Cx15' } x40

60°Cx1' }

PTEN, H-Ras, K-Ras, TP53 sequencing

For *PTEN*, *FLCN*, *H-Ras*, *K-Ras* and *TP53* sequencing specific primers are designed through the software *Primer 3* (Appendice A).

The reaction is performed using FastStart Taq DNA polymerase (Roche) for *PTEN*, *K-Ras* and *TP53*, with Master Mix Kapa 2X (*Resnova*) for *H-Ras* and with Master Mix Taq GOLD (Applied Biosystem) for FLCN.

The PCR is purified with Multiscreen Filter Plates DNA clean-up (Millipore). For the sequencing is used the Sanger technique and the Big-Dye v3.1 (Applied Biosystem) as previously described. The mutations are confirmed through sequencing the two DNA strand, and on a second PCR.

Prediction of mutation effect: PolyPhen

PolyPhen (Plymorphism Phenotyping) is a software used to predict the possible effect of an amino acid substitution in a protein. The program is based on the comparison of homologous proteins. The profile score (PSIC) that is generated for each allelic variant, represents the ratio between the probability of one amino acid in a specific site to the probability of the same amino acid if present in another site. A PSIC corresponding to 2 indicates a *damaging effect*, a score between 1,5 and 2 suggests that the variant is *possible damaging*, instead if the score is low than 1,5 the variant is *benign*.

3. AIM OF THE STUDY

γ-rays, commonly used in radiotherapy for the treatment of patients affected by several type of tumors, could activate mitochondrial biogenesis and cellular senescence. However to date, whether the two processes are related to each other has not been yet demonstrated.

The main purpose of this project is to assess whether mitochondrial biogenesis and cellular senescence can be activated in the same cells following exposure to a genotoxic stress, and if the induction of mitochondrial biogenesis may prelude the activation of cellular senescence.

The study of the oncocytic model XTC.UC1 will also enable us to determine whether the presence of mitochondrial dysfunction, induced by mtDNA mutations, may contribute to senescence activation.

Assuming that mitochondrial biogenesis and senescence are regulated by the same molecular pathway, induced by genotoixic stress, the second aim of this thesis is to determine the involvement of mTOR, and in particular of mTORC1, in the regulation of cell response to γ-rays treatment. Experimental data have indeed demonstrated the role of mTOR in regulating both mitochondrial biogenesis and certain types of cellular senescence.

Another objective of the study is to determine which mechanism induce the development of oncocytoma in patients affected by two hereditary familial syndrome, CS syndrome and BHD syndrome.

The study of CS and BHD patients suggests that the double heterozigous of the two tumor suppressor genes causative of the syndromes, respectively *PTEN* and *FLCN*, leads to mitochondrial biogenesis activation.

In fact, mutations and/or deletion in *PTEN* and *FLCN* have been found in oncoytomas developed by patients affected by CS and BHD.

Based on extant knowledge on the role of PTEN and FLCN, it has been hypothesized that the genes regulate PGC-1α through the involvement of mTORC1. In order to test this hypothesis, XTC.UC1 cell line which reproduces

in vitro what observed *in vivo* in CS and BHD oncocitomas, has been used to determine the effects of PTEN and FLCN restore expression on PGC-1α expression.

4. RESULTS

4.1 STUDY OF THE LINK BETWEEN SENESCENCE AND MITOCHONDRIAL BIOGENESIS INDUCED BY γ-RAYS TREATMENT

γ-rays are commonly used in radiotherapy for the treatment of patients affected by various types of tumors. It has been demonstrated that genotoxic stress induced in cells by γ-ray could determine the activation of mitochondrial biogenesis, which can lead to the transformation of cells towards an oncocytic phenotype.

The increase in mtDNA copy number, an index of activation of mitocondrial biogenesis, was observed in some tissues, in particular in spleen and brain of irradiated mouse models [167, 168]. Furthermore, some patients affected by colon-rectal tumors, which underwent preoperative radiotherapy, have developed relapses with oncocytic features [169, 170].

γ-rays lead to DNA damage mainly in the form of double-strand breaks and can result in the activation of cellular senescence [52, 53]. The senescence is defined as an irreversible arrest of cellular proliferation [1], and is considered, as apoptosis, a mechanism that cells can use in order to limit tumor growth [70].

Genotoxic stress can induce the activation of mitochondrial biogenesis and thus the differentiation of cells towards an oncocytic phenotype and even the activation of cellular senescence. The main objective of this thesis was to clarify if mitochondrial biogenesis and senescence could be induced in the same cell line after γ-ray treatment.

It has been hypothesized that genotoxic stress induces initially mitochondrial biogenesis which may support the activation and/or the maintenance of the senescence phenotype.

Experiments evaluating the activation of mitochondrial biogenesis in response to γ-ray treatments of the cell lines RPE1 and HCT116 had already been conducted in the laboratory of Medical Genetics.

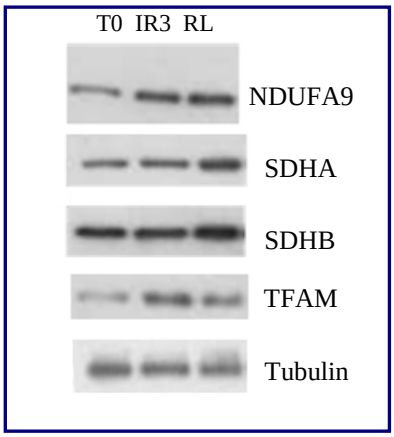
The HCT116 cell line derived from a colon-rectal carcinoma and it has been chosen for the study because the relapses with oncocytic features after radiotherapy had been observed in patients with colonrectal tumors [169, 170]. The RPE1 cell line has been selected because it is a non tumor cell line which allows to understand the effect of γ-rays on a normal tissue.

To establish the effects of genotoxic stress on mitochondrial biogenesis several indexes of biogenesis, such as changes of expression of some mitochondrial proteins and mtDNA copy numbers, have been evaluated. Figure 12 and 13 report the experiments conducted respectively on HCT116 and RPE1 cell lines.

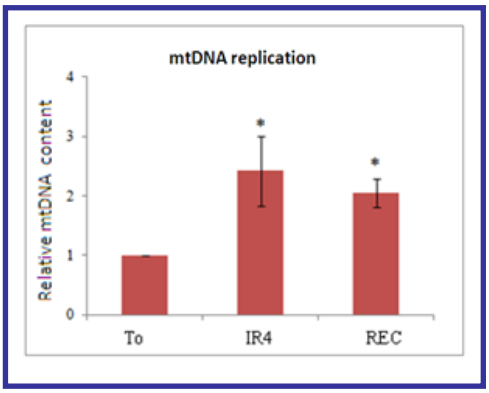
In the HCT116 cell line it has been show that genotoxic stress causes an increase in mtDNA copy number observed after 3 days of irradiation (IR3) which remains constant after 120 hours of recovery (RL). Also an increased expression of some proteins of the respiratory chain complexes, in particular NDUFA9 subunity for complex I, SDHA and SDHB for complex II and the mitochondrial transcriptional factor TFAM, has been observed.

Even in the RPE1 cell line a genotoxic stress induced the increase in mtDNA copy number (it was observed after 4 days of irradiation (IR4) and remained constant after 120 hours of recovery (RL)) as well as the increased expression of some mitochondrial proteins (NDUFA9, SDHA, SDHB and TFAM).

The increase of mtDNA copy number and the increased expression of mitochondrial proteins demonstrated that genotoxic stress induced in RPE1 and HCT116 cell lines the activation of mitochondrial biogenesis.



A



B

Fig.12: A Mitochondrial proteins expression in HCT116 cell line after γ-rays treatment **B:** mtDNA copy number after γ-rays exposure

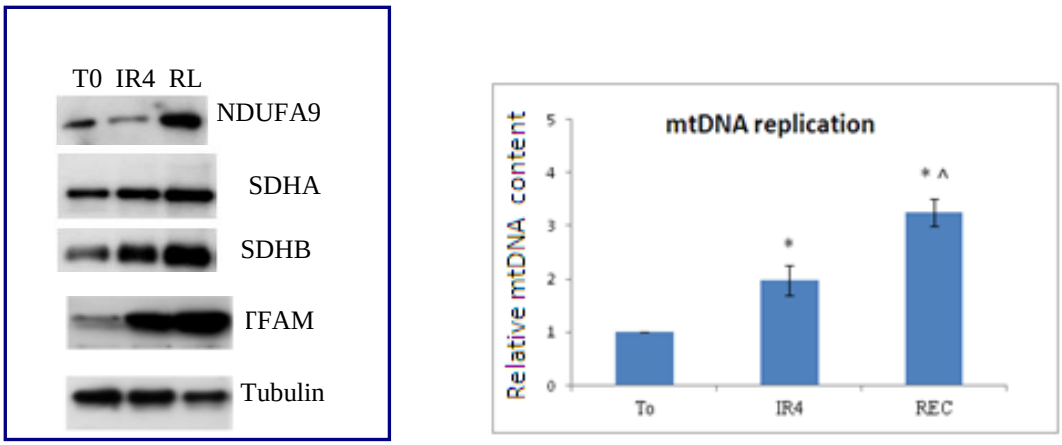


Fig.13: A Mitochondrial proteins expression in RPE1 cell line after γ-rays treatment **B:** mtDNA copy number after γ-rays exposure

4.1.1 Sequencing of TP53, PTEN, H-Ras and K-Ras genes

In order to understand how the genotype of some oncogenes and tumor suppressor genes may affect the ability of cells to activate senescence after exposure to γ-rays, RPE1 and HCT116 cell lines as well as the only existing model of oncocytic tumor, the XTC.UC1 cell line, were analyzed in order to identify mutations in some genes that regulate senescence.

The response of the XTC.UC1 cell line was studied in order to verify if mutations in mtDNA affecting the functionality of respiratory complexes could be involved in the activation of cellular senescence.

Regardless of the stimulus, senescence is regulated in most cases by the tumor suppressor gene *TP53* [2]. The *TP53* gene was therefore sequenced in cell lines in order to verify the presence of mutations that might affect the ability of these cells to induce senescence.

Senescence can also be induced by loss of function of tumor suppressor genes such as *PTEN* [46] or by mutations that activate oncogenes, as mutations in *H-Ras*

[3]. *PTEN*, *H-Ras* and *K-Ras* coding regions were sequenced with the aim of verifying the presence of mutations in these cell lines.

The results of the screening are reported in table 1.

	TP53	***PTEN***	***K-Ras***	***H-Ras***
XTC.UC1	p. P151T+LOH	Del T(67619)+LOH	wild type	wild type
RPE1	wild type	wild type	wild type	wild type
HCT116	wild type	wild type	p.G13D	wild type

Table 1: Results of the sequencing of HCT116, RPE1 e XTC.UC1 cell lines for *TP53*, *PTEN*, *K-Ras* e *H-Ras* genes.

Recently an involvement of mitochondria in the process of cellular senescence [25] has been shown in cells characterized by the presence of a mutations in *H-Ras* (*H-Ras*^{V}*12*) gene that activate the function of the proto-oncogene.

The authors have shown that the pharmacological treatment with molecules such as rotenone and oligomycin, which act as inhibitors, respectively of complex I and ATP synthase, favor the onset of the senescence phenotype [25].

The mitochondrial genome (mtDNA) of the above mentioned cell lines was sequenced with the aim to verify the presence of mtDNA mutations.

The XTC.UC1 cell line is characterized by the presence of a mutation in *TP53*. The sequencing of this gene has revealed the presence of a missense substitution in exon 5 (C13039A) which determines the substitution of a proline in position 151 with a threonine (Fig.14). The UPL copy number assay showed the loss of the wild type allele of *TP53*.

Through the use of *Polyphen* software the potential role of the mutation on protein functionality was assessed. The PSIC score assigned to the mutation was 2.731 and the substitution was defined as "*probably damaging*"

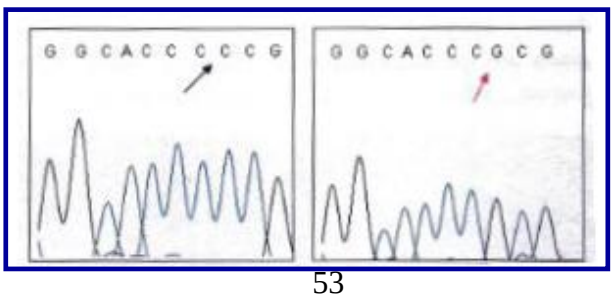


Fig. 14: Electropherogram of *TP53* mutation in XTC.UC1 cell line. The left image reported the wild type control, in the right image is reported the electropherogram of XTC.UC1 cell line. The mutation is indicated by the red arrow.

The amino acid 151 is part of the central domain of p53, also known as DNA binding domain (DBD) [58]. Since p53 acts as transcriptional factor, the presence of a mutation in this amino acid suggests that the protein loses its ability to recognize the DNA sequence and then to activate the transcription of target genes. It should be also noted that this mutation lies in exon 5 of *TP53* that is a mutational hot spot of the gene [171]. Moreover the mutated amino acid precedes and follows two amino acids, respectively Thr150 and Ser149, which are two of the three phosphorylable amino acids of DBD, whose phosphorylation has been shown to increase the protein's ability to interact with and recognize DNA sequence [58]. It can be hypothesized that the substitution of this amino acid could interfere with the ability of kinases to recognize Thr150 and Ser149, with the consequent inability of p53 to recognize target genes.

Sequencing of *PTEN* in this cell line has shown that it is characterized by the presence of a frameshift mutation (deletion of one T) in exon 4 (Fig.15). UPL copy number assay demonstrated besides the loss of the wild type *PTEN* allele. These data were confirmed by western blotting assay, which demonstrated that XTC.UC1 cell line is null for PTEN (Fig. 15). No mutations were found in *H-Ras* and *K-Ras*.

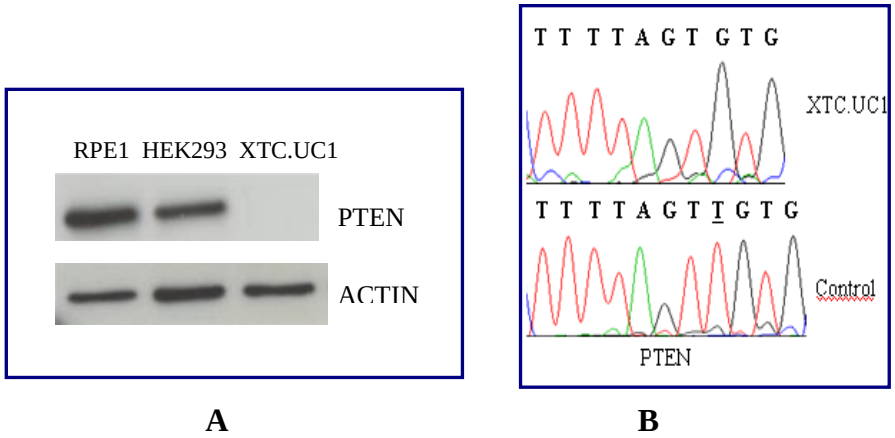


Fig. 15: A: Western blotting showing PTEN expression in a set of cell lines. XTC.UC1 do not express PTEN **B:** Electropherogram showing single base deletion in *PTEN* exon 4 of XTC.UC1

Moreover XTC.UC1 cells carry the mitochondrial mutation m.3571insC [142]. This frameshift mutation inserts a stop codon at amino acid 101 in the *ND1* subunit of complex I, thus generating a truncated protein that is hence degraded. Furthermore the XTC.UC1 cell line carries another mtDNA mutation, namely m.15557G>A, which causes a non conservative missense substitution (E271K) in cytochrome b, the only mtDNA encoded subunit of complex III. Both mutations are present in XTC.UC1 cells in a condition of heteroplasmy at 50%. The result is the alteration of the functionality of respiratory complexes and of the ability of cells to synthesize ATP.

The HCT116 cell line derived from a colon-rectal tumor; there are not mutations in *TP53* and *PTEN* genes, while the presence of mutation in exon 1 of *K-Ras* gene (G5578A), which determines the substitution of a glycine in position 13 in aspartate, has been confirmed (Fig.16). This mutation has already been reported in literature as a mutation that activates the proto-oncogenic function of this protein [172].

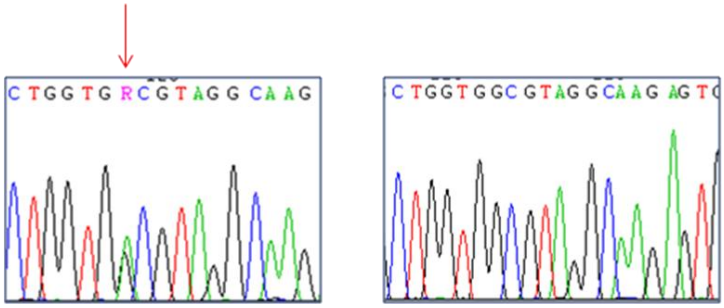


Fig. 16: Electropherogram showing the mutation in *K-Ras* exon 1 of HCT116. The mutation is reported in panel **A** and is indicated by the red arrow. **B** Wild type sequence

With regard to the mtDNA, the insertion of one C in a homopolymeric stretch of 5C's (m.5898incC) has been highlighted (Fig.17). This mutation has no functional implications because is localized in a non-coding region.

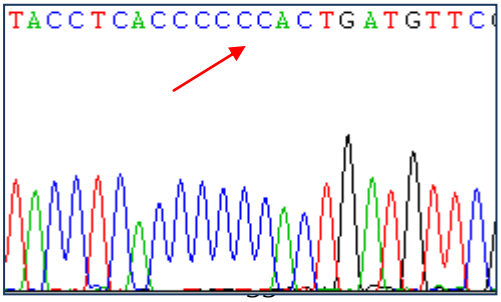


Fig. 17: Electropherogram showing the mtDNA mutation in HCT116 cell line. The insertion of one C is indicated by the red arrow.

Finally, in the RPE1 cell line no mutations were found in *PTEN*, *K-Ras*, *H-Ras* and in mtDNA

4.1.2 Cellular response to a genotoxic stress: cellular senescence

In order to determine the response of RPE1, HCT116 and XTC.UC1 to genotoxic stress in terms of activation of cellular senescence, the above mentioned cell lines have been subjected to treatment with γ-rays following the protocol previously optimized for each cell line (Table 2). The optimal dose of radiation to be administrated to cells was chosen such as to be able to induce an appreciable effect on the phenotype while maintaining cell viability.

	RPE1
T0	Untreated control
IR4	4 doses of 4 Gy
RL	4 doses of 4 Gy and 120 hours of recovery

	XTC.UC1
T0	Untreated control
IR4	4 doses of 4 Gy
RL	4 doses of 4 Gy and 120 hours of recovery

	HCT116
T0	Untreated control
IR3	3 doses of 4 Gy
RL	3 doses of 4 Gy and 120 hours of recovery

Table 2: Protocol of irradiation relative to each cell line

To date no univocal assay that can be used to determine the induction of senescence has been defined, but the marker that is commonly used to assess the activation of the process is a cytochemical assay aimed at assessing the activity of

the lisosomal enzyme β-galactosidase (SA-βGal). It has been demonstrated that senescent cells are characterized by an increased activity of this enzyme [27] whose contribution to senescence has not yet been elucidated.

Even the microscopic observation of morphological changes induced in cells after genotoxic stress was used as a parameter to determine the activation of senescence. In fact it was demonstrated that a senescent cell is characterized by the acquisition of a series of morphological changes such as: increase in size, flattened and shaped morphology and in some types of senescence cytoplasmic vacuoles may occur [10] (Fig.18).

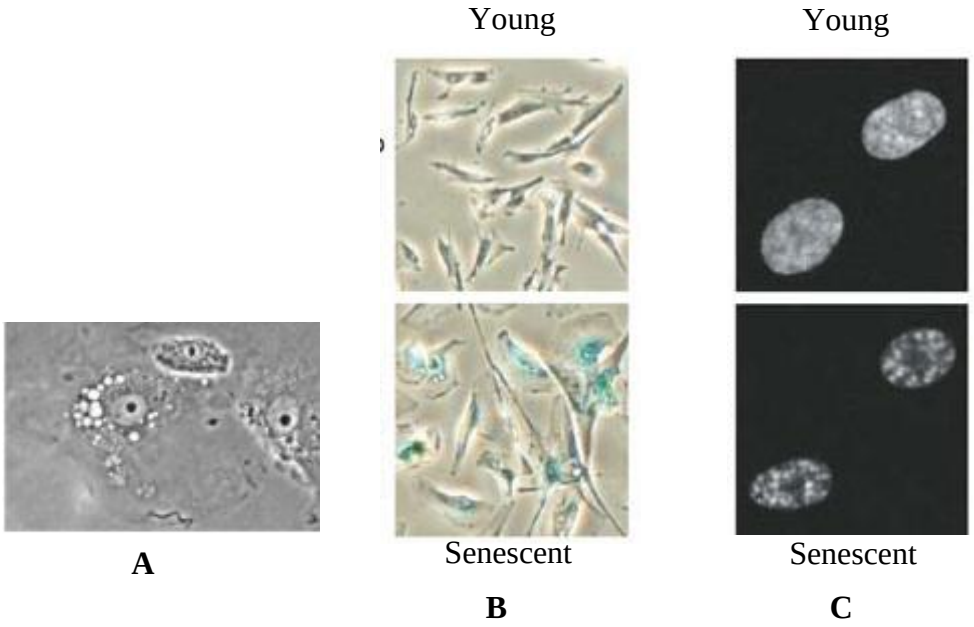


Fig.18: A: Cytoplasmic vacuoles typicall of *H-Ras* induced senescence (Moiseva et al., 2009) **B:** Fibroblats stained with SA-β-gal, senescent cells are blue(Funayama and Fuyuki 2007) **C:** Senescent cells show a large and flat morphoplogy and chromatin condensation (Funayama and Fuyuki 2007).

To confirm that genotoxic stress induces in cells a proliferative arrest, the distribution of cells in the different cell cycle phases (G0/G1-S-G2/M) was evaluated by flow cytometry. Senescent cells stop generally with a typical DNA content of G1 phase [16], although this may vary depending on the cell lines [2]. In general, the activation of senescence should result in a decrease in the percentage of S phases cells which results in a redistribution in the other two phases of the cell cycle (G0/G1-G2/M), which may vary depending on cell type.

Regardless of the stimulus that induces senescence, it was demonstrated that the pathways of p53 and $p16^{INK4A}$-pRB are the two effector pathways of senescence that determine not only the blocking of cell proliferation but also regulate the appearance of most of the morphological changes typical of a senescent cell. It has been shown that these two pathways may interact or act independently in regulating senescence [2]. In order to determine which of these two pathways was involved in regulating the activation of senescence induced by genotoxic stress, the expression of p16, p53 and p21 was evaluated.

RPE1

The images of the SA-β-Gal assay of the RPE1 cell line are reported in figure 19.

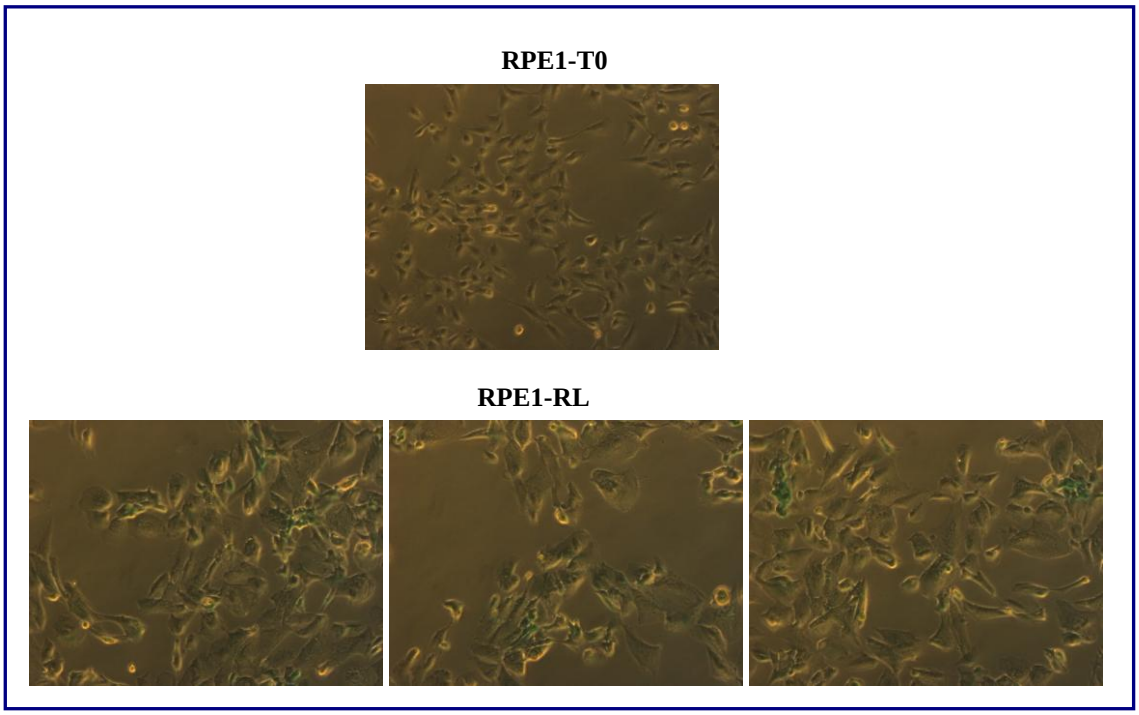
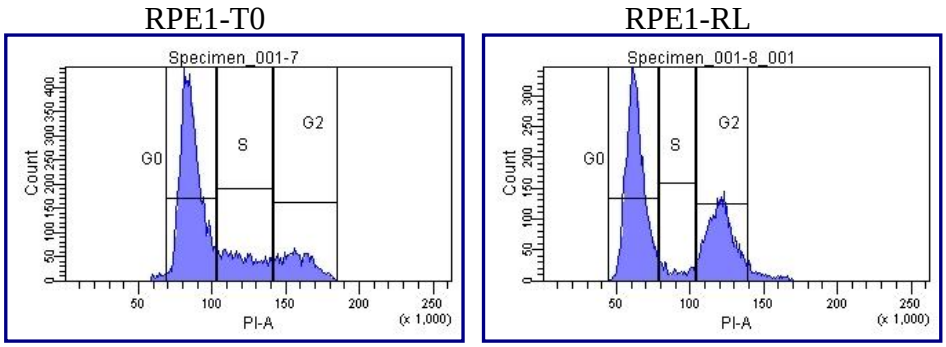


Fig.19: SA-β-Gal assay of RPE1 cell line: after γ-rays treatment cells were fixed and incubated with SA-β-Gal staining solution O/N. Cells were washed and then microphotographed (10X).
T0: untreated control **RL:** cells treated for 4 days at 4 Gy and after 120 hours of recovery.

The positivity of the irradiated RPE1 to the β-galactosidase assay showed that the genotoxic stress induces the activation of the cellular senescence. Furthermore the irradiated cells increase in size and become more flattened with respect to the untreated control.

Analysis of the cell distribution during different cell cycle phases showed that genotoxic stress caused the block of proliferation (Fig.20).

In fact, following γ-rays treatment a decrease in the percentage of cells undergoing S phase was observed (from 18% in the T0 to the 9% in the irradiated cells) as well as a re-distribution of cells between the G0/G1 phase and G2/M phases. Taken together, these results show that genotoxic stress induces in these cells the activation of the cellular senescence.



	RPE1-TO	*RPE1-RL*
GO/G1	60%	53%
S	18%	9%
G2/M	20%	30%

Fig. 20: Analysis of cells distribution in the cell cycle phases after staining with PI by flow cytometry. **T0**: untreated control **RL:** cells irradiated 4 days at 4Gy and after 120 hours of recovery.

To determine which molecular pathway regulates this process, in particular whether p53-p21 and/or p16-pRb pathways could be involved, changes in expression of these proteins were determined by western blotting assay (Fig.21).

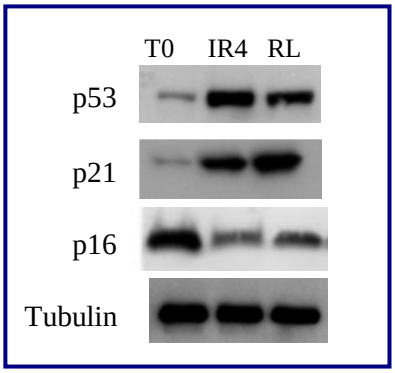


Fig. 21: Effect of γ-rays treatment on p16, p53 and p21 expression. RPE1 cells were irradiated as indicated, lysates of these cells were collected and immunoblotted with the indicated antibodies. Tubulin is used as a loading control **T0**:untreated control **IR4**: cells irradiated 4 days at 4 Gy. **RL**: cells irradiated 4 days at 4 Gy and after 120 hours of recovery.

The treatment with γ-rays of the RPE1 cell line determined an increase of p53 and p21 expression, while with regard to p16 a decrease was observed.

The increase in p53 expression, which was observed after 4 days of irradiation (IR4) and remained unchanged even after 120 hours of recovery (RL), and the consequent increase of p21 expression (which maintains the same performance of its regulator p53) leads us to conclude that senescence induced by genotoxic stress in RPE1 cell line is dependent on the activation of the p53-p21 pathway, while the Rb-p16 pathway is not involved.

HCT116

Figure 22 shows the images of the SA-β-Gal assay performed on the HCT116 cell line following γ-rays treatment.

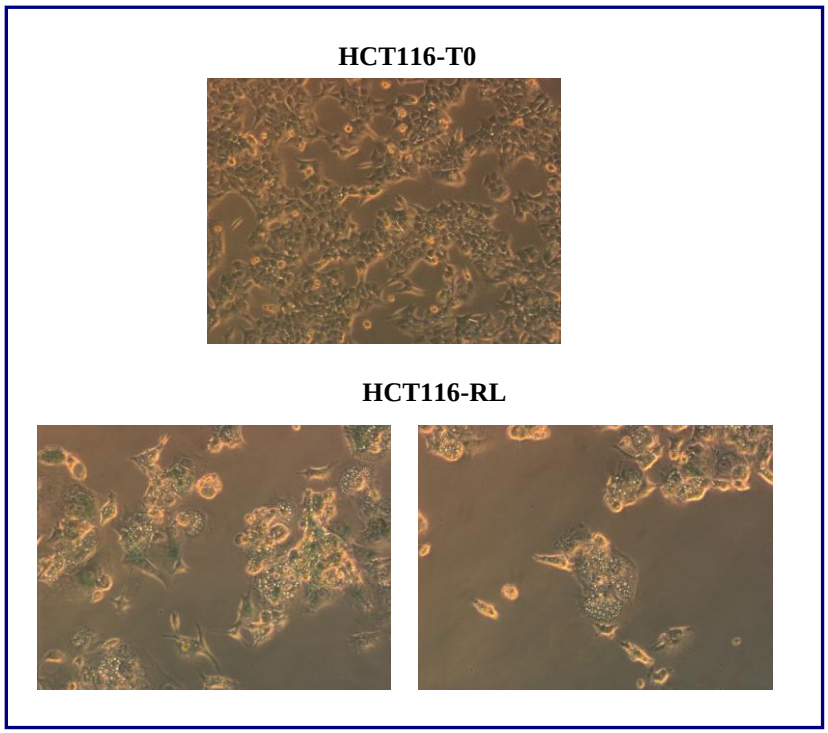


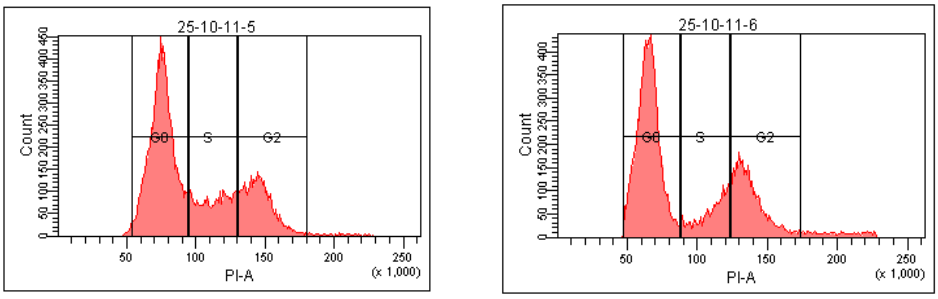
Fig. 22: SA-β-Gal assay for HCT116 cell line: after γ-rays exposure cells were fixed and incubated with SA-β-Gal staining solution O/N. Cells were washed and then microphotographed (10X). **T0:** untreated control **RL:** cells treated for 3 days at 4 Gy and after 120 hours of recovery.

Also in the HCT116 cell line, the genotoxic stress induced the activation of cellular senescence, as demonstrated by the positivity of the irradiated cell line (HCT116 RL) compared to the control (HCT116 T0) in the β-galactosidase assay. After the γ rays treatment the increase in cell size and the change in cell morfology was observed. Interestingly, the distinctive characteristic of this line was the appearance of numerous cytoplasmic vacuoles, which lead to a reduction of the cytoplasmic volume.

As previously discussed, it is possible to observe the appearance of vacuoles in *H-Ras* induced senescence (*H-Ras* ^{V}*12*) [20]. The sequencing of *K-Ras* conducted at the beginning of the study demonstrated the presence of a mutation in this gene, in HCT116 cell line, which would explain the appearance of these vacuoles after activation of the senescence program.

Subsequently, the distribution of cells in different phases of the cell cycle was analyzed after staining with PI and analysis by flow cytometry (Fig. 23).

HCT116-T0 HCT116-RL



	HCT116-TO	HCT116-RL
GO/G1	55.6%	57.1%
S	19.1%	13.2%
G2/M	22.7%	25.3%

Fig. 23: Analysis of cells distribution in the cell cycle phases after staining with PI by flow cytometry. **T0**: untreated control **RL:** cells irradiated 3 days at 4Gy and after 120 hours of recovery.

The HCT116 cell line is characterized by the presence of a frameshift mutation in the *CDKN2A* gene coding for p16. Consequently the senescence observed in this cell line probably depends on the p53-p21 pathway. To confirm that p53 and p21

are effectory of senescence induced in these cells by genotoxic stress, the expression of p53 and p21 was assessed by western blotting (Fig. 24). The treatment with γ-rays determined the increase of p53 and p21, already visible after 3 days of irradiation (IR3) which remained constant even after 120 hours after the end of treatment (RL). Therefore, in the HCT116 cell line the genotoxic stress caused the activation of cellular senescence whose induction involved the p53-p21 pathway.

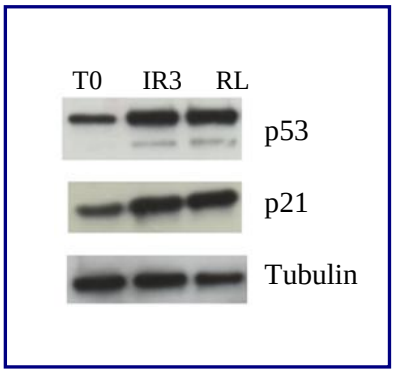


Fig. 24: Effect of γ-rays treatment on p16, p53 and p21 expression. HCT116 were irradiated, at the indicated in figure lysates of these cells were collected and immunoblotted with the indicated antibodies. Tubulin is used as a loading control. **T0**:untreated control **IR3**: cells irradiated 3 days at 4 Gy. **RL**: cells irradiated 3 days at 4 Gy and after 120 hours of recovery.

XTC.UC1

The SA-β-Gal assay of the XTC.UC1 cell line is shown in figure 25.

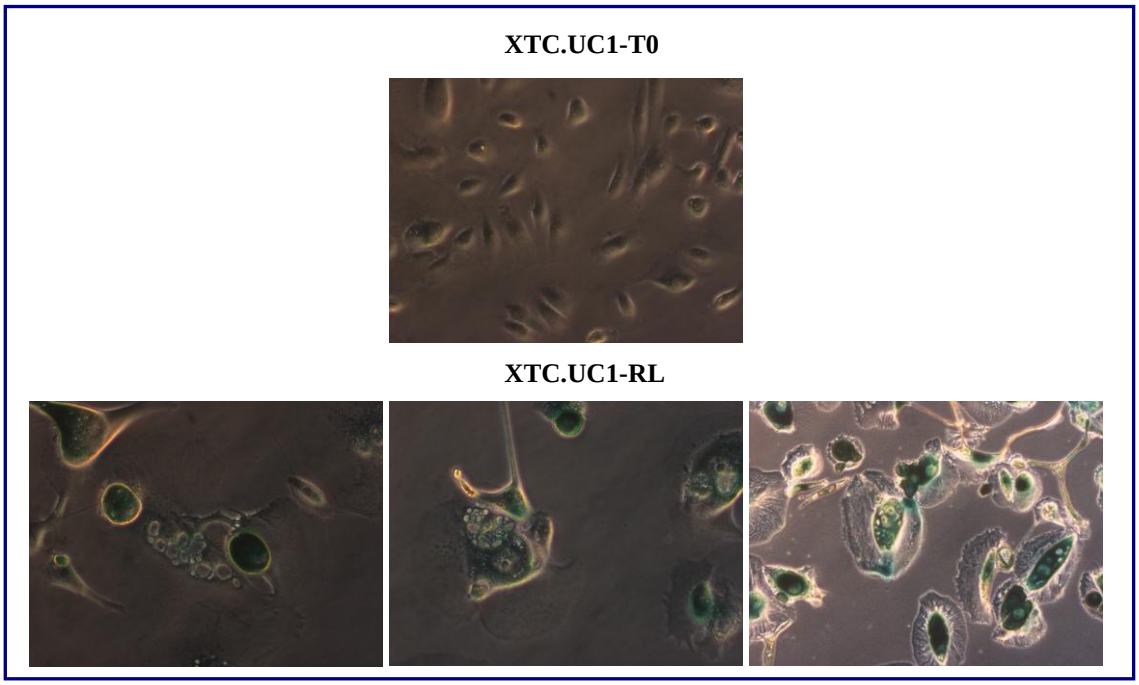


Fig.25: SA-β-Gal assay for XTC.UC1 cell line: after γ-rays treatment cells were fixed and incubated with SA-β-Gal staining solution O/N. Cells were washed and microphotographed (20X). **T0:** untreated control **RL:** cells treated for 4 days at 4 Gy and after 120 hours of recovery.

The strong positivity of XTC.UC1 subjected to treatment with γ-rays (XTC.UC1-RL) compared to the untreated control (XTC.UC1-T0) demonstrated that genotoxic stress in this cell line induced the activation of senescence. Similarly to RPE1 and HCT116, a marked alteration of cell morphology was observed in XTC.UC1 as well. Following the γ-rays treatment the cells increased in size, became more flattened and in some cases fusiform. Moreover, the appearance of numerous cytoplasmic vacuoles was observed, which in some cases occupy almost the total cytoplasmic volume.

To confirm the proliferative arrest, the distribution of cells in the different cell cycle phases will be evaluated by flow cytometry.

In order to determine the molecular pathway activated in the cells as a result of the γ-rays treatment, which could be responsible to determine the proliferative block, expression of p53, p21 and p16 were evaluated in irradiated XTC.UC1. Since this cell line is characterized, as shown from the *TP53* screening, by the presence of a mutation predicted as *probably damaging*, the hypothesis is that senescence observed in this cell line depends exclusively on the pRb-p16 pathway. Although the genotoxic stress did not cause an increase of p53 expression, an increase of p21 was observed. Instead, there were no changes in p16 expression following treatment with γ-rays (Fig.26). This result indicated that the increase of p21 expression observed in XTC.UC1 cell line is regulated by a mechanism independent from p53 action, and that only p21 is the activated senescence effector in this cell line upon genotoxic stress.

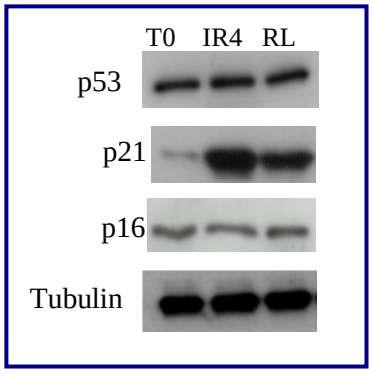


Fig. 26: Effect of γ-rays treatment on p16, p53 and p21 expression. Cells are irradiated, after the end of the treatment and after 120h of recovery, lysates of these cells were collected and immunoblotted with the indicated antibodies. Tubulin is used as aloading control. **T0**:untreated control **IR4**: cells irradiated 4 days at 4 Gy. **RL**: cells irradiated 4 days at 4 Gy and after 120 hours of recovery.

After γ-rays treatment numerous vacuoles appear in the cytoplasm of XTC.UC1.
The presence of such vacuoles has been described in certain types of senescence, such as in melamocytes in which senescence is induced as a result of the presence of mutations in tumor suppressor gene *H-Ras* (*H-Ras* ^{V}*12*) [20].
These cells are characterized by the early appearance of cytoplasmic vacuoles which are, as demonstrated by electron microscopy, the result of the endoplasmic reticulum membrane expansion . It has been shown that the presence of the *H-Ras* mutation determines the activation of the UPR (unfolded protein response) that is directly responsible for activation of senescence processes [20].
Recently, the appearance of the senescence-related cytoplasmic vacuoles has been described even in fibroblasts overexpressing the mutated form of *H-Ras*.
The authors describe these vacuoles as deposits of H_2O_2, which involvement has not yet been clarified, and introduce the hypothesis that mitochondria might be implicated in supporting the formation of a senescent phenotype [25].
In fact, just before the full activation of senescence in a cell, it is possible to observe changes in mitochondrial morphology, increase of ROS production, loss of mitochondrial potential and increase in expression of certain regulators of the mitochondrial biogenesis (NRF2, PGC-1α, PGC-1β e TFAM).
Moreover, the inhibition of the respiratory chain components by *RNA interference* or pharmacologically have been shown to activate the process of senescence, confirming the involvement of mitochondria in the induction of senescence [25].
As previously discussed, the XTC.UC1 cell line is characterized by the presence of two mtDNA mutations negatively affecting the ATP synthesis [142].
Therefore, it has been hypothesized that the presence of mitochondrial dysfunction may be involved in supporting senescence induced in XTC.UC1 following γ-rays treatment, that is mediated by activation of DNA-damage response (DDR).
The mutations that characterize the XTC.UC1, although present in heteroplasmy, cause a reduction of complex I and III activities and it is well known that

alterations in the functionality of respiratory complexes can contribute to the formation of reactive oxigen species (ROS) [142, 173-175]. It has been suggested, therefore, that in XTC.UC1 cell line the continuous production of ROS may contribute to the occurrence and maintenance of the senescence phenotype amplifying the DDR induced by genotoxic stress. In this context, vacuoles which are generated in XTC.UC1 after γ-rays treatment may have a different origin than those observed in the HCT116 cell line. In the oncocytic model the vacuoles could arise as a result of the increased ROS production. On the other hand, in the HCT116 cell line vacuoles could be formed due to the expansion of endoplasmic reticulum and after activation of UPR resulting from the presence of *K-Ras* mutations.

In order to verify whether the senescence-related vacuoles may have a different origin, and to prove whether ROS and subsequent mitochondrial mutations may be involved in regulating the activation of senescence, the accumulation of H_2O_2 was evaluated in vacuoles of HCT116 and XTC.UC1 cell lines, through the use of the probe dichlorodihydrofluorescein diacetate (DCFDA) (Fig. 27).

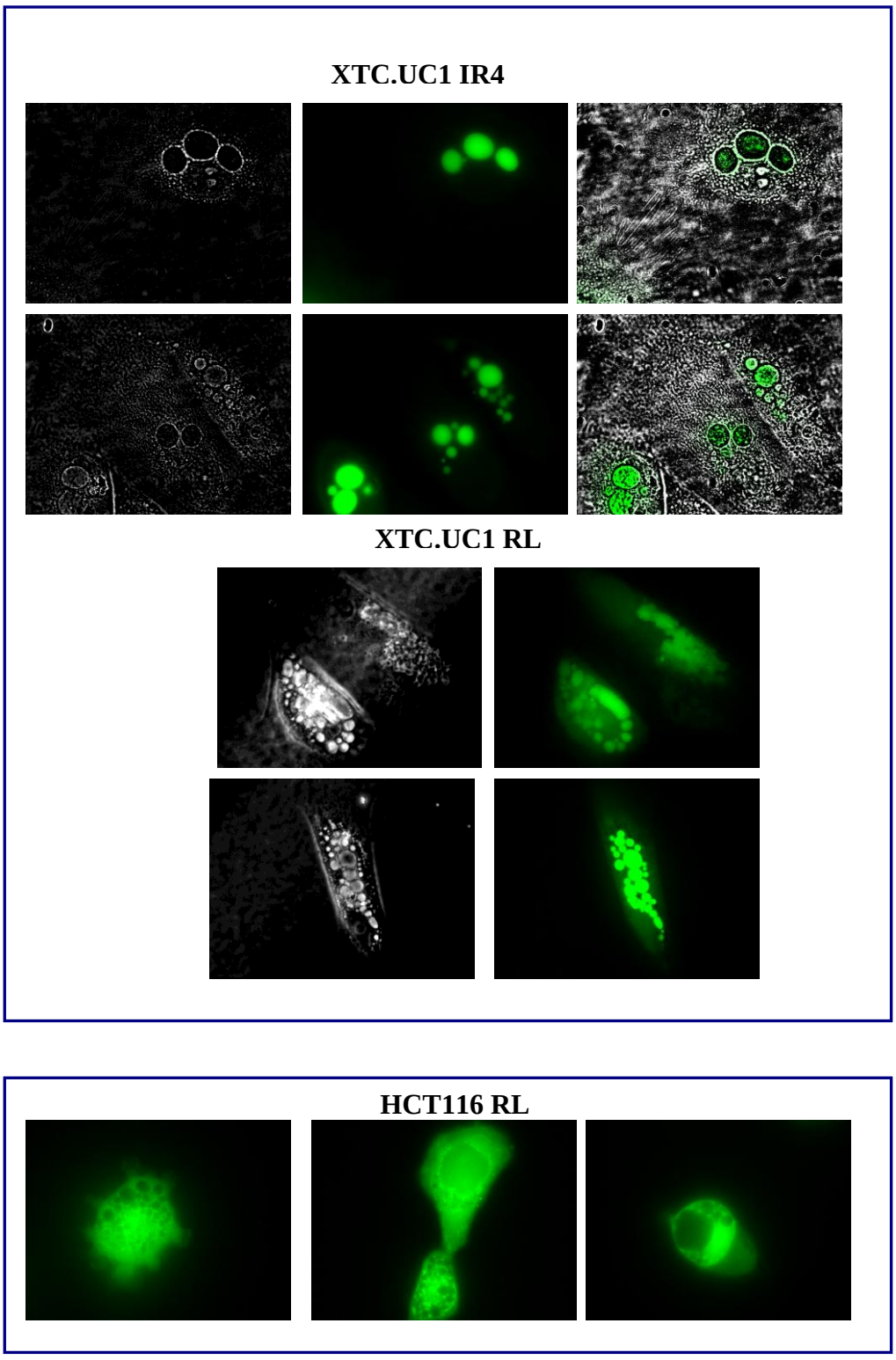


Fig.27: Immunofluorescent image (60X) to visualize the vacuoles in the cytoplasm of HCT116 and XTC.UC1 cell line stained with DCFDA. **XTC.UC1-IR4**: cells irradiated 4 days at 4Gy **XTC.UC1-RL**: cells irradiated 4 days at 4Gy and after 120h of recovery. **HCT116-RL**: cells irradiated 3 days at 4Gy and after 120h of recovery.

In XTC.UC1 cytoplasmic vacuoles were clearly visible 4 days upon irradiation (XTC.UC1-IR4) and their number increased after 120 hours of recovery (XTC.UC1-RL). The accumulation of DCFDA in these vacuoles, indicated that genotoxic stress in XTC.UC1 cell line causes an increase in ROS production.

Next, in order to establish that genotoxic stress may lead to increased ROS production and sustain the appearance of a senescence phenotype, changes in expression of cytosolic and mitochondrial antioxidant enzymes were evaluated. In particular, the expression of Manganese Superoxide Dismutase (MnSOD), Peroxiredoxin 3 (PRX3) and Catalase (CAT) were evaluated, all enzymes which catalyze the ROS detoxification (Fig.28).

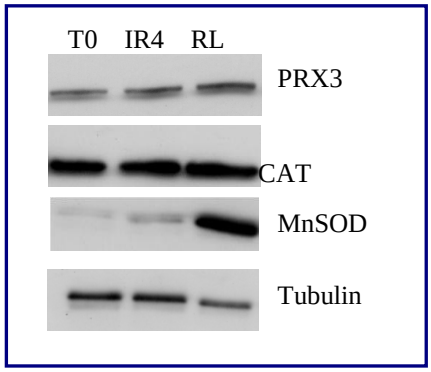


Fig. 28: Expression of antioxidant enzymes (PRX3-Catalase and MnSOD) in XTC.UC1 cell lines after γ-rays treatment. Cells were irradiated, then lysates were collected and immunoblotted with the indicated antibodies. Tubulin is used as a loading control. **T0**: untreated control **IR4**: cells irradiated 4days at 4Gy **RL**: cells irradiated 4 days at 4Gy and after 120h of recovery.

The increased expression of MnSOD, as well as the slight increase of PRX3 expression, indicating that ROS increase induced by genotoxic stress was followed by the overexpression of enzymes that are responsible of the radical detoxification.

In HCT116 vacuoles became visible 120 hours upon recovery (HCT116-RL) but did not accumulate the DCFDA probe (Fig.27), which suggested that in this cell line the activation of the senescent phenotype did not involve ROS production, but was probably determined by the activation of a different molecular mechanism. The presence of vacuoles in this cell line after exposure to γ-rays treatment, combined to *K-Ras* mutation, indicated that could be UPR activation to lead the induction of senescence phenotype. Further study will be conducted to demonstrate that these vacuoles are generated as expansion of endoplasmic reticulum.

These results lead us to conclude that in XTC.UC1 cell line, genotoxic stress can determine the activation of cellular senescence, moreover the increase ROS production and antioxidant enzyme expression, indicate that mitochondrial dysfunction contribute to the maintenance of senescent phenotype.

4.2 mTOR: A SINGLE PATHWAY MAY REGULATE SENESCENCE AND MITOCHONDRIAL BIOGENESIS?

In the first part of this study it was shown that the genotoxic stress induced by γ-rays was able to induce the activation of mitochondrial biogenesis and senescence. In order to determine which mechanism is activated following γ-rays treatment, and if the same pathway can regulate the induction of the two processes, the attention was next focused on mTOR pathway and in particular on mTORC1 complex.

mTOR is a serine-threonine kinase that regulates several cellular processes including protein synthesis, cell growth and proliferation in response to different stimuli such as growth factors, nutrients and diverse stresses and changes in energy supply [74].

Among other, experimental data has also demonstrated the involvement of mTOR in regulating certain types of cellular senescence [46, 47, 95, 96]. It has been shown that mTORC1 is involved in the induction of PICS (Pten loss Induced Senescence) [46, 47]. According to the authors, mTORC1 activated by the lack of PTEN acts by enhancing the translation of p53 and thereby supporting the activation of senescence. In this model, inhibition of mTORC1 following treatment with rapamycin would be sufficient to block the induction of the senescence processes [46, 47]. The involvement of mTORC1 in cellular senescence has also been demonstrated in human fibroblasts after inactivation of the PI3K/AKT pathway [95]. The loss of PI3K/AKT determines the induction of cellular senescence that would be controlled by mTORC1 through the enhancement of p53 translation [95].

Furthermore, mTOR, and in particular mTORC1, is responsible for the regulation of the mitochondrial metabolism [132]. It has been shown that mTORC1 binds to the transcriptional factor YY1 and with PGC-1α, forming a ternary complex which controls the transcription of several mitochondrial genes [132]. In agreement whit this notion, it was demonstrated that the lack of the principal

interacting protein of mTORC1, RAPTOR, is associated with defects of the mitochondrial biogenesis in skeletal muscle, leading to a decrease in oxidative capacity of the cells [176].

Considering the dual role of mTORC1 in the regulation of senescence and mitochondrial biogenesis, it may be hypothesized that this complex is responsible for the activation of both mitochondrial biogenesis and senescence induced upon genotoxic stress.

To verify this hypothesis RPE1, HCT116 and XTC.UC1 were treated with rapamycin, a specific inhibitor of mTORC1 before the γ-rays treatment, in order to determine alterations in senescence and mitochondrial biogenesis, resulting from inhibition of mTORC1.

To evaluate the effect of mTORC1 inhibition on the mitochondrial biogenesis variations of expression of the proteins that make part of the respiratory complexes was evaluated, such as ATPase5B, NDUFA9, SDHA, SDHB and TFAM.

To establish the effect on cellular senescence SA-β-Gal assay was used, and p21 expression, that is the senescence effector for all the cell lines, was evaluated with western blotting assay.

4.2.1 RPE1 and mTORC1

In Figure 29 the images of western blotting assay are reported through which the expression of the total and phosphorylated forms of the S6K1 kinase were evaluated. S6K1 kinase is one of the downstream target of mTORC1, which is activated after phosphorilation [90].

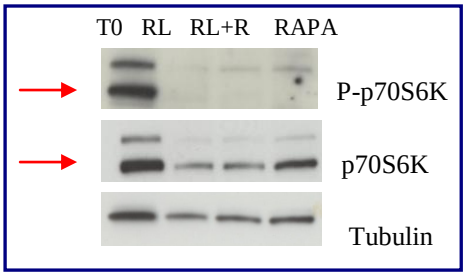


Fig. 29: Expression of total and phosphorilated form of S6K1 kinase**.** Cells were treated with rapamycin (10nM) before the irradiation.Tubulin is used as a loading control. **T0**: untreated control **RL**:cells treated 4 days at 4Gy and after 120h of recovery **RL+R**: cells treated with rapamycin (10mM), irradiated for 4days at 4Gy and after 120h of recovery **RAPA:** cells treated with rapamycin (10nM).

The absence of the phosphorylated form of the S6K1 kinase showed that the mTORC1 complex was inhibited.

RPE1 cell line: mTORC1 inhibition and cellular senescence

The images of the SA-β-Gal assay performed on the RPE1 cell line are reported in Figure 30.

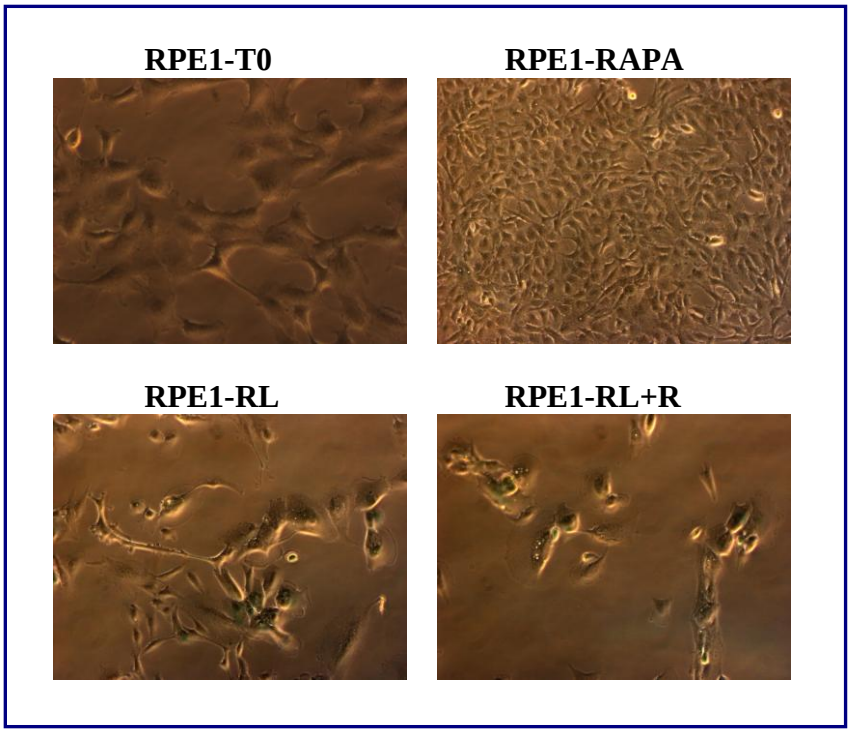


Fig. 30: SA-β-Gal assay for RPE1 cell line. Cells were treated with rapamycin and then exposed to γ-rays. After the end of the treatment cells were fixed and incubated with SA-β-Gal staining solution for ~6h. Cells were washed and then microphotographed (10X). **T0:** untreated control **RL:** cells treated for 4 days at 4 Gy and after 120 hours of recovery **RL+R:** cells treated with rapamycin (10nM) and irradiated 4 days at 4Gy and after 120h of recovery **RAPA:** control treated with rapamycin (10nM)

Conversely from what was hypothesized, the inhibition of mTORC1 in RPE1 cell line did not block the activation of cellular senescence. In fact, even the cells treated with rapamycin before the exposure to γ-rays (indicated as RL+R) were positive to the β-galactosidase assay.

As a result of the inhibition of mTORC1 a decrease of p53 and p21 expression was not observed (Fig.31). The increase in expression of p53 observed after γ-rays

treatment (RL) remains constant even in cells treated with rapamycin and consequently, the p21 expression is elevated in both conditions.

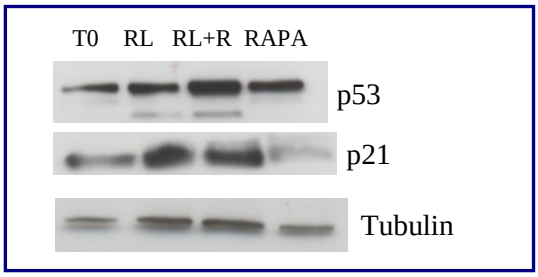


Fig.31: Expression of p53 and p21 after rapamycin and γ-rays treatment. Tubulin is used as a loading control. **T0:** untreated control **RL:** cells treated for 4 days at 4 Gy and after 120 hours of recovery **RL+R:** cells treated with rapamycin (10nM) and irradiated 4 days at 4Gy and after 120h of recovery **RAPA:** control treated with rapamycin (10nM)

The positivity to the β-galactosidase assay and the increased expression of p53 and p21 observed in RPE1 cell line after mTORC1 inhibition demonstrated that mTORC1 is not able to regulate the activation of cellular senescence induced by genotoxic stress.

RPE1 cell line: mTORC1 inhibition and mitochondrial biogenesis

In addition to determining the effect of mTORC1 on cellular senescence, its effects on mitochondrial biogenesis were evaluated.

The genotoxic stress is responsible for determining the increase in expression of mitochondrial proteins that take part of the respiratory complexes such as NDUFA9, SDHA, SDHB and of the mtDNA transcriptional factor TFAM (Fig.13). The expression of these proteins upon mTORC1 inhibition is reported in figure 32.

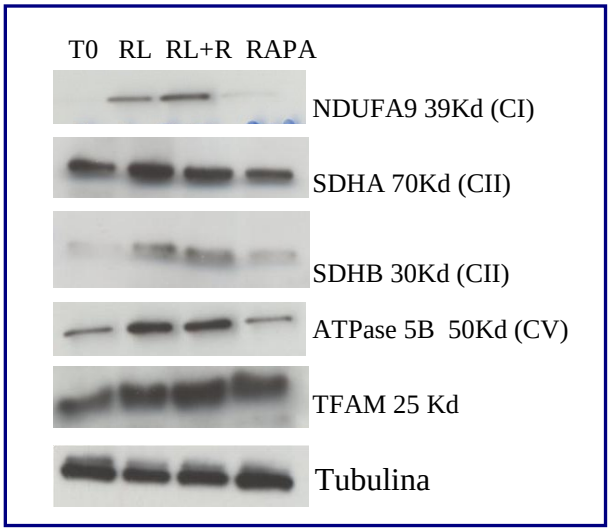


Fig. 32: Mitochondrial proteins expression after rapamycin and γ-rays treatment. **T0:** untreated control **RL:** cells treated for 4 days at 4 Gy and after 120 hours of recovery **RL+R:** cells treated with rapamycin (10nM) and irradiated 4 days at 4Gy and after 120h of recovery **RAPA:** control treated with rapamycin (10nM)

The increase in mitochondrial proteins expression upon γ-rays treatment (RL) was maintained constant even if the mTORC1 complex was inhibited (RL+R).

Taken together, the results obtained in RPE1 cell line lead us to conclude that in this cell line mTORC1 was not involved in the regulation of the cellular response to radiation treatment, nor in terms of activation of senescence nor of mitochondrial biogenesis.

In figure 29 are reported the images of western blotting assay to evaluate the expression of the phosphorilated form of S6K1 kinase. It is possible to note that γ-rays treatment determine the decrease expression of the active form of S6K1 kinase, independently of mTORC1 inhibition. mTORC1 inhibition can be induced, in this cell line, by the activation of AMPK kinase.

It has been shown that stimuli which induce DNA damage, could activate AMPK, through a p53-dependent mechanism [102,103].

Then it has been hypothesize that in RPE1 cell line, γ-rays treatment induce AMPK as a consequence of DNA damage. Preliminary data indicated that in this cell line, γ-rays exposure induced the expression of the active form of AMPK (Fig. 33).

Recently, it was shown, in skeletal muscle, that AMPK regulate mitochondrial biogenesis through the regulation of PGC-1α and PPARγ [177, 178].

It could be hypothesized that AMPK promote at least mitochondrial biogenesis induced after γ-rays; then the effect of AMPK inhibition on mitochondrial biogenesis will be investigated on RPE1 cell line treated with a specific AMPK inhibitor such as Compuond C.

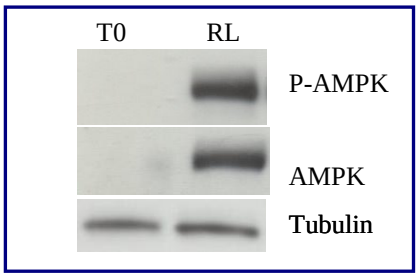


Fig.33: Expression of total and phosphorilated form of AMPK following γ-rays treatment in RPE1 cell line determined by western blotting analysis. **T0**: untreated control **RL**: cells irradiated f4 days at 4Gy and after 120h of recovery.

4.2.2 HCT116 and mTORC1

In HCT116 cell line γ-rays induced the activation of senescence and mitochondrial biogenesis (Fig. 12, 22, 23, 24). In order to establish the role of mTOR in the regulation of the response of this cell line to genotoxic stress, HCT116 were treated with rapamycin before the treatment with γ-rays.

In Figure 34 the images of western blotting assay are reported through which the expression of the total and phosphorylated forms of the S6K1 kinase were evaluated. The absence of the phosphorylated form of the S6K1 kinase showed that the mTORC1 complex was inhibited.

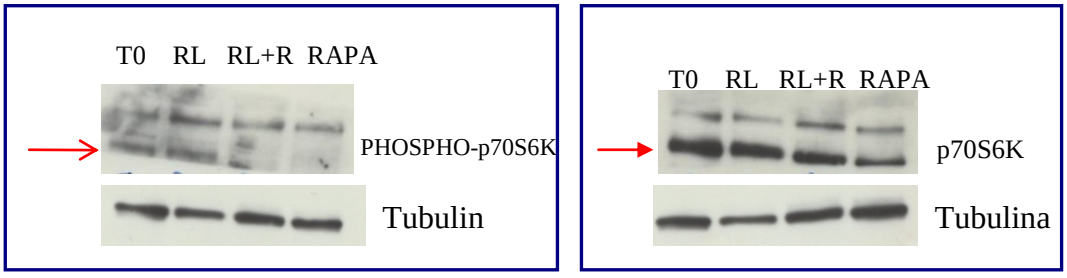


Fig. 34: Expression of total and phosphorilated form of S6K1 kinase. **T0**: untreated control **RL**:cells irradiated 3 days at 4Gy and after 120h of recovery **RL+R**:cells treated with rapamycin (10nM), irradiated 3days at 4Gy and after 120h of recovery **RAPA:** cells treated with rapamycin (10nM)

HCT116 cell line: inhibition of mTORC1 and cellular senescence

The images of the β-galalctosidase assay performed on the HCT116 cell line are reported in figure 35. In this cell line, even if mTORC1 was inhibited, the β-galactosidase assay was positive, indicating that the cellular senescence induced by genotoxic stress in HCT116 is not regulated by mTORC1. The evaluation of cellular morphology confirmed that the inhibition of mTORC1 does not affect the cellular response to γ-rays treatment; the cells increased in size and the cytoplasmic vacuoles were present, all morphological changes typical of HCT116 senescent cells.

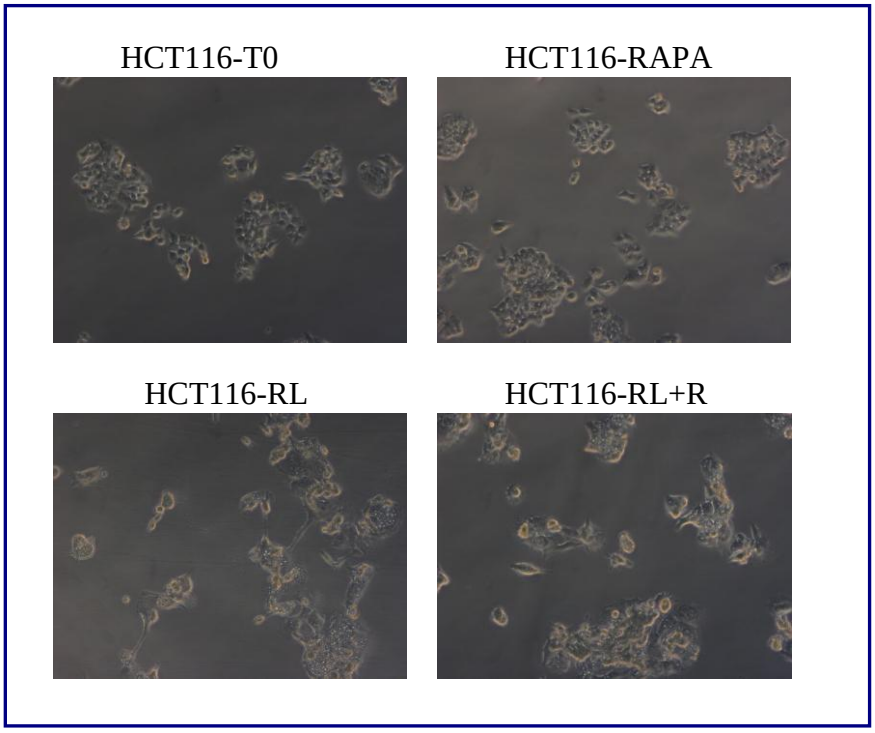


Fig 35: SA-β-Gal assay for HCT116 cell line: cells were treated with rapamycin (10nM) and then expose to γ-rays treatment. Cells were therefore fixed and incubated with SA-β-Gal staining solution for 6h. Cells were washed and then microphotographed. **T0:** untreated control **RL:** cells treated for 3 days at 4 Gy and after 120 hours of recovery **RL+R:** cells treated with rapamycin (10nM) and irradiated 3 days at 4Gy and after 120h of recovery **RAPA:** control treated with rapamycin (10nM)

The induction of senescence in this cell line after genotoxic stress was accompanied by the increase in p53 and p21 expression , which were responsible for the proliferative block observed in this cell line (Fig.24). As the mTORC1 inhibition did not cause the blocking of senescence it should not have effect on the

expression of p53 and p21. Indeed, the increase in p21 expression was observed as a consequence of γ-rays treatment despite mTORC1 was inhibited (Figure 36).

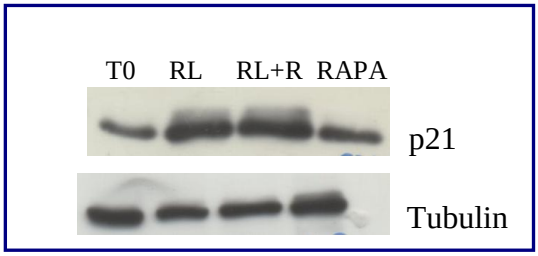


Fig.36: p21 expression after rapamycin and radiation treatment in HCT116 cell line. Tubulin were used as a loading control. **T0:** untreated control **RL:** cells treated for 3days at 4 Gy and after 120 hours of recovery **RL+R:** cells treated with rapamycin (10nM) and irradiated 3 days at 4Gy and after 120h of recovery **RAPA:** control treated with rapamycin (10nM)

Taken together, these results showed that in HCT116 cell line the inhibition of mTORC1 was not able to block the activation of cellular senescence upon genotoxic stress.

HCT116 cell line: inhibition of mTORC1 and mitochondrial biogenesis.

Next, the effect of mTORC1 inhibition on mitochondrial biogenesis was determined upon genotoxic stress in HCT116 cell line. It was previously shown that the treatment of HCT116 cell line with γ-rays induced the increase in expression of some proteins of respiratory complexes (NDUFA9, SDHA and SDHB) and of TFAM (Fig.12). In order to determine the effect of mTORC1 inhibition on mitochondrial biogenesis variations of expression of these proteins were evaluated (Fig.37).

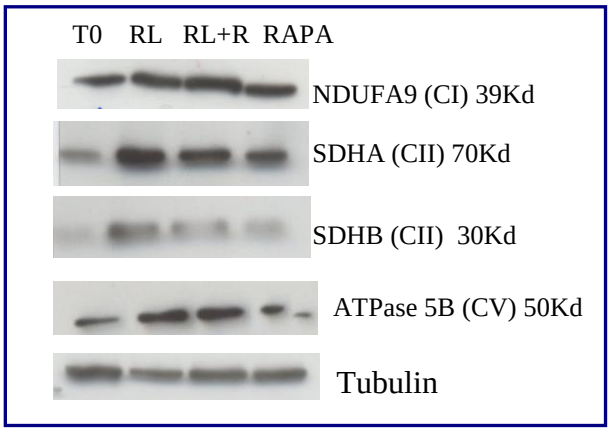


Fig.37: Mitochondrial proteins expression after rapamycin and γ-rays treatment **T0:** untreated control **RL:** cells treated for 4 days at 4 Gy and after 120 hours of recovery **RL+R:** cells treated with rapamycin (10nM) and irradiated 4 days at 4Gy and after 120h of recovery **RAPA:** control treated with rapamycin (10nM)

The increase expression of mitochondrial proteins NDUFA, SDHA, SDHB, ATPase5B and TFAM induced after γ-rays treatment (RL) remained unchanged after inhibition of mTORC1 (RL+R). This result demonstrated that mTORC1 inhibition is not implicated in the response of this cell line to irradiation. In fact, the rapamycin treatment did not block the activation of mitochondrial biogenesis observed in this cell line.

Therefore, it may be concluded that mTORC1 does not control the activation of senescence and mitochondrial biogenesis induced by genotoxic stress in HCT116. However, conversely from what was seen in RPE1, in HCT116 cell lines the phosphorylated form of S6K1 kinase was maintained after γ-rays treatment. Further studies are needed in order to clarify the molecular pathways activated after radiation treatment which could regulate the induction of both senescence and mitochondrial biogenesis. The involvement of AMPK kinase that may be activated in a p53-dependent manner could be a valid hypothesis.

4.2.3 XTC.UC1 and mTORC1

The XTC.UC1 cell line responds to genotoxic stress by inducing cellular senescence (Fig.25, 26), but γ-rays treatment not induced an increase of mitochondrial biogenesis (Fig. 38). As reported in figure 38, no change in proteins expression were observed. This is because XTC.UC1 is a model of oncocytic tumor, and then is characterized by a basal mitochondrial hyperproliferation. The transcriptional factors and the co-activator of mitochondrial biogenesis, as well as protein synthesis, are in this cell line iperactivated.

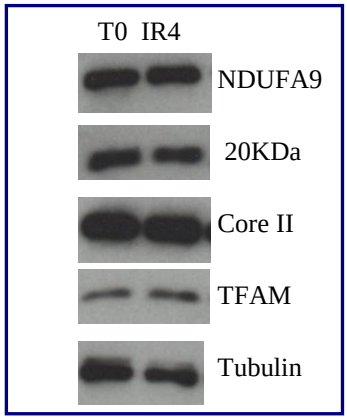


Fig.38: Mitochondrial proteins expression in XTC.UC1 cell line after γ treatment. Tubulin is used as a loading control. **T0:** untreated control **IR4:** cells irradiated 4 adys at4Gy

The response of this cell line to mTORC1 inhibition is evaluated, either in terms of senescence or mitochondrial biogenesis.

Western blotting assay by which the expression of the phosphorilated and total forms of S6K1 kinase were determined is presented in figure 39. The absence of the phosphorilated form of the kinase demonstrated that the rapamycin treatment has determined the inhibition of mTORC1.

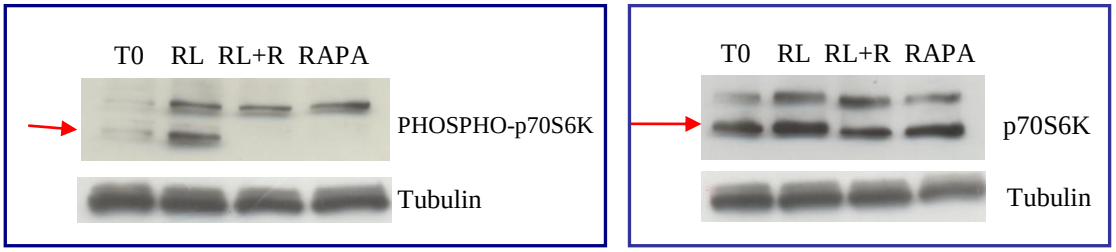


Fig. 39: Expression of total and phosphorilated form of S6K1 kinase. **T0**: untreated control **RL**:cells irradiated 4 days at 4Gy and after 120h of recovery **RL+R**:cells treated with rapamycin (10nM), irradiated 4days at 4Gy and after 120h of recovery **RAPA:** cells treated with rapamycin (10nM)

An interesting data that emerges from the evaluation of the phosphorylated form of S6K1 kinase is the increase expression of the active form of this kinase following exposure to γ-rays (RL), which shows that genotoxic stress determines, in this cell line, the activation of mTORC1. This result supports the hypothesis that mTORC1 regulate the response of this cell line to genotoxic stress at least in terms of activation of cellular senescence.

XTC.UC1 cell line: mTORC1 inhibition and cellular senescence

The SA-β-Gal assay of the XTC.UC1 cell line is shown in figure 40.

Unlike what has been observed in other cell lines, inhibition of mTORC1 is responsible for blocking the activation of cellular senescence. In fact XTC.UC1 treated with rapamycin before exposure to γ-rays (RL+R) are negative to the β-galactosidase assay unlike the XTC.UC1 cell line treated only with γ-rays (RL).

The observation of cellular morphology confirms the data obtained by β-galactosidase assay. In fact, after mTORC1 inhibition, the cells does not increase in size and is not possible to observe the appearance of cytoplasmic vacuoles.

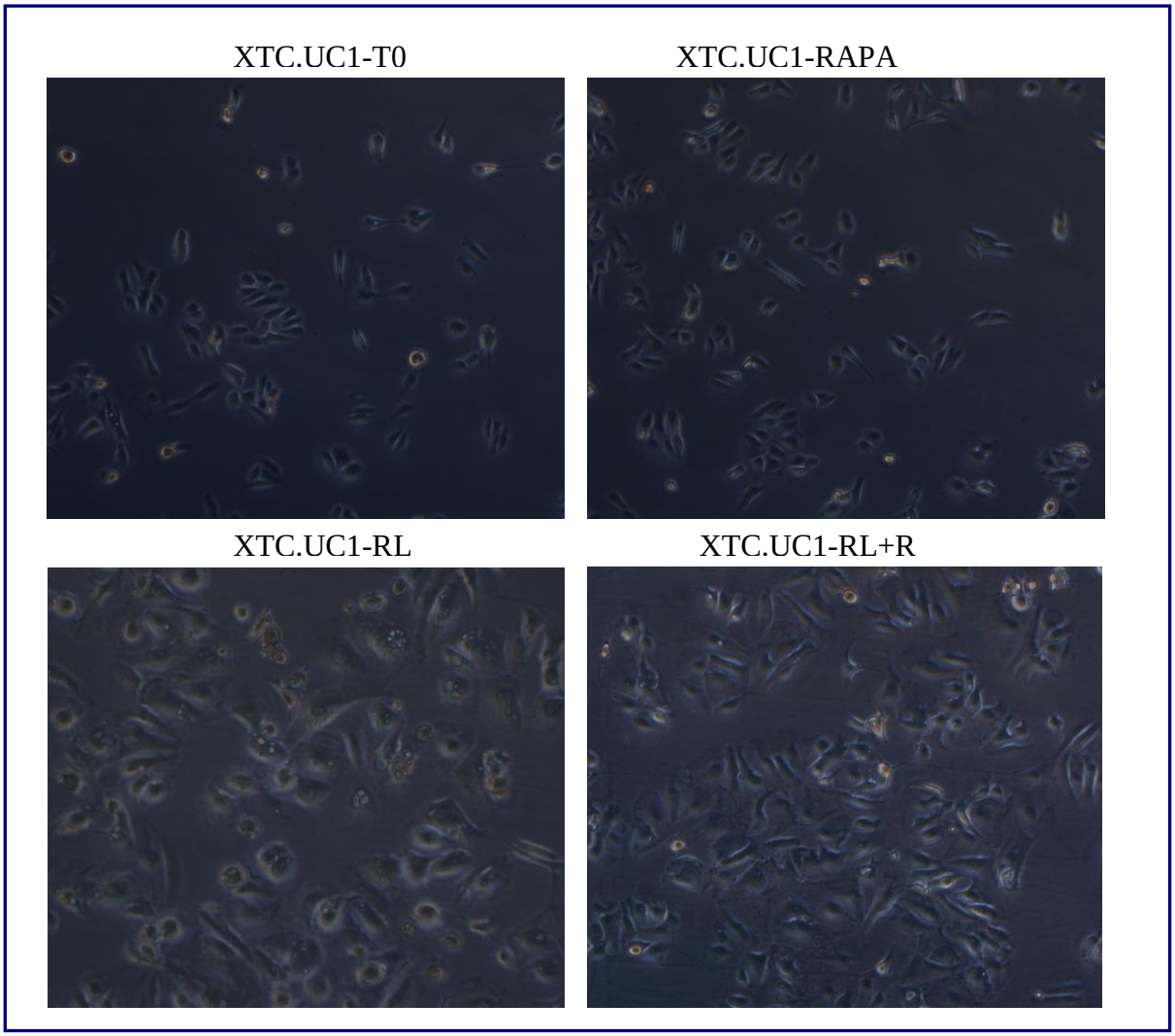


Fig.40: Sa-β-Gal assay for XTC.UC1 cell line: cells are treated with rapamycin (10nM) and then irradiated. After the end of the treatment cells were fixed and incubated with SA-β-Gal staining solution for ~6h. Cells were washed and then microphotographed (10X) **T0:** untreated control **RL:** cells treated for 4 days at 4 Gy and after 120 hours of recovery **RL+R:** cells treated with rapamycin (10nM) and irradiated 4 days at 4Gy and after 120h of recovery **RAPA:** control treated with rapamycin (10nM)

In order to determine whether mTORC1 act by inhibiting the increase of p21, observed in XTC.UC1 cell line after γ-rays treatment and that is involved in the senescence activation, changes in p21 expression was evaluated by western

blotting (Fig.41). The mTORC1 inhibition determines a decrease of p21 expression reaching levels close to those of non-irradiated control (T0).

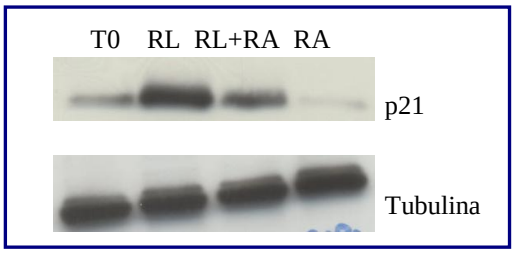


Fig.41: p21 expression after rapamycin and γ-rays treatment. **T0:** untreated control **RL:** cells treated for 4 days at 4 Gy and after 120 hours of recovery **RL+R:** cells treated with rapamycin (10nM) and irradiated 4 days at 4Gy and after 120h of recovery **RAPA:** control treated with rapamycin (10nM)

XTC.UC1 cell line: inhibition of mTORC1 and mitochondrial biogenesis

Genotoxic stress seems not induce the activation of mitochondrial biogenesis in XTC.UC1 cell line (Fig. 38). It is plausible suppose that a slightly increase in mitochondrial biogenesis was induced in this cell line, but it is difficult to determine as a consequence of the mitochondrial hyperproliferation characteristic of this line. To verify this hypothesis, the effect of mTORC1 inhibition on mitochondrial biogenesis was evaluated.

Therefore, the expression of some mitochondrial proteins (NDUFA), SDHA; SDHB, ATPase5B and TFAM) after mTORC1 pharmacological inhibition and γ-rays treatment was evaluated.

Unlike the hypothesis, it was no observed a decrease expression of mitochondrial proteins after the combined treatment (Fig. 42).

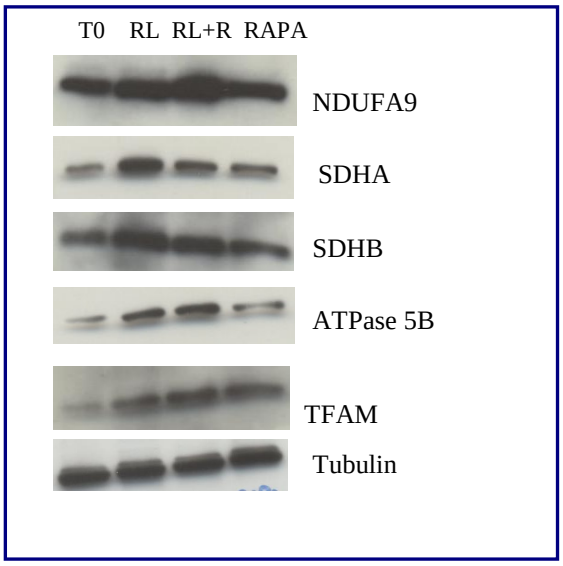


Fig.42: Western blotting relativo all'espressione delle proteine mitocondriali **T0**:controllo non trattato **RL**: cellule irradiate 4 giorni a 4Gy e lasciate 120h in recupero **RL+R**: cellule trattate con rapamicina (10 nM), irradiate 4 giorni a 4Gy e lasciate 120 h in recupero **RAPA:** controllo trattato con rapamicina (10nM)

These results lead us to conclude that γ-rays treatment did not induce a further increase in mitochondrial biogenesis in XTC.UC1 cell line. The most interesting data that emerges in this study is that senescence observed in XTC.UC1 cell line after exposure to a genotoxic stress is blocked after inhibition of mTORC1. Therefore it is possible to conclude that mTORC1 is a regulator of senescence induce in this cell line.

In the first part of this study it has been demonstrated that in RPE1 and HCT116 cell line genotoxic stress induced the activation of mitochondrial biogenesis and senescence. Further studies will be then conducted in these cell lines to evaluate if mitochondrial biogenesis will be involved in the activation of senescence.

In these cell line has been shown that mTORC1 is not involved in regulation of senescence and mitochondrial biogenesis, and it was hypothesized a role for AMPK. Then experiments to evaluate the effect of AMPK inhibition will be conducted.

The response of XTC.UC1 cell line subjected to treatment with γ-rays has demonstrated the involvement of mitochondrial dysfunction caused by mtDNA mutations in the maintenance of senescence induced by genotoxic stress. Based on

the experiments conducted seem that ROS production is directly involved in the regulation of senescence. In this cell line it was also demonstrated that mTORC1 inhibition is responsible to block the activation of senescence.

4.3 mTOR AND THE ONCOCYTIC TUMORS OF THE HEREDITARY CANCER SYNDROME

The previous part of this study has demonstrated the involvement of mTORC1 in the regulation of senescence induced by γ-rays treatment in XTC.UC1 cell line; the results obtained in RPE1 and HCT116 cell line, have ruled out the involvement of mTOR in the regulation of mitochondrial biogenesis following γ-rays treatment.

XTC.UC1 cell line is characterized by an aberrant mitochondrial hyperplasia that is not increased by genotoxic stress exposure (Fig.38). This result is expected as this cell line is a model of oncocytoma and is characterized by a constitutive activation of mitochondrial replication and protein synthesis.

As previously discuss, it has been reported that mTORC1 regulates mitochondrial metabolism [132] by interacting with and activating the transcriptional factor YY1 and PGC-1α.

It has been suggested that the stimulation of mitochondrial biogenesis, which characterizes XTC.UC1 cell line, could be sustained by a nuclear factor namely a tumor suppressor gene inactivation, which lead to activation of mTORC1.

This hypothesis is based on the fact that XTC.UC1 represent the *in vitro model* of what occurs *in vivo* in patients affected by two hereditary cancer syndrome: Cowden syndrome (CS) [145] and Birt-Hogg-Dubé syndrome (BHD) [153].

Patients affected by these syndromes show similar cutaneous manifestations and are predisposed to the development of multiple tumors that in some cases may be oncocytic. BHD and CS patients develop rarely oncocytic tumors which mainly occur in the kidney and the thyroid respectively. The cause of oncocytic transformation associated to inherited tumor syndromes has not been elucidated.

It has been hypothesized that the activation of mTORC1 pathway could induce mitochondrial biogenesis and then lead to the oncocytic transformation in this patients. This hypothesis was formulated according to the function of the main causative genes of these two syndromes: namely *PTEN* for CS patients [147] and *FLCN* for BHD [153]. PTEN acts as a negative regulator of mTOR as a consequence of its inhibition of AKT [150]; FLCN function is yet to be

elucidated but it has been hypothesized that FLCN could act as a negative regulator upstream mTORC1 pathway as a consequence of its interaction with AMPK, that mimicking TSC1/TSC2 complex function [155].

We hypothesized that PTEN and FLCN could exert their functions in a common pathway, which when deregulated may cause mTOR activation, subsequent increase of PGC-1α and mitochondrial biogenesis.

The study of oncocytomas associated to CS and BHD suggested that *PTEN*/ *FLCN* double heterozigosity could cause the oncocytic transformation in inherited syndromes.

Indeed the analysis of thyroid oncocitoma developed by a CS patient come to the attention of the unit of medical genetics, has demonstrated the loss of one of the two alleles of *PTEN*; causative event in the development of pathology, and a deletion of 242bp in respiratory complex I subunit *ND1*. Also it has been verified the loss of one FLCN allele in thyroid oncocytomas [179].

In the meantime, a BHD patient, who developed a rare parotid oncocytic tumors, was also analyzed. First, direct sequencing of *FLCN* resulted in the discovery of a germline heterozygous mutation, namely c.347dupA. The subsequent analysis of the pathologic tissue did not show loss of the wild-type *FLCN* allele. The oncocytic tumor was then characterized by UPL for *PTEN* deletion. The analysis demonstrated the loss of one *PTEN* allele (Fig.43) compared to DNA extracted from peripheral blood of two different healthy controls.

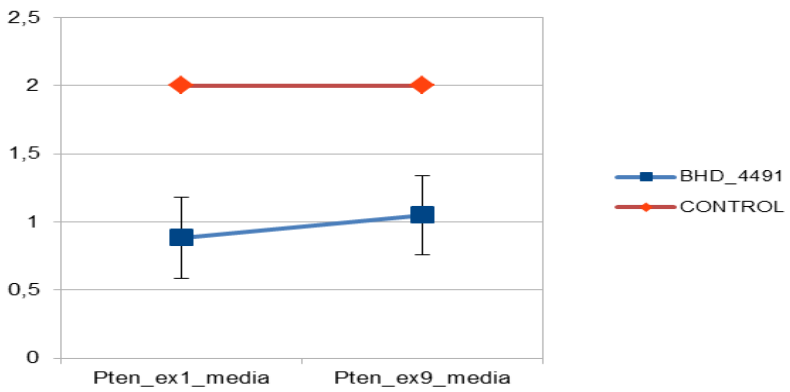


Fig.43: Copy number assay using UPL showing loss of one *PTEN* allele in the oncocytic lesion of BHD patient compared to the medium of two healthy controls.

These results revealed the existence of a "mirrored" molecular profile in the BHD and the CS patients' oncocytic tumors and suggested that the two syndromes may be related and that *FLCN* and *PTEN* probably converge in a common pathway leading to oncocytic phenotype.

In order to verify if mutations, deletions or *PTEN* /*FLCN* double heterozigosity could be responsible of mitochondrial biogenesis activation through the regulation of PGC-1α expression, experiments of complementation of FLCN and PTEN in XTC.UC1, the most suitable *in vitro* model, were carried out.

This cell line is resulted the most suitable in vitro model to verify our hypothesis; in fact do not express PTEN (as resulted by the sequencing conducted in the first part of this thesis), and furthermore was characterized for the loss of one allele of *FLCN* as verified by UPL copy number assay (Fig. 44).

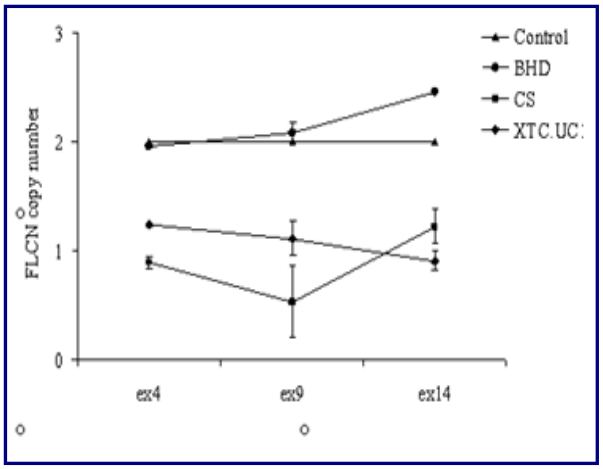


Fig. 44: Copy number assay by UPL technique for FLCN which shows XTC.UC1 monoallelic expression.

We performed a specific semi-quantitative PCR and RT-PCR experiment comparing XTC.UC1 PGC-1α expression to the expression in HEK293, HPS11 and RPE1 cell lines. Semi-quantitative PCR shows an increase of PGC-1α expression in XTC.UC1 cell line compared to the other cell lines (Fig.45).

This data was confirmed by quantitative real time PCR (qRT-PCR) which compared PGC-1α expression XTC.UC1 cell line to its expression in HPS11. HPS11 was selected as a control for PGC1a expression as it is one of the rare cell lines which shows a detectable PGC-1α expression at basal conditions (Fig. 45). The qRT-PCR experiment showed a thirty-fold increase of PGC-1α expression in

XTC.UC1 compared to HPS11. In order to verify if the increase of PGC-1α expression level was accompanied by the increase of its co-activators, namely PGC-1β and PRC, expression levels of these co-activators were measured in XTC.UC1 cell line. The experiment has showed not differences in the expression of PGC-1β and PRC.

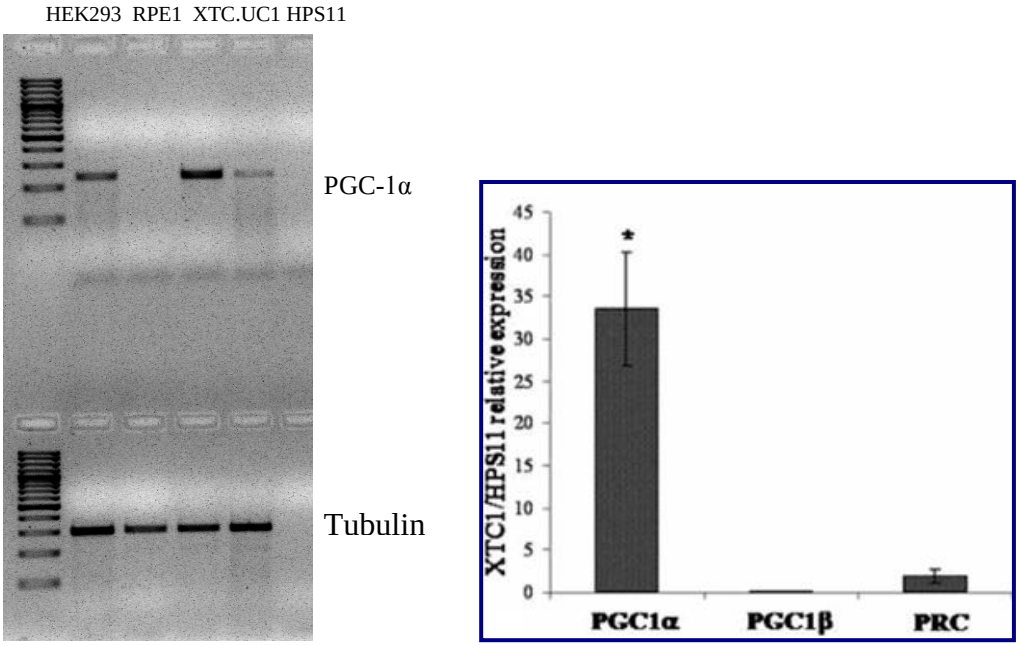


Fig. 45: A: Semi-quantitative real time PCR for PGC-1α (above) in different cells normalized on tubulin expression (below) **B:**qRT-PCR for PGC-1α, PGC-1β and PRC expression in XTC.UC1 and HPS11 cell lines.

All these findings support the hypothesis that mitochondrial biogenesis can be regulate by PTEN and FLCN, and suggest that the consequent deregulation of mTOR pathway could lead to the activation of mitochondrial biogenesis through the regulation of PGC-1α expression.

To investigate the effect of PTEN and/or FLCN restored expression on PGC-1α transcription the two genes are complemented in XTC.UC1 cell line.

To this aim we have cloned both *PTEN* full lenght cDNA and *FLCN* full lenght cDNA into two plasmid vectors, namely pcDNA 3.1 (-) PURO and pcDNA 3.1 (+)ZEO.

First, we have investigate the effect of PTEN complementation.

PTEN expression was verified by western blotting (Fig. 46) which demonstrated the presence, in XTC.UC1 cell line transfected with PTEN expressing vector, of a band comparable in intensity to that of RPE1 cell line, used as a positive control.

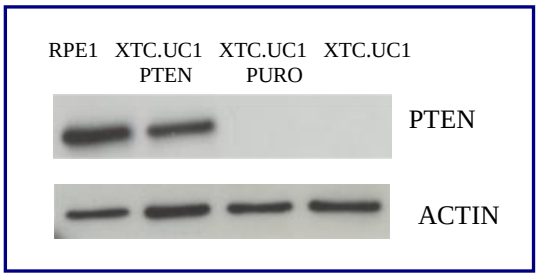


Fig.46: Western blotting for PTEN expression on XTC.UC1 cell line after PTEN complementation.

The effect of PTEN complementation on PGC-1α expression were verified by qRT-PCR. In figure 47 are reported PGC-1α expression of PTEN-transfected XTC.UC1 (PTEN) and mock-trasfected XTC.UC1 (PURO).

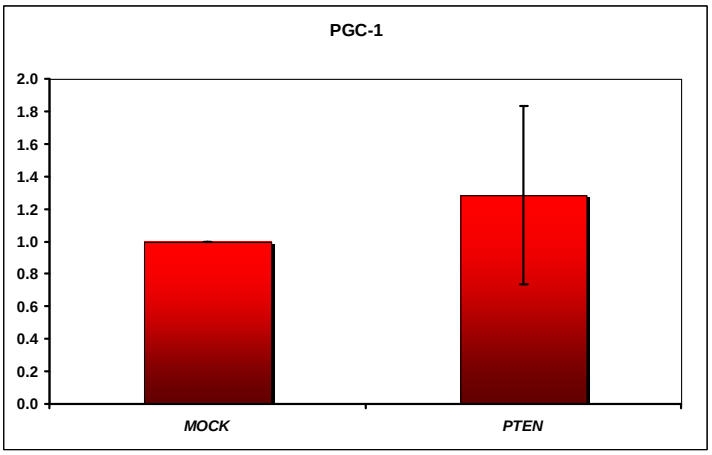


Fig.47: qRT-PCR for the evaluation of PGC-1α expression on XTC.UC1 cell line after PTEN complementation.

It was observed a slightly increase of PGC-1α expression level after restoration of PTEN expression. As the sample analyzed was too small and the data are not statistically significant, they should be confirmed on a larger set.

This result seems to disagree with the hypothesis that PTEN expression, resulting in mTOR inhibition, should reduce PGC-1α expression and arrest mitochondrial biogenesis.

As the role of PTEN as an inhibitor of mTORC1 has been demonstrated it was expected that PTEN restored expression should inhibit mTORC1 activation.

In order to establish if PTEN is functionally active, the expression of the phosphorilated and total form of S6K1 kinase was evaluated. The decrease expression of phosphorilated S6K1 after PTEN restored expression, shows that the protein is active and therefore able to inhibit mTORC1 complex (Fig. 48).

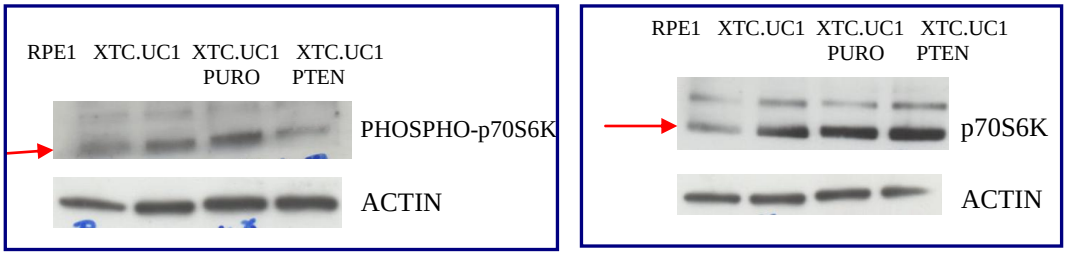


Fig.48: Expression of the total and phosphorilated form of S6K1 kinase in XTC.Uc1 cell line after PTEN complementation. Action is used as normalization gene

The presence of a week band of the phosphorilated form of S6K1 indicates that mTORC1 is not completely inhibited by PTEN expression.

However a possible explanation of this apparent contradictory result could be justified by the fact that these experiments were carried out on XTC.UC1 cell line which has been complemented only for PTEN expression but not for FLCN.

It could be speculated that a compensatory signal is activated and is responsible to keep PGC-1α expression level up-regulated.

It was hypothesized that PTEN expression induced a slightly increase of PGC-1α expression as a consequence of the induction of a compensatory signal. Therefore the expression of genes controlled by YY1-PGC-1α, that is inhibited in absence of mTORC1, was evaluated.

Preliminary experiments demonstrated that PTEN expression induced a slightly increase in the expression of two mitochondrial proteins namely Porin and TFAM (Fig. 49). This result, which should be confirmed even by evaluation of expression of the other regulator of mitochondrial biogenesis (for example NRF1 and PGC-1β), supports the result obtained by PTEN complementation.

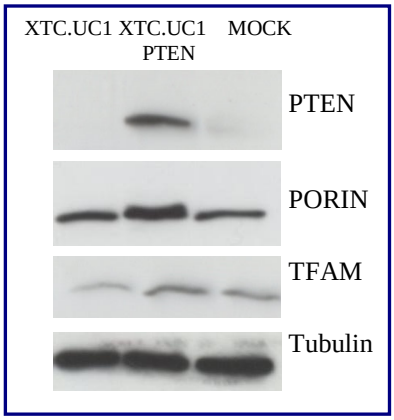


Fig. 49: Expression of TFAM and Porin after PTEN complementation. Tubulin is used as housekeeping gene for normalization.

In order to confirm the data obtained after PTEN restored expression, XTC.UC1 cell line was treated with rapamycin, a specific inhibitor of mTORC1.

Figure 50 are shows the western blotting assay to evaluate the efficiency of mTORC1 inhibition. Subsequently the expression of PGC-1α was evaluated by qRT-PCR (Fig.51).

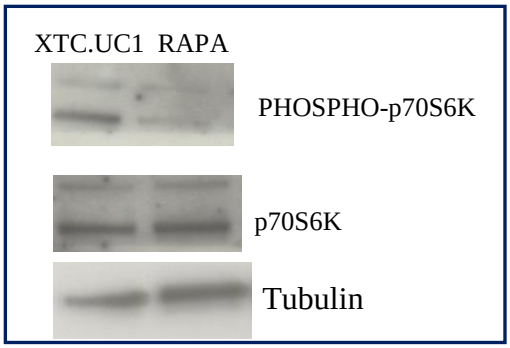


Fig.50: Pharmacological inhibition of mTORC1 through rapamycin treatment.

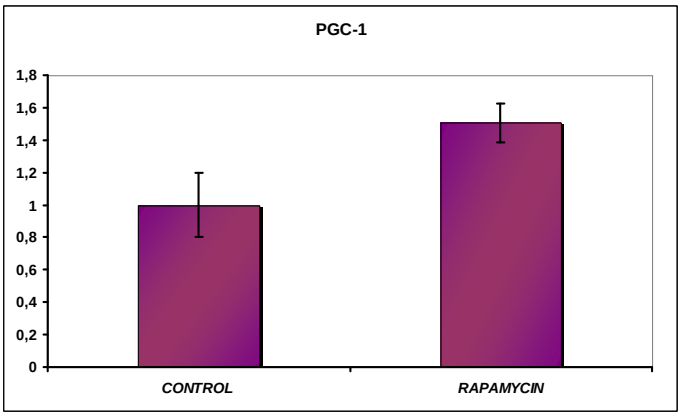


Fig.51: PGC-1α expression in XTC.UC1 cell line after rapamycin traetment (10nM).

mTORC1 inhibition induces a slightly increase of PGC-1α expression, comparable to the data obtained after PTEN restored expression.

These results seems to rule out the function of PTEN as a negative regulator of PGC-1α through mTORC1 repression. A possible explanation on this apparent contradictory result is that we have restored only PTEN expression but not FLCN, while the initial hypothesis is that *PTEN* and *FLCN* double heterozigous leads to PGC-1α expression. Stable clones in which both PTEN and FLCN expression will be restored should give a complete idea of the role of these two tumor suppressor genes on PGC-1α expression through mTORC1 pathway.

Finally, the effects of FLCN restored expression on the regulation of PGC-1α were evaluated. FLCN expression were initially evaluated by western blotting analysis (Fig.52).

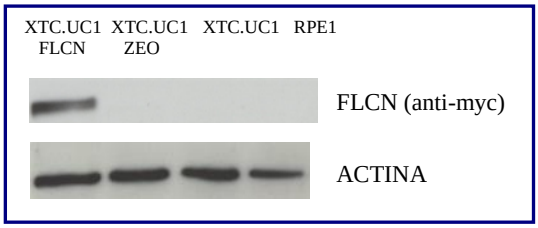


Fig.52: Western blotting for FLCN expression in XTC.UC1 cell line complemented with *FLCN* . Western blotting assay was performed using an anti-myc antibody

Figure 53 reports PGC-1α expression level determined by qRT-PCR on mock transfected XTC.UC1 and FLCN-transfected XTC.UC1.

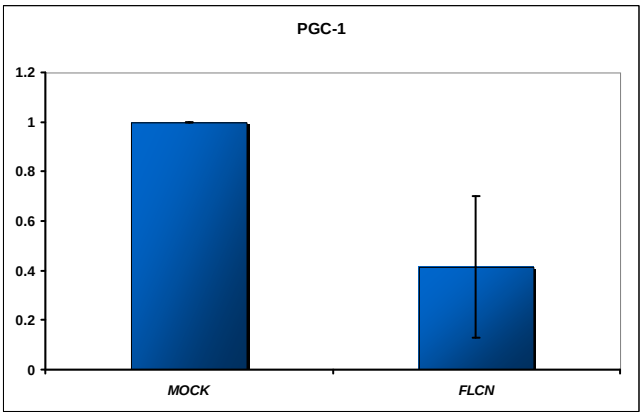


Fig. 53: PGC-1α expression after FLCN restored expression.

FLCN restored expression induced a slightly decrease of PGC-1α expression level in XTC.UC1 cell line, which should be confirmed increasing the number of samples analyzed and in stable clones expressing both FLCN and PTEN.

This result seems to demonstrate that FLCN is involved in PGC-1α expression, but the previously data seems to shown that mTORC1 is not involved in PGC-1α expression. Then, the pathway that links FLCN to PGC-1α is yet to be elucidated.

FLCN interacts with AMPK [155-158], and recently it has been demonstrated that AMPK should regulate mitochondrial genes expression [177-178]. It is possible to speculate that AMPK is involved in the regulation of PGC-1α and then in that of mitochondrial biogenesis. The role of AMPK in the regulation of mitochondrial biogenesis will be investigated by the use of specific AMPK inhibitors such as Compound C.

As previously discuss, before to rule out the involvement of PTEN and mTORC1 in the regulation of PGC-1α expression, stable clones in which both PTEN and FLCN expression will be restored should give a complete idea of the role of these two tumor suppressor genes on PGC-1α expression through mTORC1 pathway.

5. DISCUSSION

Radiotherapy, as well as chemotherapeutic agents, can induce the activation of cellular senescence in tumoral tissue [52, 53]. Senescence is considered, like apoptosis, an efficient mechanism that cells activate in order to limit tumor growth [70]. Several reports indicate that some molecules secreted by senescent cells could stimulate the immune system that is responsible for their elimination [70]. These data supported the idea of activating senescence as the treatment of cancer patients. In fact, various therapeutic studies are currently under development, directed to activate cellular senescence based on the use of molecules that act by enhancing senescence-related pathways [2, 47]. Moreover, in order to enhance treatment effectiveness, therapeutic protocols involving the use of these molecules in combination with standard procedures, such as radiotherapy and chemiotherapy, are in phase of research.

On the other hand, several reports have demonstrated an increase of mtDNA copy number after radiation treatment in certain tissues of γ-irradiated mice [167,168]. Moreover, experimental data show that patients, affected by colon cancer, following radiation treatment, may develop tumor relapses with oncocytic features [169, 170]. Recently, the increase of mitochondrial biogenesis, ROS accumulation and decrease of ATP synthesis have been demonstrated as effectory pathways of *H-Ras* induced senescence [25]. It was therefore hypothesized that the activation of mitochondrial biogenesis and thus the differentiation towards an oncocytic phenotype observed after γ-rays treatment may be involved in the activation of cellular senescence.

Taken together, the data obtained in this study allow us to conclude that, in our cellular models, genotoxic stress induced activation of mitochondrial biogenesis and senescence. It is not possible to establish whether mitochondrial biogenesis is activated before senescence or if it is one of senescence-related consequences. Therefore, further experiments must be carried out in order to determine if inhibition of mitochondrial biogenesis could affect the activation of cellular senescence.

The most interesting result that emerges from this study concerns the oncocytic model XTC.UC1. It has been demonstrated that in this cell line the genotoxic stress is able to determine the activation of cellular senescence and that the

mitochondrial dysfunction, typical of this cell line, contributes to the maintenance of senescent phenotype.

The involvement of mitochondrial dysfunction in senescence had already been hypothesized by the group of Moiseva in fibroblasts that underwent *H-Ras* induced senescence, through pharmacological inhibition of complexes I and III and by *RNA-interference* experiments [25]. The results obtained in this study demonstrated that mtDNA mutations leading to mitochondrial dysfunction contribute to senescence phenotype in γ-rays induced senescence.

The increase of ROS production and the increase in antioxidant enzyme expression observed in XTC.UC1 cell line following γ-rays treatment, demonstrated that mitochondrial dysfunction contributes to the maintenance of senescent phenotype through oxidative stress. In fact, ROS causing DNA damage, are responsible for maintenance active DDR that is involved in the activation of this type of senescence.

Furthermore, it was shown that the molecular pathway regulating the response of the oncocytic model to γ-rays treatment is different from the pathway that is activated in the other cell lines. The involvement of mTOR and in particular of mTORC1 has been shown responsible for determining senescent phenotype in XTC.UC1 cell line. In fact, mTORC1 inhibition in this cell line prevents the activation of cellular senescence, while the same inhibition did not have any effects on senescence process in the other cell lines.

The difference in senescent response of XTC.UC1 compared to the other cell lines analyzed in this study can be attributed mainly to the presence of *TP53* mutations. Preliminary data have shown that in RPE1 cell lines, which do not bear *TP53* mutations, γ-rays treatment induced the activation of AMPK kinase which inhibits mTOR. Consequently, AMPK activation observed in this cell line justifies the finding that mTORC1 is not responsible for regulating the response of these cell lines to radiation treatment.

AMPK activation following γ-rays treatment has already been demonstrated [181], and it has been verified that this kinase is activated in a p53-dependent mechanism [181]. Therefore, due to the presence of *TP53* mutations, genotoxic stress can not induce AMPK activation in XTC.UC1 cell line. Moreover, it has been demonstrated that γ-rays are responsible to activate mTORC1 in oncocytic cell line, which is already active in basal conditions, due to the loss of PTEN.

It has been shown that cells carrying mutations in *ND5* gene which codify for one subunit of respiratory complex I are characterized by activation of AKT pathway, compared to control cells with wild-type protein [182]

The recovery of complex I functionality, achieved after introducing a yeast NADH quinone oxidoreductase (*NDI1*) gene led to a decrease in the phosphorilation of AKT. According to the authors, AKT activation depends on ROS production [182]. It is possible to hypothesize that in XTC.UC1 cell line, where mTORC1 complex is already active as a consequence of PTEN loss, the mitochondrial dysfunction contributes to activation of mTORC1 observed following γ-rays treatment, probably due to enhanced AKT activation induced by increased ROS production.

Thus, mitochondrial dysfunction could contribute to the maintenance of senescent phenotype acting by a dual mechanism that involves ROS. ROS produced following γ-rays treatment may be responsible to amplify the DDR and on the other hand contribute to mTORC1 activation that seems directly responsible, in this cell line, to control cellular senescence.

In order to demonstrate the involvement of mitochondrial dysfunction in regulating cellular senescence, HXTC-UC1 cybrid cell line were subjected to treatment with γ-rays. This cell line was obtained from the fusion of enucleated XTC.UC1 which served as a donor of cytoplasm, and *143B TK*-osteosarcoma cell line deprived of their mtDNA (Rho0), as nuclei donor.

If mitochondrial dysfunction is a general mechanism responsible for control of γ-rays induced senescence, genotoxic stress should activate senescence pathway in HXTC.UC1 cell line as well.

Oncocytic tumors have been well characterized in the laboratory of Medical Genetics directed by Prof. Romeo, where it was demonstrated that these tumors are in most cases benign, having a low proliferative potential and are not aggressive [135, 138-144]. Furthermore, in these tumors the presence of mutations in respiratory complex I is associated to a destabilization of HIF-1α (Hypoxia-inducible factor) which is responsible for mediating the metabolic adaptation of cells during the progression from benignity towards malignancy. Even in hypoxic condition mitochondrial dysfunction determines HIF-1α destabilization as a consequence of the increase ratio of α-ketoglutarate/succinate [140]. Taken together, these data demonstrated that the presence of complex I

disruptive mutations in mtDNA contributes to tumor growth reduction and to maintainance of benign state of the tumors [138].

The data obtained by this study lead us to speculate that the low proliferative potential of oncocytoma could be attributed to activation of cellular senescence, and if senescence is activated in oncocytoma this may depend on mitochondrial dysfunction. XTC.UC1 cell line is characterized by the presence of *TP53* mutations and complete absence of PTEN. It will be therefore necessary to sequence both genes in a panel of oncocytic tumors to investigate the potential occurrence of mutations. This data will allow verification that the oncocytoma-related senescence is activated following the pattern described in XTC.UC1.

In the second part of this study it has been verified the involvement of mTORC1 in the regulation of PGC-1α expression. This experiment has been conducted in order to verify which mechanism induce the development of oncocytoma in patients affected by two hereditary familial syndrome, CS syndrome and BHD syndrome. The study of CS and BHD patients lead us to hypothesized that the double heterozigous of the two tumor suppressor genes causative of the syndromes, respectively *PTEN* and *FLCN*, leads to mitochondrial biogenesis activation through the involvement of mTORC1. The XTC.UC1 cell line was used as model to verify the hypothesis because do not express PTEN and is characterized by loss of one FLCN allele. Taken together the results obtained in XTC.UC1 cell line seem to indicate that mTOR is not involved in the regulation of PGC-1α expression. This result it has been demonstrated after complementation of PTEN and after pharmacological treatment of XTC.UC1 cell line with rapamycin, a specific inhibitor of mTORC1. In contrast with what hypothesized, the results of these experiments seem to demonstrate that mTORC1 acts in cells as an inhibitor of mitochondrial biogenesis as the increase of PGC-1α expression was observed after inhibition of mTORC1.

Further studies will be conducted in order to demonstrate if mTORC1 activation leads to inhibition of mitochondrial biogenesis, even in XTC.UC1 which stably express PTEN.

The experiments of reciprocal complementation of FLCN and PTEN in XTC.UC1 indicated that the two genes have two opposite functions. While PTEN expression induced a slightly increase of PGC-1α expression, a decrease of PGC-1α was observed in cells complemented with FLCN. PTEN acts as a negative regulator of

mTORC1. Thus, it can be assumed that if mTORC1 is not involved in regulating mitochondrial biogenesis, PTEN loss is not involved in regulating PGC-1α expression through mTORC1.

At the same time, FLCN should not act through mTORC1 in regulating PGC-1α expression.

The role of FLCN as an inhibitor of mTORC1 has been deduced on the basis on the functions of its interactors FNIP1 and FNIP2, which also interact with AMPK that inhibits mTORC1 [155-158]. It may be speculated that FLCN acts regulating PGC-1α expression through AMPK. In fact, it has been demonstrated in skeletal muscle, that AMPK regulates mitochondrial biogenesis through controlling PGC-1α and PPARγ expression [177, 178].

The hypothesis of AMPK involvement in regulation of mitochondrial biogenesis is in apparent disagreement with the basal activation of mTORC1 observed in XTC.UC1. Further studies will be conducted in order to establish how loss of FLCN and PTEN may control mitochondrial biogenesis, and to elucidate the pathway that links FLCN to PGC-1α. It is also needed to verify whether the increased expression of PGC-1α observed after PTEN complementation can be attributed to the activation of a compensatory mechanism.

It was initially hypothesized that it was *FLCN* and *PTEN* double heterozigous to induce mTORC1 activation and then mitochondrial biogenesis. The study presented in this thesis are conducted on XTC.UC1 cell line complemented with FLCN or PTEN and before ruling out the involvement of PTEN and mTOR in regulation of mitochondrial biogenesis, it will be necessary to generate stable clones which are complemented with PTEN and FLCN in order to verify the effect of the double heterozygous on mTOR and PGC-1α expression.

6. CONCLUSIONS

The results obtained in the first part of this thesis, allowed us to demonstrate that exposure to genotoxic stress is responsible for activating cellular senescence in oncocytic model. Based on these results, it has been speculated that senescence induced in this cell line is supported by the presence of mitochondrial dysfunction. Mitochondrial dysfunction contributes to the maintenance of senescence phenotype through the constant production of ROS.

It was also demonstrated that in XTC.UC1 cell lines mTOR, and in particular mTORC1 is responsible to regulate the activation of cellular senescence, in contrast to what observed in other cell lines.

This study's results, together with data from literature, lead us to hypothesize that ROS produced following γ-rays treatment are on the one hand responsible for keeping the DDR, directly involved in inducing senescence, active. On the other hand, they contribute to mTORC1 activation that is shown to regulate cellular senescence in this cell line.

These data lead us to speculate on what happens *in vivo* in oncocytoma. These tumors are benign and have a low proliferative potential. On the basis of data observed in XTC.UC1 cell line, it is possible to conclude that the typical benign state of oncocytoma is imputable to the activation of cellular senescence, which blocks proliferation. It is possible to hypothesize that senescence is activated in oncocytoma through a mechanism that involves mitochondrial dysfunction.

In the second part of this thesis we have investigated the role of mTORC1 in sustaining the activation of mitochondrial biogenesis that characterizes XTC.UC1 cell line. Exploring this question allows us to understand if loss of tumor suppressor gene can determines the development of oncocytomas in CS and BHD patients.

Unlike literature data, the results obtained seem to exclude the involvement of mTOR in regulating PGC-1α expression, and instead suggest that mTOR acts as a PGC-1α inhibitor.

Besides, PTEN and FLCN complementation experiments seem to indicate that the two genes have opposite functions in regulating PGC-1α expression. In fact, PTEN complementation determines an increase in PGC-1α expression, while FLCN reconstruction seems to lead to a decrease in PGC-1α expression. The

initial hypothesis of this study was that PTEN and FLCN double heterozigous are responsible for sustaining PGC-1α expression. Additional studies will be therefore conducted to investigate more closely the effect of PTEN and FLCN restore expression on PGC-1α and to evaluate the involvement of mTORC1.

7. BIBLIOGRAPHY

1. Hayflick, L., *Exp. Cell Res*. **37**: 614-636 (1965)
2. Campisi, J., and d'Adda di Fagagna, F., Nature Review 8: 729-740 (2007)
3. Serrano, M., Lin, A. W., McCurrach, M. E., Beach, D. and Lowe, S. W., *Cell* **88**: 593-602 (1997)
4. Collado, M. and Serrano, M., *Nature Reviews* **10**: 51-57 (2010)
5. Rodier, F. and Campisi, J., *J. Cell Biol* (192) **4**: 547-556 (2011)
6. Nardella, C., Clohassy, J. G., Alimonti, A., Pandolfi, PP., *Nature Reviews* **11:** 503-511 (2011)
7. Beauséjour, CM., Krtolica, A., Salimi, F., Marita, M., Lowe, SW., Yaswen, P. and Campisi, J., *EMBO J* **22**: 4212-4222 (2003)
8. Dirac, A. M. G., Bernards, R., J Biol Chem 278: 11731-11734 (2003)
9. Kuilman, T., Michaloglou, C., Vredeveld, L.C.W, Douma, S., van Doorn, R., Desmet, C. J., Aarden, L. A., Mooi, W. J. and Peeper, D.S., *Cell* **133:** 1019-1031 (2008)
10. Kuilman, T., Michaloglou, C., Mooi, W. J. And Peeper, D. S., *Gen & Dev* **24:** 2463-2479 (2010)
11. Sherr, C. J. and DePhnho, R. A., *Cell* **102**: 407-410 (2000)
12. Ramirez, R. D., et al., *Genes Dev*. **15 :** 398-403 (2011)
13. Fusenig, N.E. and P. Boukamp., *Mol. Carcinog.* **23:** 144-158 (1998)
14. 70C+F
15. Campisi, J., *Curr. Opin Gen & Dev* **21:** 107-112 (2011)
16. DiLeonardo, A., Linke, S. P., Clarkin, K. & Wahl, *Genes Dev.* **8**, 2540-2551 (1994).
17. Wada, T. et al., *Nature Cell Biol.* **6**, 215-226 (2004).
18. Di Micco, R. et al., *Nature* **444**, 638-642 (2006).
19. Zhu, J., Woods, D., McMahon, M. & Bishop, J. M. *Genes Dev.* **12**, 2997-3007 (1998).
20. Denoyelle, C., Abou-Rjaily, G., Bezrookove, V., Verhaegen, M., Johnson, T. M., Fullen, D. R., Pointer, J. N., gruber, S. B., Su, L. D., et al., *Nat. Cell Bi*ol **8:** 1053-1063 (2006)
21. Parrinello, S., Camper, E., Krtolica, A., Goldestein, J., Melow, S., and Campisi, J., *Nat Cell Biol* **5:** 741-747 (2003)
22. Chen, Q., Fischer, A., Reagan, J.D., Yan, L. J., Ames, B. N., *Proc Natl Acad Sci* **92:** 4337-4341 (1995)
23. Chen, Z., Trotman, L. C., Schaffer, D., Lin, H-K., Dotan, Z. A., niki, M., Koutcher, J. A., Scher, H. I., Ludwig, T., Gerald, W., et al *Nature* **436:** 725-730 (2005)
24. Michaloglou, C., Vredeveld, L. C. M., Soengas, M. S., Denoyelle, C., Kuilmann, T., van der Horst, C. M. A. M, Majoor, D. M., Shay, J. W., Mooi, W. J. And Peeper, D. S., *Nature* **436:** 720-724 (2005)
25. Moiseeva, O., Bourdeau, V., Roux, A., Deschenes-Simard, X., and Ferbeyre, G., *Mol Cell Biol.* **16 :** 4495-4507 (2009)
26. Debacq-Chainiaux, F., Erusalimski, J. D., Campisi, J., and Toussaint, O., *Nature Protocols*, **4 (12):** pp. 1798-1806
27. Lee, B. Y., et al., *Aging Cell* **5 :**187-195 (2006)
28. Kurt, D. J., Decary, S., Hong, Y., and Erusalimsky, J. D., *J. Cell Sci*. **113 :** 3613-3622 (2000)
29. Narita, M., and Reddel, R.R., *Human Mol Genet* **14:** R191-R196 (200)
30. Coppé, j. P., Desprez, P.Y., Krotolica, A., and Campisi, J., Ann. Rev. Pathos. Mech. Dis. **5:** 99-118 (2010)
31. Rodier, F., Coppé, J. P., Pathil, C. K., Hoeijmakers, W. A. M., Munoz, D. P., Raza, S. R., Freund, A., Campeau, E., Davalos, A. R. and Campisi, J., *Nat Cell Biol* **11:** 973-979 (2009)
32. D'Adda di Fagagna, F., *Nat Rev Cancer* **8:** 512-522 (2008)

33. Campisi, J., *Cell* **120:** 513-522 (2005)
34. Ben-Prath, I., and Weinberg, R. A., *Int J Biochem Cell Biol* **37:** 961-976 (2005)
35. Masutomi, K., Yu, E. Y., Khurts, S., Ben-Porath, I., Currier, J., Metz, G. B., et al *Cell* **114:** 241-253 (2003)
36. Shay, J.W., and Bacchetti, S., Eur J Cancer 33: 787-791 (1997)
37. Muntoni, A., and Reddel, R. R., Hum Mol Genet 14: R191-R196 (2005)
38. Kamijo, T., Zindy, F., Roussel, M.F., Quelle, D. E., Downing, J.. R., Ashmun, R. A., Grosveld, G., and Sherr, C. J., Cell 91: 649-659 (1997)
39. Michaloglou, C., Vredeveld, L. C., Soengs, M. S., Denoyelle, C., Kuilmann, T., van derHorst, C. M., Majoor, D. M., Shay, J.W., Mooi, W. J., and Peeper, D. S., *Nature* **436:**720-725 (2005)
40. Collado, M., Gil, J., efeyan, A., Guerra, C., Schuhmacher, A. J., Barradas, M., Benguria, A., Zaballos, A., Flores, J. M, et al., *Nature* **436:** 642 (2005)
41. Braig, M., Lee, S., Loddenkemper, C., Rudolph, C., Peters, A. H., Schlegelberger, B., Stein, H., Dorken, B., Jenuwein, T., and Schmitt, C. A., *Nature* **436:** 660-665 (2005)
42. Che, Z., Trotman, L. C., Schaffer, D., Lin, H. K., Dotan, Z. A., Niki, M., koutcher, J. A., Scher, H. I., ludwig, T., et al *Nature* **436:** 725-730 (2005)
43. Davies, H., Bignell, G. R., Cox, C., Stephens, P., Edkins, S., Clegg, S., Teague, J., Woffendin, H., Garnett, M. J., et al *Nature* **417:**949-954 (2002)
44. Michaloglou, C., Vredeveld, l. C., Mooi, W.J, and Peeper, D. S., *Oncogene* (2007)
45. Mooi, W.J., and Peeper, D. S., *N. Engl J. Med* **355:**1037-1046 (2006)
46. Alimonti, A., Nardella, C., Chen, Z., Clohessy, J. G., Carracedo, A., Trotman, L. C., Cheng, K., Vermeh, S., Kozma, S. C., Thomas, G., Rosivatz, E., Woscholski, R., Cognetti, F., Scher, H. I, and Pandolfi, P. P., *J.Clin. Invest.* **120:** 681-693 (2010)
47. Nardella, C., et al *Sci. Signal* 2 ra2(2009)
48. Chen, Z., et al, *Sci. Signal* 2, ra 44(2009)
49. Palmero, I., Pantoja, C., and Serrano, M., Nature 395: 125-126 (1998)
50. Song, M. S., et al., Cell 144:187-199 (2011)
51. DiLeonardo, A., Linke, S. P., Clarkin, K. & Wahl, *Genes Dev.* **8**, 2540-2551 (1994).
52. Parrinello, S. et al., *Nature Cell Biol.* **5**, 741-747 (2003).
53. Roninson, I. B., *Cancer Res.* **63**, 2705-2715 (2003)
54. Roberson, R. S., Kussick, S. J., Vallieres, E., Chen, S. Y. & Wu, D. Y., *Cancer Res.* **65**, 2795-2803 (2005).
55. Schmitt, C. A. et al., *Cell* **109**, 335-346 (2002).
56. te Poele, R. H., Okorokov, A. L., Jardine, L., Cummings, J. & Joel, S. P., *Cancer Res.* **62**, 1876-1883 (2002).
57. Olsen, C. L., Gardie, B., Yaswen, P. & Stampfer, M. R., *Oncogene* **21**, 6328-6339 (2002).
58. Bode, M. A., and Dong, Z., Nature Review 4:793-805
59. Jacobs, J. J. & de Lange, T., *Curr. Biol.* **14**, 2302-2308 (2004).
60. Stein, G. H., Drullinger, L. F., Soulard, A. & Dulic, V., *Mol. Cell. Biol.* **19**, 2109-2117 (1999).
61. Narita, M. et al., *Cell* **113**, 703-716 (2003).
62. Ohtani, N. et al., *Nature* **409**, 1067-1070 (2001).
63. Jeyapalan, J. C., Ferreira, M., Sedivy, J. M. & Herbig, U., *Mech. Ageing Dev.* **128**, 36-44 (2007).
64. Price, J. S. et al., *Aging Cell* **1**, 57-65 (2002).
65. Vasile, E., Tomita, Y., Brown, L. F., Kocher, O. & Dvorak, H. F., *FASEB J.* **15**, 458-466 (2001).
66. Collado, M. et al., *Nature* **436**, 642 (2005).
67. Braig. M, et al., *Nature* **436**, 660-665 (2005).
68. Chen, Z. et al., *Nature* **436**, 725-730 (2005).

69. Castro, P., Giri, D., Lamb, D. & Ittmann, M., *Prostate* **55**, 30-38 (2003).
70. Prieur, A., and Peepe, D. S., *Curr. Opin. Cell. Biol* **20:**150-155 (2008)
71. Coutois-Cox, S., et al., *Cancer Cell* **10:** 459-472 (2006)
72. Gray-Schopfer, V., *Cancer Cell* **10:** 496-505 (2006)
73. Rakhra, K., et al *Cancer Cell* **18:**485-498 (2010)
74. Wullschleger, S., Loewith, R., and Hall, M., *Cell* **124:** 471-484 (2006)
75. Kunz, J., Henriquez, R., Schneider, U., Deuter-Reinhard, M., Movva, N. R., and Hall, M. N., *Cell* **73:** 585-596 (1993)
76. Hellivell, S. B., Wagner, P., Kunz, J., deuter-Reinhard, M., Henriquez, R., and Hall, M. N., *Mol. Biol. Cell* **5:**105-118 (1994)
77. Keith, C. T., and Schreiber, S. L., *Science* **270** (1995) 50
78. Abraham, R. T., *DNA Repair* **3**(2004) 883
79. Dunlop, E.A., Tee, A. R., Cell. Sign. 21: 827-835 (2009)
80. van Slegtenhorst, M., de Hoogt, R., Hermana, C., Nellist, M., Janssen, B., Verhoef, S., Lindhout, S., van den Ouweland, A., Halley, D., young, J., Burley, M., Jeremiah, K., et al *Science* **277:** 805 (1997)
81. European Tuberous Sclerosis Consortium, *Cell* **75:** 1305 (1993)
82. Xu, G. F., O'Connel, D., Viskochill, R., Cawthon, R., Robertson, M., culver, M., Dunn, D., Stevensen, R., Gesteland, R., White, R., and Weiss, R., *Cell* **62 :** 599 (1990)
83. Hemminki, A., Merkie, D., Tomlinson, E., Avizienyte, E., Roth, S., Loukola, A., Bignell, G., Warren, W., Aminoff, A., Hoglund, P., et al *Nature* **391:** 184 (1998)
84. Liaw, D., Marsh, D. J., Li, J., Dahia, P. L., Wang, S. I., Zheng, Z., Bose, S. M., Call, K.M., Tsou, H.C., Peacocke, M., Eng, R., and Parsons, R., *Nat. Genet* **6:** 599 (1997)
85. Nelen, M. R., van Staveren, W. C., Peeters, E. A., Hassel, M. B., Gorlin, R. J., Hamm, H., Lindboe, C.F., Fryns, J.P., Sijmons, R. H., Woods, D. G., Mariman, E.C., Padberg, H., and Kremer, H., *Hum.Mol.Genet*. **6:** 1383 (1997)
86. Baba, M., Hong, S.B., Sharma, N., Warren, M.B., Nickerson, M. L.,Iwamatsu, A., Esposito, D., Gillette, W. K., et al., *Proc. Natl. Acad. Sci.* **103:** 15552 (2006)
87. Hasumi, H., Baba, M., Hong, S. B. Hasumi, Y., Huang, M., Yao, M., Valera, V.A ., Linehan, W. M., and Schidt. L. S., *Gene* **415:** 60 (2008)
88. Takagi, Y., Kobayashi, T., Shiono, M., Wang, L., Piao, X., Sun, g., Zhang, D., Abe, M., Hagiwara, Y., Takahashi, K., and Hino, O., *Oncogene* **27:** 5339 (2008)
89. Hay, N., and Sonenberg, N., *Genes & Devel.* **18:** 1926-1945 (2004)
90. Zoncu, R., Efeyan, A., and Sabatini., D.M., *Mol. Cell. Biol.* **22:** 21-35 (2011)
91. Yip, C. K., Murata, K., Walz, T., Sabatini, D. M., and Kang, S. A., *Cell* **38:** 768-774 (2010)
92. Sarbassov, D. D., et al., *Cell* **22:** 159-168 (2006)
93. Noda, T., and Ohsumi, Y:, *J. Biol. Chem.* **273:** 3963-3966 (1998)
94. Thoreen, C. C., et al., *J. Biol. Chem.* **284:** 8023-8032 (2009)
95. Castilho, R. M., Squarize, C. H., Chodosh, L.A., Williams, B. O., and Gutkind, J. S., Cell Stem *Cell* **5:** 279-289 (2009)
96. Astle, M. V., Hannan, K. M., Ng, P. Y., Lee, R. S., Gorge, A. J., Hsu, A. K., Haupt, Y., Hannan, R. D., and Pearson, R. B., *Oncogene* 1-14 (2011)
97. Manning, B.D., et al., *Mol Cell* **10**(1):151-62 (2002)
98. Potter, C.J., et al., *Biochem Soc Trans* **31**(Pt 3): 584-586 (2003)
99. Tee, A.R., Anjum, R., and Blenis, J., *J Biol Chem* **278**(39): 37288-37296 (2003)
100. Garami, A., et al., Mol Cell **11**(6): 1457-1466 (2003)
101. Kovacina , K,., et al., *J.Biol. Chem* **278:** 10189-10194 (2003)
102. Gwinn, D. M., et al, *Mol. Cell* **30 :** 214-226 (2008)
103. Feng, Z., et al., *Cancer Res.* **67 :** 3043-3053 (2007)
104. Jones, R. G., et al, *Mol Cell* **18 :** 737-749 (2005)
105. Budanov, A.V., and Karin, M., *Cell* **134:** 451-460 (2008)

106. Gao, X., et al., *Nat Cell Biol* **4**(9): 699-704 (2002)
107. Attardi, G., Shatz, G., *Annu. Rev. Cell Biol.* **4:** 289-333 (1988)
108. Scarpulla, R.C., et al J. Biol. Chem. 279: 39593-39603 (2004)
109. Van Houten, B., Woshner, V., Santos, J. H., *DNA Repair* **5:**145-152 (2006)
110. Scarpulla, R., *Physiol Rev* **88:** 611-638 (2008)
111. Passarella, S., and Atlante, A., *Mitochondrion* **2:** 319-343 (2003)
112. Schlamen, M., and Ren, M., *Biochim. Biophysi. Acta* **1788:** 2080-2083 (2009)
113. Frey TG e Mannella CA *Trends Biochem Sci* **25**: 319-324 (2000).
114. Anderson S, Bankier AT, Barrell BG, de Bruijn MH, Coulson AR, Drouin J, Eperon IC, Nierlich DP, Roe BA, Sanger F, Schreier PH, Smith AJ, Staden R, Young IG. (1981) *Nature*. **290(5806):**457-465
115. Macreadie, G., Novitskic, E., Maxwell, R. J., John, U., Ooi, B. G., McMullen, B. L., Lukins, H. B., Linnane, A. W., and Nagley, O., *Nucleic Acids Res* **11:** 4435-4451 (1983)
116. Chomin, A., Cleeter, M. W., Ragan, C. I., Riley, M., Doolittle, M. T., and Attardi, G., *Science* **234:** 614-618 (1986)
117. Ojala, D., Monttoya, J., and Attardi, G., *Nature* **290:** 470-474 (1981)
118. Holt, I.J., He, J., Mao, C. C., Boy, D., Kirkup, J. D., Martinsson, P., Sembonghi, H., and Spelbrink, J. N., *Mitochondrion* **7:** 311-321 (2007)
119. Malaka, F., Lombès, A., and Rojo, M., *Biochim. Et Biophys Acta* **1763:** 463-472 (2006)
120. Takamatsu, C., Umeda, S., Ohsato, T., Ohno, T., Abe, Y., Fukuoh, A., Shinagawa, N., and Kang, D., *EMBO* Rep **3:** 451-456 (2002)
121. Alam, T. I., Kanki, T., Muta, T., Ukaji, K., Abe, Y., Nakayama, H., Takio, K., Hamasaki, N., Kang, D., *Nucl. Acid. Res* **31:** 1640-1645 (2003)
122. Sutovski, P., McCauley, T. C., Sutovsky, M., and Day, B. M., Biol. Reprod. 68: 1793-1800 (2003)
123. Sutovsky, P., Moreno, R. D., Ramalo,-Santos, J., Dominko, T., Simerly, C., and Scatten, G., Nature 402: 371-372 (1999)
124. DiMauro, S., and Davidson, G., Annu. Med. 37: 222-232 (2005)
125. Fernández-Silva P, Enriquez JA, Montoya J Replication and transcription of mammalian mitochondrial DNA. *Exp Physiol.* **88:** 41-56(2003)
126. Gleyzer, N., Vercauteren, N., and Scarpulla, R.C., *Mol. Cell. Biol* **25:** 1354-1366 (2005)
127. Scarpulla, R.C., 2006 *J. Cellul. Biochem* **97:** 673-683 (20069
128. Wu, Z:, Ouigserver, P., Andersson, U., Zhang, C., Adelmant, G., Mootha, V., Troy, A., Cinti, S., Lowell, B., Scarpulla, R. C., and Spiegelman, B. M., *Cell* **98:** 115-124 (1999)
129. Scarpulla R.C.,
130. Hardie, D. G., and Sakamoto, K., *Physiology* **21:** 48-60 (2006)
131. Kahn, B. B., Alquier, T., Carling, D., and Hardie, D. G., *Cell Metab* **1:** 15-25 (2005)
132. Puigserver, P., et al., *Science* **286**(5443): 1368-71 (1999).
133. Cunningham, J.T., Rodgers, J., Arlow, D. H., Vazquez, F., Mootha, V. K., and Puigserver, P., Nat Lett **450(29):** 736-741(2007)
134. Schieke, S. M., McCoy, P. J., Aponte, A. M., Shen, R-F., Balaban, R.S., and Finkel, T., *J. Biol. Chem* **37:** 27643-27652 (2006)
135. Tallini, G., *Virchows Arch* **433**(1): 5-12 (1998)
136. Gasparre, G., Romeo. G., Rugolo, M., Porcelli, A. M., Biochim Biophys Acta 1807: 633-642 (2010)
137. Ambu, R., et al., *Ultrastruct Pathol* **24**(4): 211-219 (2000).Bio
138. Riva, A. and Tandler, B., *Ultrastruct Pathol* **24**(3): 145-150 (2000).
139. Gasparre, G., Kurelac, I., Capristo, M. A., iommarini, L., Ghelli, A., Ceccarelli, C., Nicoletti, G., Nanni, P., De Giovanni, C., Scotlandi, K., Betts, C., Carelli, V., Lollini, P. L., Romeo, G., Rugolo, M and Porcelli, A. M., Cancer Res. 71:6220-6229 (2011)

140. Gasparre, G., Hervouet, E., de Le, Demont, J., Pennisi, L. F., colombel, M., et al, Hum Mol Genet 17 : 986-995 (2008)
141. Gasparre, G., Iommarini, L., Porcelli, A. M., Lang, M., Ferri, G. G., Kurelac, I., et al., Hum Mutat 30: 391-396 (2009)
142. Gasparre, G., Porcelli, A. M:, Bonora, E., pennini, L. F., Toller, M., Iommarini, L., et al, Proc Natl Acad Sci U S A 104: 9001-9006 (2007)
143. Bonora, E., Porcelli, A. M., Gasparre, G., Biondi, A., Carelli, V., et al Cancer Res 66: 6087-6096 (2006)
144. Gasparre, G., Bonora, E., Tallini, G., Romeo, G., Mol Cell Endocrinol 321: 67-76 (2010)
145. Porcelli, A. M., Ghelli, A., Ceccarelli, C., Lang, M., Capristo, M. A., Hum Mol Genet 19: 1019-1032 (2010)
146. Hobert, J.A. and Eng, C., *Genet Med* **11**(10): 687-694 (2009).
147. Nelen, M.R., et al., *Nat Genet*, **13**(1): 114-116 (1996).
148. Cantley, L.C. and B.G. Neel, Proc Natl Acad Sci U S A **96**(8): 4240-4245 (1999).
149. Di Cristofano, A. and P.P. Pandolfi, Cell **100**(4): 387-390 (2000).
150. Lee, J.O., et al., Cell, **99**(3): 323-334 (1999).
151. Blanco-Aparicio, C., et al., *Carcinogenesis* **28**(7): 1379-1386 (2007).
152. Planchon, S.M., K.A. Waite, and C. Eng, J *Cell Sci* **121**(Pt 3): 249-253 (208).
153. Shen, W.H., et al., *Cell* **128**(1): 157-170 (2007).
154. Birt, A.R., G.R. Hogg, and W.J. Dube, *Arch Dermatol* **113**(12): 1674-1677 (1977).
155. Nickerson, M.L., et al., *Cancer Cell* **2**(2): 157-164 (2002).
156. Baba, M., et al., *Proc Natl Acad Sci* U S A **103**(42): 15552-15557 (2006).
157. Gwinn, D.M., et al., *Mol Cell* **30**(2): 214-226 (2008).
158. Hasumi, H., et al., *Gene* **415**(1-2): 60-67 (2008).
159. Takagi, Y., et al., *Oncogene* **27**(40): 5339-5347 (2008).
160. Wang, L., et al., *FEBS Lett* **584**(1): 39-43 (2010).
161. Piao, X., et al., *Biochem Biophys Res Commun* **389**(1): 16-21 (2009).
162. Hartman, T.R., et al., *Oncogene* **28**(13): 1594-1604 (2009).
163. Baba, M., et al., *J Natl Cancer Inst* **100**(2): 140-154 (2008).
164. Chen, J., et al., *PLoS One* **3**(10): e3581 (2008).
165. Preston, R.S., et al., *Oncogene* **30**(10): 1159-1173 (2010).
166. Warburg, O., *Science* ***1*****24**(3215): 269-270 (1956).
167. Klomp, J.A., et al., *BMC Med Genomics* **3**: 59 (2010).
168. Zhang, Maguire, Swartz, Sun, Yang, Wang, Liu, Zhang, Zhang, Zhang, Keng, Zhang, Okunieff., *Adv Exp Med Biol* (2009)
169. Malakhova., Bexlepink, Antipova, Ushakova, Fomenko, Sirota, Gaziev:; *Cell and Mol. Biol. Lett*, 2005, **10:** 721-735
170. Ambrosini-Spaltro, A., et al. *Virchows Arch*, **448:** 442-448 (2006)
171. Rouzbahman, M., serra, S., and Chetty, R., *J Clin Pathol* **59:** 1039-1043 (2006)
172. Soussi, T., and Béroud, C., *Nature Review* **1 :** 233-240 (2001)
173. Bos, J. L., *Cancer Res.* 49(17) : 4682-4689 (1989).
174. Brandon, M., Baldi, P., and Wallace, D., *Oncogene 25:*4647-4662 (2006)
175. Ishikawa, K., Takenaga, K., Akimoto, M., Koshikawa, N., Yamaguci, A., Imanishi, H., Nakada, K., Honma, Y., and Hayashi, J., *Science* **320:** 661-664
176. Park, J. S., Sharma, L. K., Li, H., Xiang, R., Holstein, D., wu, J., Lechleiter, J., Naylor, S. L., Deng, J. J., Lu, J., et al., *Hum Mol Gen* **18:** 578-1589 (2009)
177. Bentzinger, C. F., et al., *Cell Metab*. **8:** 411-424 (2008)
178. Jager, S., et al., *Proc Natl Acad Sci USA* **104(29):**12017-12022 (2007)
179. Narkar, V. A., *Cell* **134(3):** 405-415 (2008)
180. Pradella, L. M., Zuntini, R., Magini, P., Ceccarelli, C., Neri, I., Cerasoli, S., Graziano, C., Gasparre, G., and Turchetti, D., *J. Med. Genet* **48(11):** 779-782 (2011)

181. Sanli, T., Storozhuk, Y., Linher-Melville, K., Bristol, R. G., Laderout, K., Viollet, B., Wright, J., Singh, G., and Tsakiridis, T., *Rad. and Oncol*. (2012)
182. Sharma, L. K., Fang, H., Liu, J., Vartak, R., Deng, J., Bai, Y., *Hum. Mol. Genet.* **20(3):** 4605-4616
183. Di Cristofano, A., et al., *Science* **285**(5436): 2122-2125 (1999).
184. Di Cristofano, A., et al., *Nat Genet* **27**(2): 222-224 (2001)

APPENDIX A

PRIMER REAL TIME

GENE	PRIMER FW	PRIMER RV
PGC-1α	5'-TGAGAGGGCCAAGCAAAG-3'	5'-ATAAATCACACGGCGCTCTT-3'
TUBULINA	5'ATACCTTGAGGCGCAAAA-3'	5'-CTGATCACCTCCCAGAACTTG-3'

PRIMER RT

GENE	PRIMER FW	PRIMER RV
PGC-1α	5'-TTGCCCAGATCTTCCTGAAC-3'	5'-AAATGAGGGCAATCCGTCTT-3'
TUBULINA	5'-GAACGTGATGGTGTCCACAG-3'	5'-CGCTTACGCAGCTTGTCATA-3'

PRIMER PTEN ($MgCl_2$: 2.5mM- Fast Start Taq (Roche))

	PRIMER FW	PRIMER RV
Promoter	5'-GCGTGGTCACCTGGTCCTTT-3'	5'-GCTGCTCACAGGCGCTGA-3'
Exon 1	5'-AAGTTTGAGAGTTGAGCCG-3'	5'-GTGTTGGAGGCAGTAGAA
Exon 1a	5'-ATTTCCAGGGCTGGGAA-3'	5'-GAAAGGTAAAGAGGAGCA-3'
Exon 2	5'-TGTCTTTTCAGGCAGGTGTC-3'	5'-GGTGACCAGCATTTTATGGAC-3'
Exon 3	5'-TTTGATGGGAAAATGATGTCTG-3'	5'-CATGAATCTGCCAACAATG-3'
Exon 4	5'-GCATGGAAGCACCTGAATTTAC-3'	5'-TGTTTCTCCCTGTGATTGC-3'
Exon 5	5'-TTGCATTGAGAGTCCTGACG-3'	5'-AAGAAACCCAAAATCTGTTTTCC-3'
Exon 6	5'-GCATAGCTGTAGGGCAGAGG-3'	5'-TTGGCTTCTTTAGCCCAATG-3'
Exon 7	5'-TCAAGATTGCAGATACAGAATCC-3'	5'-GGCCTTTTCCTTCAAACAGG-3'
Exon 8	5'-TGTCATTTCATTTCTTTTTCT-3'	5'-TCAAGCAAGTTCTTCATCCAGC-3'
Exon 9	5'-TGTTCATCTGCAAAATGGAATAA-3'	5'-GGTAATCTGACACAATGTCC-3'

Temperatur	***Time***	***Cycle***
95°C	5 min	1
96°C	30 sec	35
58°C	30 sec	
72°C	1 min	
72°C	7 min	1

PRIMER K-Ras (MgCl2: 1.5mM-Fast Start Taq (Roche))

	PRIMER FW	PRIMER RV
Exon 1	5'-TATTAAAAGGTACTGGTGG-3'	5'-ATAAGTACTCATGAAAATGG-3'
Exon 2	5'-AACAATGTCTTTTCAAGTCC-3'	5'-TGGCATTAGCAAAGACTC-3'
Exon 3	5'-TACATTGTTTTCTTTCAGCC-3'	5'-TAGTATAGCATAATTGAGAG-3'
Exon 4a	5'-TCATAATCTCAAACTTCTTG-3'	5'-AAGTAGTTCTAAAGTGGTTG-3'
Exon 4b	5'-GCCTGAAGAGAAACATAAAG-3'	5'-GGTAATGTAAAACAAATGC-3'

Temperatur	*Time*	*Cycle*
95°C	5 min	1
95°C	30 sec	35
54°C	30 sec	
72°C	30 sec	
72°C	7 min	1

PRIMER H-Ras (Taq : KAPA 2X (Resnova))

	PRIMER FW	PRIMER RV
Exon 2	5'-GGTCATTAAGAGCAAGTGGG-3'	5'-AGAGGAAGCAGGAGACAGG-3'
Exon 3/4	5'-AGAGGCTGGCTGTGTGAACT-3'	5'-GTGTCAAGGGAGAGGGTCAG-3'
Exon 5/6	5'-AAGTGGCTGGTGGTGGAGTCG-3'	5'-GGTTCCGACATACCTCATGC-3'

Temperatur	*Time*	*Cycle*
96°C	10 min	1
96°C	30 sec	45
58°C	30 sec	
72°C	30 sec	
72°C	7 min	1

PRIMER TP53 ($MgCl_2$ 2.5mM- Fast Start Taq (Roche))

	PRIMER FW	PRIMER RV
Exon 2/3	5'-ATCCCCACTTTTCCTCTTGC-3'	5'-AGCCCAACCCTTGTCCTAC-3'
Exon 4	5'-CCTGGTCCTCTCTGACTGCTCT-3'	5'-CAGGCATTGAAGTCTCATGG-3'
Exon 5	5'-CTGTCTCCTTCCTCTTCCTACAG-3'	5'-AACCAGCCCTGTCGTCTCT-3'
Exon 6	5'-CAGGCCTCTGATTCCTCACT-3'	5'-CTTAACCCCTCCTCCCAGAC-3'
Exon 7	5'-CTCATCTTGGGCCTGTGT-3'	5'-TGGAAGAAATCGGTAAGAGGTG-3'
Exon 8	5'-GGGACAGGTAGGACCTGATTT-3'	5'-ATAACTGCACCCTTGGTCTCC-3'
Exon 9	5'-GGGACAGGTAGGACCTGATTT-3'	5'-TCAGGCAAAGTCATAGAACCA-3'
Exon 10	5'-AACTTGAACCATCTTTTAACTCAGC-3'	5'-GGAATCCTATGGCTTTCCAAC-3'
Exon 11	5'-GTCATCTCTCCTCCCTGCTTC-3	5'-CACAACAAAACACCAGTGCAG-3'

Temperatur	*Time*	*Cycle*
95°C	10 min	1
94°C	30 sec	14+35 (Touch down)
60-53°C	30 sec	
72°C	45 sec	
72°C	7 min	1

PRIMER FLCN (Master Mix Taq GOLD (Applied Biosystem))

	PRIMER FW	PRIMER RV
Exon 4	5'-GGGAGGTTTCATGGAGTCAA-3'	5'-ACTGCAGGGATCACAAAACC-3'
Exon 5	5'-ACTGCAGGGATCAACAAAACC-3'	5'-GAGCACCTGGGAGCATG-3'
Exon 6	5'-TGGTGTCACTAAGCGCGGAA-3'	5'-TGTAAGCCAGAGGGGAAGACG-3'
Exon 7	5'-TTAAAGAGGCCATCCCTTCC-3'	5'-CCTAAGAGATATGCCAAAAGC-3'

Exon 8	5’-GTGAGCGTCAGGTTTGCTTT-3’	5’-CCTCCCTCAGCGATTCCT-3’
Exon 9	5’-TTGGGCTGAAGTCACAGGAT-3’	5’-GCTCTCCTCCTGAGCTCCT-3’
Exon 10	5’-AGAAAAACCATTTCTAGTC-3’	5’-ACCTTGGCATCCCCACCT-3’
Exon 11	5’-CGTGTGGGGTTTGGGTAGTA-3’	5’-TTCCACTTTGGGCCTGAG-3’
Exon 12/13	5’-CACGGGCCTTGTGTTGTTAC-3’	5’-CCTCACCCACACTGTTGCTT-3’
Exon 14	5’-GGATTGTGCTGTGGTGTCTG-3’	5’-AGCTCCTTCCAGCAGTTGAG-3’

Temperatur	***Time***	***Cycle***
95°C	5 min	1
94°C	30 sec	35
56°C	45 sec	
72°C	45 sec	
72°C	10 min	1

ABSTRACT

The first aims of this study were to demonstrate if mitochondrial biogenesis and senescence can be induced simultaneously in cell lines upon exposure to a genotoxic stress, and if the presence of mtDNA mutations which impair the functionality of respiratory complexes can influence the ability of a cell to activate senescence. The data obtained on the oncocytic model XTC.UC1 demonstrated that the presence of mitochondrial dysfunction is involved in the maintenance of a senescent phenotype induced by γ-rays treatment. The involvement of mTORC1 in the regulation of senescence has been shown in this cell line. On the other hand, in cells which do not present mitochondrial dysfunction it has been verified that genotoxic stress determines the activation of both mitochondrial biogenesis and senescence. Further studies are necessary in order to verify if mitochondrial biogenesis sustains the activation of senescence.

The second aim of this thesis was to determine the involvement of mTORC1 in the regulation of PGC-1α expression, in order to verify what is the cause of the development of oncocytoma in patients affected by two hereditary cancer syndromes; Cowden and Birt-hogg-Dubé . The study of oncocytic tumors developed by patients affected by these syndromes suggested that the double heterozigosity of the two causative genes, *PTEN* and *FLCN* respectively, induce the activation of mTORC1 and therefore the activation of PGC-1α expression. On XTC.UC1 cell line, the most suitable *in vitro* model, experiments of complementation of PTEN and FLCN were conducted. To date, these results demonstrated that mTORC1 is not involved in the regulation of PGC-1α expression, and PTEN and FLCN seem to have opposite effect on PGC-1α expression functions

PUBLICATIONS

1. Fusco, D., Vargiolu, M., Vidone, M., **Mariani, E.**, Pennini, L. F., Bonora, E., Capellari, S., Dimberger, D., Baumeister, R., Mrtinelli, P., and Romeo, G., *Hum Mol Genet* (2010)

2. Porcelli, A. M., Angelin, A., Ghelli, A., **Mariani, E.,** Martinuzzi, A., Carelli, V., Petronilli, V., Bernardi, P., and Rugolo, M., *J. Biol. Chem* (2009)

3. Gasparre, G., Iommarini, L., Porcelli, A. M., Lang, M., Ferri, G. G., Kurelac, I., Puntini, R., **Mariani, E**., Pennini, L. F., Pasquini, E., Pasquinelli, G., Ghelli, A., Bonora, E., Ceccarelli, C., Rugolo, M., Salfi, N., Romeo, G., and Carelli, V., *Hum. Mutat* (2009)

4. Porcelli, A. M., Ghelli, A., Iommarini, L., **Mariani, E.**, Hoque, M., Zanna, C., Gasparre, G., and Rugolo, M., *Cell Mol Life Sci* (2008)

PROCEEDINGS

1. L.M Pradella, **E. Mariani,** G. Gasparre, L. Amato, A. Lanzoni, C. Ishioka, K. Saijo, G. Rossi, G. Romeo, D. Turchetti *"A novel missense mutation in the PTEN gene in a patient with multiple melanoma and feature of Cowden Syndrome"* AMERICAN SOCIETY OF HUMAN GENETICS (**ASHG**) 2010

2. D. Fusco, M.Vargiolu, M.Vidone, L.Pennisi, E. Bonora, **E.Mariani**, S.Capellari, P.Martinelli, G. Romeo *"The RET51/FKBP52 complex and its involvement in Early Onset-Parkinson disease."* EUROPEAN SOCIETY OF HUMAN GENETICS (**ESHG**) 2010

3. **E. Mariani**, D. Fusco, E. Bonora, M.Vargiolu, M.Vidone, G. Romeo "*Identification of novel proteins interacting with the RET9 receptor using the Split-Ubiquitin Yeast Two Hybrid System*." EUROPEAN SOCIETY OF HUMAN GENETICS (**ESHG**) 2009

4. M. Vargiolu, D. Fusco, I. Kurelac, L. F. Pennini, **E. Mariani**, M. Vidone, D. Dirnberger, R. Baumeister, I. Morra, A. Melcarne, R. Rimondini, G. Romeo, E. Bonora. *"The tyrosine kinase REt interacts in vivo and in vitro with AIP."* EUROPEAN SOCIETY OF HUMAN GENETICS (**ESHG**) 2009

5. A.M Porcelli, A. Ghelli, L. Iommarini, **E. Mariani**, M. Hoque, G. Gasparre, C. Zanna, M. Rugolo. "Actin cleavage in cells with detective respiratory complex is inhibited by Bcl-2 through an antioxidant function." GRUPPO ITALIANO DI BIOMEMBRANE E BIOENERGETICA (**GIBB**) 2008

6. A.M. Porcelli, A. Ghelli, L. Iommarini, **E. Mariani**, M. Hoque, G. Gasparre, C.Zanna, M. Rugolo. *"Bcl-2 antioxidant function in cells with detective complex I."* INTERNATIONAL SYMPOSIUM ON "MITOCHONDRIAL PHYSIOLOGY AND PATHOLOGY" 2008

DOTTORATO DI RICERCA IN BIOCHIMICA
Estratto del verbale della seduta del 9 febbraio 2012

L'anno 2012, addì 9 del mese di febbraio in Bologna, nell'aula del Dipartimento di Biochimica "G.Moruzzi" alle ore 15.00 si è riunito il Collegio dei Docenti del Corso di Dottorato di ricerca in Biochimica.

Risultano presenti i Professori: A.Falasca, S.Hrelia, S.Iotti, G.Lenaz, A.Pagliarani, G.Solaini, V.Tugnoli.

Risultano assenti giustificati i Professori: D.Fiorentini, F.Flamigni, C.Guarnieri, .

Risulta assente il Professore: E.Carpenè.

Risultano presenti i Professori a titolo personale: C.Muscari, C.Stefanelli.

Risultano assenti giustificati i Professori a titolo personale: A.Baracca, R.Fato, C.Prata, G.Romeo, B.Tantini, F.Trombetti.

Risultano assenti i Professori a titolo personale: R.Agati, A.Bordoni, E.D.Giordano, G.Isani, P.Parchi, A.Pession, B.Vaira.

Presiede la seduta il Prof. Giorgio Lenaz.

Assume le funzioni di Segretario il Prof. Giancarlo Solaini.

Il Prof. Lenaz riconosce valida la seduta e la dichiara aperta per trattare, come dagli avvisi di convocazione, il seguente ordine del giorno:

1. Comunicazioni.
2. Presentazione Dottorandi XXIV Ciclo da allegare alla tesi di Dottorato.
3. Designazione docenti per la composizione della Commissione Giudicatrice per il conferimento del titolo di Doctor Europaeus
4. Varie ed eventuali.

----------OMISSIS----------

2. Presentazione Dottorandi XXIV Ciclo da allegare alla tesi di Dottorato.

Il Prof. Lenaz fa presente al Collegio dei Docenti che i dottorandi, iscritti all'ultimo anno di corso, hanno provveduto a presentare, nei termini previsti, le dissertazioni finali scritte.

Il Collegio è chiamato a redigere, per ciascuno di essi, la "presentazione" da allegare alla tesi finale.

Si invitano, a tal fine, i componenti del Collegio, che prevalentemente hanno guidato le attività di ricerca dei dottorandi, a voler illustrare i contenuti delle predette tesi ed i risultati conseguiti dagli allievi.

Dopo ampia discussione, il Collegio dei Docenti decide, unanime, di approvare le "presentazioni" di seguito riportate le quali illustrano la personalità di ciascun dottorando e l'attività scientifico-formativa svolta durante il corso, mettendone in luce gli aspetti positivi.

----------OMISSIS----------

Dott.ssa **Elisa Mariani.** Tema di Ricerca: Biochimica di cellule, organi e tessuti e dei mediatori cellulari. Titolo tesi Dottorato: "Ruolo dei mitocondri nella regolazione mTOR dipendente della senescenza indotta dalle radiazioni gamma". Tutore Prof. Giovanni Romeo (allegato n° 3).

----------OMISSIS----------

Le deliberazioni prese in questa seduta sono state redatte, lette, approvate e sottoscritte seduta stante.

La seduta è tolta alle ore 16.

Il Segretario

Prof. Giancarlo Solaini

Il Coordinatore

Prof. Giorgio Lenaz

Allegato n° 3
Seduta del 9/2/2012

DIPARTIMENTO DI SCIENZE GINECOLOGICHE OSTETRICHE E PEDIATRICHE

CATTEDRA DI GENETICA MEDICA

Presso Pad. 11 - Policlinico S. Orsola-Malpighi

Via Massarenti 9 - 40138 Bologna

Tel. 051/306474 – Fax 051/6364004

Bologna, 01/02/2012

Alla Cortese attenzione

Membri del Consiglio di Dottorato in Biochimica

Presentazione del candidato: dr.ssa Elisa Mariani

La dr.ssa Elisa Mariani ha svolto la attività di ricerca inerenti al suo progetto di dottorato presso il laboratorio di Genetica Medica, Dipartimento di Scienze Ginecologiche Ostetriche e Pediatriche, dell'Ospedale S.Orsola di Bologna.

Durante questi anni la dr.ssa Mariani si è occupata principalmente di studiare la risposta di una serie di linee cellulari di origine tumorale e non, opportunamente selezionate sulla base del loro genotipo, al trattamento con radiazioni gamma in termini di attivazione del processo di senescenza. Con il termine di senescenza ci si riferisce all'arresto della proliferazione cellulare che può essere indotto nelle cellule da stimoli di diversa natura. A tale fine la dr.ssa Mariani ha utilizzato tecniche di genetica al fine di verificare l'eventuale presenza di mutazioni a livello di alcuni dei geni che sono coinvolti nel regolare il processo di senescenza: PTEN, p53, K-Ras ed H-Ras. A seguito del trattamento delle linee cellulari con radiazioni gamma, l'attivazione del processo di senescenza è stato verificato mediante saggi citochimici e analisi per western blot delle variazioni dei livelli d'espressione di alcuni regolatori della senescenza.

In una fase successiva, la dr.ssa Mariani ha cercato di verificare l'eventuale coinvolgimento di mTOR nel regolare il processo di senescenza, a tale scopo le linee cellulari sono state sottoposte ad un trattamento combinato irradiamento+rapamicina (inibitore specifico di mTOR), e l'eventuale attivazione del processo di senescenza è stato verificato utilizzando le tecniche precedentemente descritte.

Il trattamento con raggi gamma può determinare la comparsa di recidive di natura oncocitica, caratterizzata da un iperplasia mitocondriale. Gli oncocitomi sono tumori prevalentemente di natura benigna e resistenti al trattamento radioterapico, l'ipotesi di partenza è che la resistenza al trattamento che caratterizza gli oncocitomi, sia in realtà dovuto all'attivazione del processo di senesceza. La dr.ssa Mariani ha cercato di verificare se il processo di senescenza possa essere correlato al processo di biogenesi. Dal momento che dati in letteratura dimostrano che mTOR potrebbe essere coinvolto nella regolazione del metabolismo mitocondriale, l'ipotesi che la Dott.ssa Mariani ha cercato di verificare è se mTOR possa essere il denominatore comune che regola sia il processo di senescenza che quello di biogenesi indotto dal trattamento radiante.

In una seconda parte del progetto, la dr.ssa Mariani ha cercato di verificare se la biogenesi mitocondriale che caratterizza gli oncocitomi possa essere sostenuta da un hit nucleare, come ad esempio l'inattivazione di geni oncosoppressori. Tale ipotesi nasce dall'osservazione che alcuni

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pazienti affetti da sindromi tumorali familiari, quali la Cowden e la BHD, che si caratterizzano per lo sviluppo di tumori multipli possono sviluppare anche degli oncocitomi. Il dato interessante è che queste due sindromi colpiscono dei geni, rispettivamente PTEN e FLCN, che agiscono da regolatori negativi del pathway di mTOR, che come precedentemente affermato potrebbe regolare il metabolismo mitocondriale. L'ipotesi che si è cercato di verificare è se effettivamente mTOR agisca regolando il processo di biogenesi mitocondriale. A questo scopo, linee cellulari nulle per i geni PTEN e FLCN sono state trasfettate con vettori contenenti i suddetti geni, e mediante western blot e real time si sono verificate eventuali variazioni d'espressione di alcuni geni a codifica mitocondriale. Il trattamento delle cellule con la rapamicina è servito per verificare se effettivamente mTOR possa regolare la biogenesi mitocondriale osservata negli oncocitomi.

Durante il triennio di dottorato la dr.ssa Mariani ha lavorato con perseveranza ed entusiasmo ad un progetto che ha ampiamente contribuito a personalizzare e a far crescere in maniera indipendente. La padronanza della varietà di tecniche che ha ottimizzato ed utilizzato ai fini sperimentali è stata prontamente e completamente acquisita insieme alla capacità di fare critico *troubleshooting* e di re-indirizzare la linea di ricerca su nuove ipotesi, con coraggio e determinazione. La dr.ssa Mariani ha mostrato un impegno costante nel raggiungimento degli obiettivi di volta in volta più ambiziosi ed ha arricchito il team di ricerca grazie alla sua capacità di lavorare in squadra e di collaborare contribuendo con le sue expertise anche alle linee di ricerca altrui. La sua crescita professionale è stata esponenziale in particolare nel corso degli ultimi due anni del programma di dottorato, durante i quali ha dimostrato capacità analitica ed ha acquisito abilità complementari a quelle più prettamente tecniche, quali la redazione di progetti di ricerca e di richieste di finanziamento, sia in lingua italiana che inglese. Ha partecipato attivamente alle attività gestionali del laboratorio mostrando maturità nell'affrontare problemi sia tecnici che sperimentali, ha seguito molti studenti più giovani e ne ha curato la preparazione con meticolosa attenzione, affermandosi come punto di riferimento importante.

Infine, la dr.ssa Mariani è coautrice di 4 lavori con impact factor ed attualmente sta lavorando alla stesura di un lavoro inerente ai dati raccolti durante il triennio di dottorato.

In fede,

Prof. Giovanni Romeo

Giovanni Romeo

Visto: il Coordinatore del Dottorato
(p. il Collegio dei Docenti)

Prof. Giorgio Lenaz