NCOA3-

a prognostic marker in primary cutaneous melanoma

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Table of content:

1. Abstract:

Nuclear receptor coactivator 3 (NCOA3) is a member of the steroid receptor coactivator family that mediates the transcriptional function of nuclear receptors and other transcriptional factors, thereby influencing their gene expression. NCOA3 is known to be overexpressed in several cancers, especially in breast cancer where it has been shown that the deregulation of its pathway can lead to the initiation and progression of breast cancer.
The role of NCOA3 in melanoma, however, is still unknown. Recently our lab has shown NCOA3 to be a novel, independent marker of melanoma outcome with potential to regulate proliferation of melanoma cells in culture through deregulation of cell cycle checkpoint proteins, especially the DNA damage response.

The data shown in this study suggests that NCOA3 has a major effect on the cell cycle machinery in melanoma cell lines, DO4 and C8161.9. In these cell lines systematic targeting of NCOA3 using shRNA resulted in marked reduction of proliferation, which was shown to have a direct effect on the deregulation of the cell cycle and activation of apoptotic pathways.

The involvement of NCOA3 in cell cycle progression was further examined by analyzing the expression levels of several cyclins, such as cyclin D1, cyclin B1 and cyclin B2. Melanoma cell lines demonstrate typically an increase in the expression levels of several cyclins, while melanoma cell lines with reduced expression of NCOA3 showed a significant decrease in the expression levels of different cyclins.

The results suggest that the role of NCOA3 in melanoma progression occurs via the control of the cell cycle by modulating the expression of cyclines and the activation of apoptotic pathways.

2. Introduction:

2.1. History:

Cancer is the second leading cause of death in the world after cardiovascular disease [1]. About one half of men and one third of women will develop cancer through their lifetime in the United States[2]. Due to improvement in early diagnosis and treatment of cancer, a lot of patients can extend their lives.

The knowledge about the underlying mechanisms of cancer are still not very well understood, although cancer is a disease which was already found in ancient Egypt dated back to about 3000 BC[2]. In a copy of an ancient Egyptian textbook about trauma surgery, 8 cases of tumors of the breast were described which were removed in a surgery by cauterization. Already the Egyptians claimed that there is no treatment.

Hippocrates used the terms carcinos and carcinoma for the description of non-ulcer-forming and ulcer-forming tumors. Both of those words refer to a crab as the spreading projections from a cancer reminded him of the shape of a crab. Celsus, a romain physician translated the Greek word into the Latin word cancer. But also the Greek word oncos, which means swelling, was used to describe tumors[2]. Nowadays this term is for example used in the description of genes causing cancer, called oncogenes.

In 1761, Giovanni Morgagni of Padua was the first scientist who did autopsies and related the patient's illness to pathological findings after death[2]. Later on, John Hunter, a Scottish surgeon, proposed that it may be possible to cure some cancers by removing the tumor in a surgery. When anesthesia was developed, classical cancer operations as John Hunter suggested, evolved[2].

During the 19th century the modern microscopes developed which made it possible to study tissues from disease. Rudolf Virchow correlated microscopic pathology to diseases which did not only lead to a better understanding of cancer, but also helped in the development of cancer surgery. As it was possible to examine the removed body tissue under the microscope, more precise diagnosis could be made and it allowed telling the surgeon if the tumor was removed completely[2].

Already during the 17th and 18th century scientists were interested in understanding the causes, distribution and control of disease, called epidemiology. In 1620, Thomas Venner of London pointed out about the dangers of using tobacco in his “Via Recta”. In 1761John Hill wrote a book with the title “Cautions Against the Immoderate Use of Snuff”, these first observations linking tobacco and cancer led to the research in the 20th century where it was shown that smoking can lead to lung cancer[2]. Today, researchers still study molecular epidemiology by studying the impact and interactions of external factors on genes.

2.2. Hallmarks of cancer:

Although there are many different types of cancers, they all have in common that they develop from normal cells which accumulate mutations and become cancer cells. These mutations can be caused by different factors, both external influences i.e. caused by environmental factors such as UV light, as well as genetic factors are possible[3,4].

In order to understand the biology of cancer several hallmarks have been identified which enable tumor growth and metastatic dissemination[5]. Six core hallmarks have been identified which get acquired during the multistep development of tumors. These six core hallmarks allow cancer cells to survive, proliferate and disseminate which are some of the key functions of cancer. Progress in the last decades of research have added four other hallmarks which play an important role in cancer development, namely genome instability, inflammation, change in energy metabolism and escaping immune destruction.

Fig. 1: Different hallmarks of cancer

2.2.1. *Sustain proliferative signaling:*

The fundamental capability of cancer cells is that they can sustain chronic proliferation. Normal cells control growth promoting signals that control the entry and progression through the cell growth and division cycle and ensure a homeostasis of cell number[5]. As cancer cells can deregulate these signals, they are able to progress through the cell cycle and proliferate without control.

Cancer cells have several possibilities to keep their proliferative signals. First of all, cancers cells are able to produce growth factor ligands by themselves and respond to them via the expression of cognate receptors, which leads to an autocrine proliferative stimulation[6]. Moreover, cancer cells are able to stimulate normal cells, within the tumor-associated stroma, by sending signals to them in order that they supply the cancer cells with growth factors.

Thirdly, cancer cells may be independent of growth factors, as they can activate components of a signaling pathway operating downstream of these receptors[5]. In some human tumors it was identified that somatic mutations occur which lead to the activation of signaling pathways which are normally triggered by activated growth factor receptors. 40% of melanomas reveal mutations which affect the structure of the B-Raf protein leading to the activation of the MAP-kinase pathway[7].

2.2.2. Evading Growth Suppressors:

Cancer cells are not only able to sustain proliferative active by inducing positively acting growth-stimulatory signals, but they can also evade cell death programs which get induced by tumor suppressor genes. The most important tumor suppressor genes are retinoblastoma (Rb) and p53. Both of them are involved as central control nodes in the control of the cell cycle, by regulating the decision of cell proliferation or the activation of senescence or apoptosis pathways[5]. Defects in Rb pathway function, lead to the absence of a gatekeeper in the progression of the cell cycle and thus to uncontrolled cell proliferation. Rb receives its signals from extracellular as well as intracellular sources and decides in response whether a cell should proceed in cell proliferation or not. p53 responds to stress and abnormality signals from intracellular systems. Due to suboptimal levels in glucose, oxygenation, growth promoting signals or excessive damage to the genome, p53 can cause a halt in the cell cycle progression until the optimization of the different conditions[5]. If the damage cannot be repaired anymore, p53 can trigger pathways leading to apoptosis. As p53 is not only involved in apoptosis, but also in cell-cycle control and in maintaining of genetic stability, it is not surprising that p53 is mutated in about half of all human cancers[8].

2.2.3. Resisting cell death:

In order to sustain cell proliferation it is not only necessary to keep cells in the division, but also to keep them from committing suicide by apoptosis[8]. Apoptosis helps to maintain a normal balance between cell proliferation and cell loss in different tissues. But not only keeps apoptosis the balance between cell death and cell division, but also it is involved in cellular reaction to damage and changes and forms a natural barrier to

cancer development. Meaning that cells go into apoptosis when severe defect, for example massive DNA damage, occurs. The counterbalancing between pro- and antiapoptotic members of the Bcl.2 family triggers apoptosis. Antiapoptotic members of the Bcl-2 family inhibit apoptosis by binding to the proapototic members Bax and Bak[5].

Therefore it is a very important characteristic of cancer cells to avoid apoptosis. By means of avoiding cell death, it is not only possible for malignant cells to proliferate, but they can also accumulate more mutations which can lead to cancer[8].

2.2.4. <u>Enabling replicative immortality:</u>

Vertebrate cells have a limited potential of proliferation, meaning that when cells reach their limit of proliferation called Hayflick limit, they go into senescence, an irreversible entrance into a nonproliferative but viable state, or go into cell death[5,9].
However, in order to generate tumors, cancer cells need unlimited replicative potential. Telomeres, a repetitive DNA sequences and associated proteins are capping the ends of the chromosome. An enzyme called telomerase synthesizes and maintains these DNA sequences. In most normal human cells, except the germ lines and some stem cells, the gene coding for the catalytic subunit of the telomerase is switched off or only active to some extent. This results in telomeres that get shorter after each cell division in these cells. When the shortening of the telomeres reaches a certain point, a danger signal is generated which leads to the arrest of the cell cycle, resulting in senescence or cell death[8,10]. The telomerase, which is mostly inactive in normal human cells, is expressed in functionally significant levels in most immortalized cell lines, including human cancer cells. The highly active telomerase of cancer cell lines, leads to infinite cell division, as telomere erosion and the result in senescence or cell death do not occur[5].

That's why, the shortening of telomeres has become one of the most important machineries which control the limited replicative potential of normal cells and thus is one that must be overcome by cancer cells.

2.2.5. Inducing angiogenesis:

For cancer cells, as for normal tissues, the supply with nutrients and oxygen as well as the disposal of waste products is vital. Angiogenesis is the process which generates tumor-associated neovasculature which addresses these needs[5].
In normal tissues, the development of the vasculature involving the formation of new endothelial cells, their tube formation, called vasculogenesis and the sprouting of new vessels from existing ones, called angiogenesis, is active in embryogenesis and becomes largely quiescent after the morphogenesis. In adults, angiogenesis is only turned on in specific physiologic processes such as wound healing or the female reproductive cycling. But in tumor progression, an “angiogenic switch” is almost always activated, leading to continually sprouting of new vessels of normally quiescent vasculature which help sustain expanding neoplastic growths[5,11].
Not only is angiogenesis crucial for the tumor growth, but also the formation of metastasis depends on it[12].

2.2.6. Activating invasion and metastasis:

The ability of spreading to adjacent or distant organs makes cancer life threatening. Tumor cells have the ability to penetrate blood or lymphatic vessels, circulate through the intravascular stream and proliferate at other sites of the body. As already mentioned above, angiogenesis is necessary for the spreading of cancer tissue[12]. In order to be able to invade tissue and form metastasis, tumor cells have to alter their morphology by changing from a highly differentiated morphology to a migratory and invasive phenotype[13]. The change in morphology makes it possible for tumor cells to permeate the basal lamina and invade surrounding tissue. Due to the downregulation of specific adhesion molecules, e.g. E-cadherin, cell-cell contacts get lost and cell motility is gained[5]. This process is known as epithelial-mesenchymal transition (EMT) and gets induced by several growth factors which are produced either by the cancer cells themselves or by stromal cells, e.g. TGF-β, EGF, IGFs etc. [13].
Not only the expression of genes coding for cell-cell adhesion, but also the expression of genes coding for cell-to- ECM adhesion molecules gets changed, meaning the ones

favoring the inhibition of cell growth are down-regulated while the ones associated with cell migration occurring during embryogenesis or inflammation get up-regulated[5].

All these six hallmarks mentioned above are described as the core hallmarks of cancer which make it possible for cancer cells to survive, proliferate and disseminate. In the last years of research, four other hallmarks of cancer were added to the already existing six core hallmarks, namely genome instability, tumor promoting inflammation, reprogramming energy metabolism and the escape of immune destruction. Although the functions of proliferation, survival and dissemination get obtained in different tumor types through different mechanisms and various time points, two characteristics enable their acquisition. Namely the development of genome instability which lead to random mutations including also chromosomal rearrangement resulting in genetic changes which can orchestrate hallmark capabilities[5]. As well as the inflammatory state of premalignant and malignant lesions driven by the immune system, which sometimes serve promoting tumor progression.

2.2.7. Genome Instability and Mutation:

All the hallmarks mentioned above are often dependent on alterations in the genome of neoplastic cells, as some mutant genotypes result in selective advantage of subclonal cells giving them eventually dominance in certain tissue environment.
In normal cells, the extraordinary system of the genome maintenance system, allows the cell to detect and resolve defects in the DNA and ensures that spontaneous mutations are kept low during cell division. Cancer cells tend to accumulate more and more mutations with each cell division in order to acquire the mutant genes needed to facilitate tumorigenesis[4,5].
Several forms of genomic instability are known, but the most prominent one occurring in cancer is called chromosomal instability (CIN), referring to the high rate by which chromosome structure and number changes over time in cancer cells compared to normal cells. Another form of genomic instability described is microsatellite instability (MSI), a form of genomic instability that is characterized by the expansion or contraction of the number of oligonucleotide repeats present in microsatellite sequences[14].

2.2.8. Tumor-Promoting inflammation:

In the last years, studies have shown that chronic inflammation predisposes to different forms of cancer, for example inflammatory bowel disease can lead to colorectal cancer. But even cancers which are not related to inflammations, e.g. breast cancer build up an inflammatory microenvironment. Meaning that in most of neoplastic tissues an inflammatory component is present[15].

It is known that tumor-associated inflammatory response can enhance tumorigenesis and progression as immune cells, mostly of the innate immune system, have a functionally important tumor-promoting effect on neoplastic progression.
Furthermore, inflammation can contribute to many hallmark capabilities by supplying the tumor microenvironment with bioactive molecules, such as growth factors to sustain proliferative signaling, survival factors to escape cell death, proangiogenic factors and signals which lead to the activation of EMT resulting in invasion and the formation of metastasis[5]. The capability of releasing reactive oxygen species which are actively mutagenic facilitates cancer cells to accumulate genetic mutations in order to get highly malignant[16].

2.2.9. Reprogramming Energy Metabolism:

Over 80 years ago, it was already observed by Otto Warburg that cancer cells generate ATP through the glycolytic pathway rather than through the tricarboxylic acid (TCA) cycle, even in the presence of oxygen. This phenomenon is called “Warburg effect”[17].
The Warburg effect is a characteristic which can be found in most of malignant tumors independent of their carcinogenic origin[17,18].
Normally, when oxygen is present, most cells primarily metabolize glucose in glycolysis and then further oxidize it in the TCA cycle to carbon dioxide, whereby oxygen is the final acceptor in the electron transport chain. Through the generation of an electrochemical gradient the production of ATP is facilitated. During the TCA cycle a net gain of 34 molecules of ATP can be generated of one glucose molecule while during the process of glycolysis only 2 molecules of ATP per glucose molecule can be generated.

Of course, the difference of net gain between these two processes raised the question why cancer cells prefer glycolysis with a less efficient ATP production[17].

Due to the changes of cancer cells in cell proliferation also the energy metabolism needs to be adjusted in order to fuel cell growth and division. Therefore cancer cells reprogram their glucose metabolism and energy production by limiting it mostly to glycolysis, called “aerobic glycolysis”[5]. But still the functional reason for cancer cells preferring glycolysis with a low production of ATP must be explained. A recently refined hypothesis explains that an increase in glycolysis allow the distribution of glycolytic intermediates into several biosynthetic pathways, which generate nucleotides and amino acids which further facilitate the generation of macromolecules and organelles in order to build up new cells[19]. Therefore reprogramming the energy metabolism mostly to glycolysis allows cancer cells to proliferate more rapidly.

2.2.10. Evading Immune Destruction:

The immune system constantly controls and monitors cells and tissue in the body. Tumors can be recognized by the immune system and their proliferation can be stopped and controlled long term, a process which is known as immunosurveillance[20]. Due to the immunosurveillance the majority of abnormal cells gets recognized and eliminated, nevertheless, solid tumor cells must have managed to somehow circumvent the detection by the immune system or to limit the extent of immunological killing in order to survive[5]. Cancer cells have the ability to modify the immune regulation to their advantage. The change in the regulation leads to a prevention of tumor-antigen helper and cytotoxic T cells and a stimulation of the production of proinflammatory cytokines and other factors which results in the accumulation of cells in the tumor microenvironment that inhibit instead of promote immunity[20,21].

Once cancer has achieved in gaining all the functions mentioned, especially the function to form metastasis, it is nearly impossible to remove cancer, as the formation of metastasis allows cancer to spread throughout the whole body. The metastatic spread of primary tumors is responsible for approximately 90% of all cancer deaths, as the effect of surgery and radiation therapies decreases rapidly[13].

2.3. Melanoma:

Melanoma is the most aggressive form of skin cancer and the cases of melanoma have increased dramatically over the last years[22]. The highest number of cases recorded is in Queensland, Australia with about 56 cases per 100000 per year per men and 41 cases per 100000 per year for women. Not only in Australia a lot of melanoma cases are recorded, but among Europe a high number of cases was recorded in Switzerland, Austria and Norway[23]. Although the survival rate of melanoma patients has increased over the last decades, the prognosis of patients affected by metastatic melanoma is still low, with a 5 year survival of approximately 10% and an average survival of 6 to 9 months[24,25].
Metastatic melanoma caused about 7600 deaths in 2003[22].

Identifying the cause of melanoma is still difficult and requires investigations in mechanisms underlying melanoma. It is known that UV exposure and melanoma are causally related. Increased exposure to sun of pale white skin to natural UV radiation may also be the main reason for an increase in melanoma cases[23]. Melanoma risk depends not only on environmental influences, such as UV light, but also on genetic factors. 10% of cutaneous melanoma occurs in families where already other close relatives were affected.

But how does melanoma develop?

Melanoma can arise in every part of the body occupied by melanocytes.
Melanocytes are located in the basal layer of the epidermis, where they occur in a stable ratio with keratinocytes. The growth and behavior of melanocytes gets normally controlled by keratinocytes through a system of paracrine growth factors and cell-cell adhesion molecules. For the proliferation melanocytes need to decouple from the basement membrane and from the keratinocytes, divide, migrate along the basement membrane and re-couple again with the keratinocytes and the matrix[26]. When melanoma develops, changes in this system need to be done in order to sustain uncontrolled proliferative active. The changes lead to altered expression of cell to cell adhesion molecules and to a change in cell communication molecules favoring the

development of melanoma. Melanoma cells escape the control system of keratinocytes through different mechanisms. First of all, melanoma cells down-regulate receptors, such as E-cadherin, which are important for the cell communication with keratinocytes. The expression of signaling molecules and receptors important for melanoma-melanoma interactions get up-regulated and a change of the extracellular matrix (ECM) leads to the loss of binding to the basement membrane[26].

Although cutaneous melanoma, developed from epidermal melanocytes of the skin, represents the most common site of the formation of melanoma, noncutaneous melanoma, arising in the choroidal layer of the eye, respiratory system, gastrointestinal system or the genitourinary mucosal surface may occur. Therefore melanoma is divided into several subgroups, including superficial spreading melanoma, nodular melanoma, acral lentiginous melanoma and lentigo maligna melanoma[27].

For the development of melanoma, mutations in several genes need to occur. The most famous gene mutations in cutaneous melanoma are BRAF, NRAS, PTEN and CDKN2A. The mutations of the relevant genes have an impact on several signaling transduction pathways including the RAS/RAF/MEK/ERK mitogen-activated protein (MAP) kinase pathway and the phopshoinositol 3 kinase (PI3K/AKT) pathway[28].

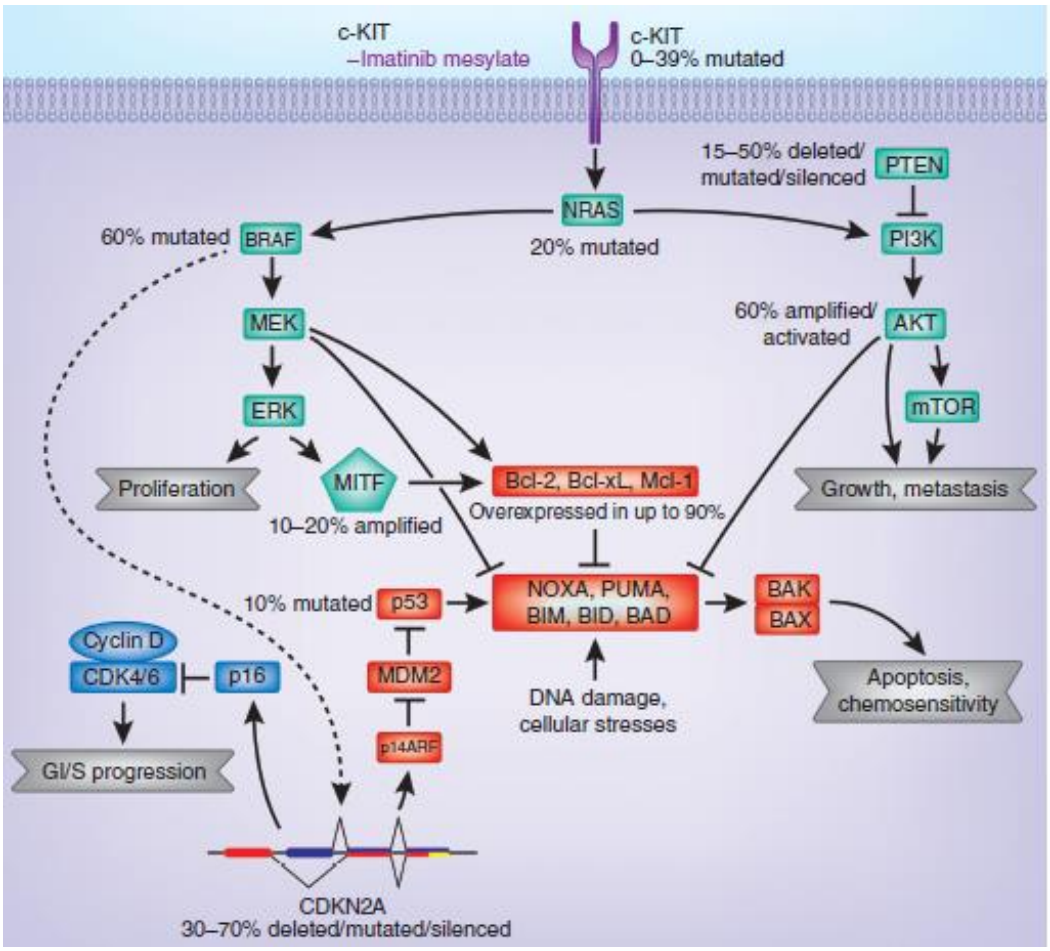


Fig. 2: Signaling networks in melanoma: Illustrating the 3 major genetic networks involved in melanoma tuorigenesis, survival and senescence. Shown are the NRAS network (green) which has an influence on the MAPK and the PI3Kinase/AKT pathways, which are important for melanoma survival and proliferation. The CDKN2A locus encoding two separate tumor suppressors (p16 and p14ARF) which play a major role in senescence and tumor growth restriction. The Bcl-2 signaling network (red) which contributes to melanoma apoptosis and chemosensitivity which gets regulated by the oncogenic melanoma pathways.

The complex network of RAS regulates cell growth, invasion and survival through two distinct signaling pathways, namely the Ras mitogen-activated protein kinase (MAPK) and the Ras/phosphatidylinositol-3-kinase (PI3K) signaling pathway[28]. Alterations in the RAS signaling cascade are therefore almost uniformly present in melanoma. Mostly not RAS itself is mutated, but in up to 90% of melanomas active mutations in genes lying downstream of RAS cascade occur. The most frequent mutations occur in BRAF on the MAPK pathway and PTEN or NRAS lying on the PI3/AKT pathway[29,30]. In general, melanoma carries a mutated NRAS, a mutated BRAF or concurrent BRAF and PTEN mutations [31].

2.3.1. NRAS:

One third of cancers carry mutations in the three rat sarcoma (RAS) family members HRAS, KRAS or NRAS. All RAS proteins are molecular switches mediating signals from ligand activated receptor tyrosine kinases (RTK) to the nucleus through downstreaming cascades. NRAS, KRAS and HRAS share similar functions, based on their distinctive subcellular localization the predominance of certain RAS mutations in different cancer types may be explained.

NRAS, the predominant mutated RAS isoform in melanoma, was among the first oncogenes discovered to be mutated in melanoma and is mutated in about 20% of melanoma, causing changes in signaling in several downstream cascades [32].

Therefore NRAS is accepted as a key oncogene in human melanoma. The most common mutation occurs at codon 61 in Glutamine, substitutions at this codon damage the enzymatic activity of RAS to cleave GTP into GDP. Cleavage of GTP into GDP would normally result in terminating the downstream growth. As NRAS remains in its active, GTP-bound state, it drives cell proliferation, survival and motility of melanoma shown in Fig.3 [32–34]. NRAS can activate both the MAPK as well as the PI3K pathway. In the MAPK pathway, NRAS mutations lead to the activation of CRAF instead of BRAF leading to a dysregulation of cAMP signaling and allowing CRAF to stimulate MEK which further signals to ERK and induces uncontrolled proliferation [34]. Mutations in NRAS lead also to the constitutive activation of the PI3K pathway through the activation of AKT which results in cell survival.

It is known that mutations of BRAF and NRAS occur mutually exclusive. In contrast to BRAF mutations, NRAS mutations occur also mutually exclusive of changes in PTEN, as NRAS alone can activate both MAPK and PI3K pathways [34].

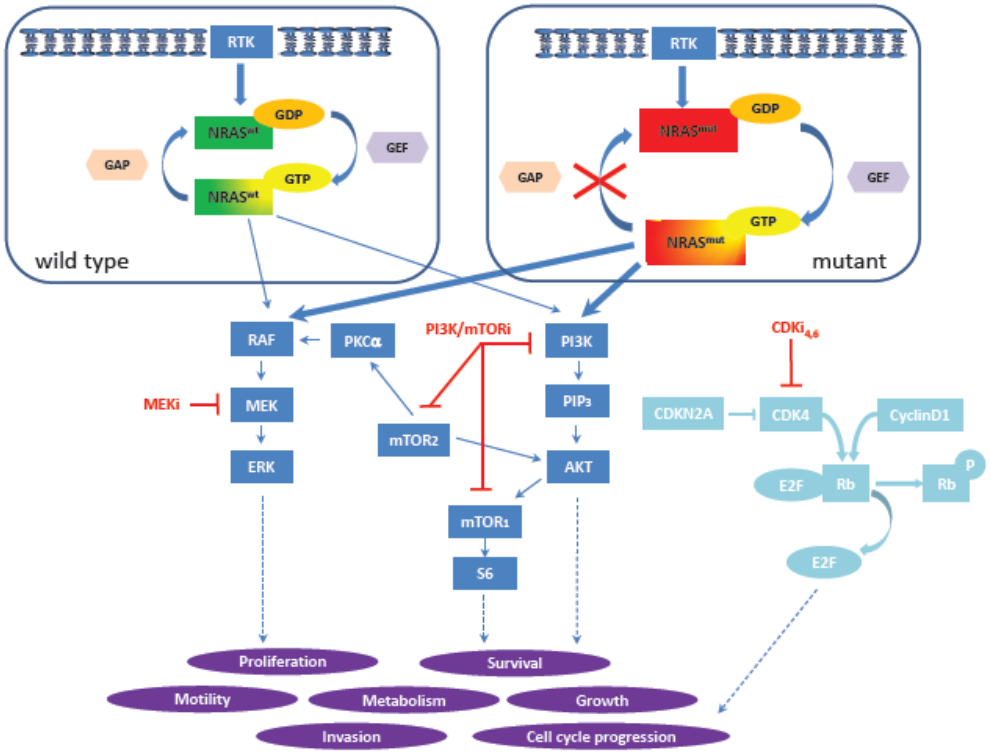


Fig. 3: Model of NRAS signaling in melanoma: Wild type NRAS (top left) cycles between it's in acitve GDP-bound and it's active GTP-bound state. Mutations in NRAS (top right) lead to the blockage of NRAS in it's active GTP-bound state resulting in continous independent downstream signaling in MAPK and Pi3K pathway leading to permanent cell proliferation and survival.

2.3.2. BRAF:

BRAF is a serine/threonine kinase activating the MAPK /ERK signaling pathway. Although BRAF is only mutated in about 20% of most cancers, it is mutated in around 60% of all melanomas and therefore BRAF is the most commonly mutated gene in melanoma [29].

In about 90% of melanoma with BRAF mutation, the BRAF mutation occurs at codon 600, over 90% of those mutations result in the substitution from glutamic acid to valine in a single nucleotide change, called BRAF ^{V600E} [35,36].

80% of benign nevi carry also a BRAF mutation which suggests that a BRAF mutation alone is not sufficient to induce the formation of melanoma. BRAF mutation alone has been shown to result in a senescence-like state. Therefore, the full oncogenic potential

of a BRAF mutation seems to be dependent on the presence or absence of other genes, most often by the cooperation with a mutation or loss in PTEN [29,31].

If a BRAF mutation leads to the formation of melanoma, it leads to its constitutive activation and therefore to the constitutive activation of the extracellular-regulated kinase (ERK) of the MAPK pathway [37]. The activation of BRAF leads to the phosphorylation of MEK which in turn activates ERK by phosphorylation. Activation of ERK leads to the promotion of cell proliferation and to the inhibition of apoptosis, which results in the formation of melanoma [35]. Furthermore, mutations in BRAF lead to angiogenesis through the activation of HIF-1α, which is dependent on MEK, and VEGF and result in the formation of metastasis as well as the evasion of immune response through the up-regulation of proteins involved in migration, cell contractility and tumor- and microenvironment-derived interleukin 8 [36].

As already mentioned before a mutation in BRAF alone normally does not lead to the development of melanoma. Studies suggested that PTEN, a tumor suppressor gene, is often mutated or lost in melanoma and cooperates with a BRAF mutation [31].
PTEN encodes a protein that is involved in the control of apoptosis through the PI3K pathway. PTEN can lead to the termination of the PI3K pathway, to the up-regulation of cell-cycle arrest and expression of proapoptotic proteins, as well as to the down-regulation of antiapoptotic proteins of the Bcl2-family [29]. Studies support the importance of PTEN in melanoma as it serves as a critical player in the activation of PI3K, in melanoma lacking a mutation in NRAS. As PTEN and NRAS both lead to the activation of the PI3K pathway, they may be functionally equivalent. Several studies have shown that either a mutation in NRAS alone, about 19%, a mutation in BRAF alone, about 60% or a concurrent PTEN loss and BRAF mutation, about 80%, occur in melanoma [29,31].
The expression of PTEN is decreased or lost in about 15-50% of melanoma and occurs almost always with a BRAF mutation.
The co-occurrence of a mutation in BRAF and a loss of PTEN, demonstrates the importance of the coactivation of the MAPK and the PI3K pathway in melanoma development [29].

2.3.3. CDKN2A:

10% of cutaneous melanoma cases occur due to a family history of melanoma which would implicate that melanoma is a genetic disease. Hereditary melanoma is often associated with multiple cases of melanoma in a family, multiple primary melanomas in one individual or an early onset of the disease.
In hereditary melanoma often the locus CDKN2A (cyclin-dependent kinase inhibitor 2A), in about 40% of family melanoma cases, is involved. CDKN2A encodes two separate tumor suppressor genes, namely p16I^{NK4a} and p14^{ARF} , which play a major role in the regulation of cell cycle and apoptosis [27,29,38].
p16^{INK4a} binds to and inhibits CDK4/6 in G1 phase of the cell cycle, which prevents the phosphorylation of Rb protein by CDK4/6. Hyperphosphorylation of Rb would lead to the release of E2F1, a transcriptional inducer of G1 to S-phase transition. Loss of the p16^{INK4a} protein triggers hyperphosphorylation of Rb and the resulting release of E2F1 encouraging the G1 to S-phase transition and the re-entry of the cell cycle.
p14^{ARF} binds to the HDM2 protein at the N terminal and helps to degrade the HDM2 protein rapidly. HDM2 is a protein which phosphorylates p53 for ubiquitination leading to the destruction of p53. When p14^{ARF} is lost, destabilization and ubiqitination of p53 occurs and results in the loss of DNA damage response [27].

Studies have shown that the risk in a mutation in p16^{INK4a} is higher than in p14^{ARF} [38].
The risk of developing cutaneous melanoma among CKDN2A mutation carriers has been analyzed and in family cases it has been shown that at the age of 50 the risk of developing cutaneous melanoma is about 30%. With an increase in age, also the risk in developing melanoma rises and at an age of 80 the risk lies already at about 67%. In countries with sunnier climes the risk is even higher, for example in Australia the risk at age 80 is about 91%, while in Europe the risk is about 58% at the same age [39].

Melanoma is a difficult cancer to treat, given the fact that this type of cancer is resistant to standard chemotherapy. The consensus in dealing with melanoma is early detection and “personalized” therapy using molecular techniques to identify specific mutations within each patient.
Ongoing research focuses on the identification of molecules acting as markers for

melanoma progression which allow predictions of survival rates. Based on immunohistochemistry multimarker assays the different expression of these markers can be analyzed and allows the diagnosis and prognosis at the molecular level of melanoma with providing information about the survival chances of patients[40].

Our project, therefore, focuses on the understanding of a novel, so far unknown gene in melanoma, called nuclear receptor coactivator-3, short NCOA3.

2.4. NCOA3:

NCOA3 is a member of the p160 SCR family (SRC1, SCR2 and SCR3= NCOA3). NCOA3 occurs under a lot of different names, for example SCR3; AIB1, p/CIP, RAC3, ACTR and TRAM1 [41]. All three of the steroid receptor co-activators mediate transcriptional functions of nuclear receptors and other transcriptional factors. Several studies have shown that the SRC genes are often amplified or overexpressed in various human cancers [42]. The focus of investigating the role of NCOA3 lies especially on human breast cancer, and is very well understood in this field.
SRC genes in general contain three structural domains, a helix-loop-helix domain which is required for the protein-protein interaction and can also interact with several transcription factors. The central region three LXXLL (in which X is any amino acid) motifs, this region is responsible for the interaction with nuclear hormone receptors (NRs).The carboxyl terminus contains two transcriptional activation domains, called AD1 and AD2. AD1 is responsible for the binding of the CREB-binding protein and also contains the histone acetyltransferase p300, both of them get recruited by chromatin and are essential for the transcriptional activation mediated by the SRCs. The role of AD2 is to interact with different histone methyltransferase [42,43]. The HAT activity domain at the C terminal, allows NCOA3 to recruit additional coregulators and general transcription factors, which results in chromatin remodeling [42,44]. As NCOA3 is also a co-activator of other transcriptional factors, it has also an influence on NF-κB, Smads, E2F1, STATs, HIF1, p53 and Rb [42,45]. The activity of SRCs is dependent on their posttranslational modifications, such as phosphorylation, ubiquitylation, acetylation and methylation, and have crucial roles in the protein stability and the transcriptional activity

of SRCs. Deregulation in posttranslational modifications can lead to the development of cancer [42].

Especially in breast cancer, the role of NCOA3, often found as AIB1, has been investigated a lot. Human breast cancer is known to be promoted by enhanced activity of the estrogen receptor (ER) pathway. Studies have shown that estrogens can directly cause proliferation of breast cancer [45]. It has been found that NCOA3 amplification in breast cancer can be correlated with estrogen and progesterone receptor positivity and tumor size [45]. These findings suggest that amplification and overexpression of NCOA3 is a contributing factor promoting ER-dependent signaling in the mammary gland and in breast cancer [41,43,45,46]. This also provides further evidence of NCOA3 being an oncogene in breast cancer as it has an impact on cell proliferation, apoptosis and tumor growth [43,47].
Not only is NCOA3 known to play an important role in breast cancer, but also in several other cancers, such as ovarian cancer, lung cancer, prostate cancer and pancreatic cancer [46,48–51].

It is known that NCOA3 has an effect on the cell cycle in breast cancer.
The cell cycle is a precisely programmed series of events that is controlled by the cell cycle clock and allows cells to duplicate. The different steps of the cell cycle are controlled by changing the levels and availability of cyclins. Cyclins control the cyclin dependent kinases (CDKs) and activate them by activating their catalytic subunit. CDK inhibitors provide a further control in the cell cycle. The levels of D cyclins are controlled by extracellular signals. Once the D cyclins are activated and late G1 phase is reached, the remaining cyclins get activated in a preordained schedule. Cell cycle checkpoints are another protective mechanism in the cell cycle machinery and ensure that the transition from one phase to another is only accomplished when the preceding step has been completed correctly and the genome is not damaged. In several cancer types, at least one cell cycle checkpoint is inactivated, in order to accumulate more mutations and alter the genome so that it favors tumor growth. In most of cancers the restriction point (R point) is altered as this is the point where the decision is made if a cell should proceed the cell cycle or should stay in G1 phase and go into the quiescent state. G_0.

The R point is controlled by the retinoblastoma protein which in turn is controlled by D and E cyclins. Hypophosphorylation of pRb blocks the passage into S phase, while hyperphosphorylation of pRb permits the transition through the R point. Moreover, hyperphosphorylation of pRb leads to the release of E2F transcription factors associated with promoters of genes that allow the transition of cells from late G1 into S phase [52].

Researchers focused on the identification of the role of NCOA3 in the control of breast cancer cell proliferation and found that NCOA3 directly interacts with E2F1. Overexpression of NCOA3 in breast cancer up-regulates several key cell cycle genes, such as genes for cyclin E1, cyclin A2, CDK2 and E2F1. In the study it is suggested that endogenous NCOA3 directly controls the expression of genes critical for the initiation of DNA replication and is needed for G1 into S phase transition [53].

A recently published article suggested that defects in cell cycle checkpoint may act as drivers in melanoma progression [54]. The control of cell cycle checkpoints is tightly associated with repair mechanisms, such as DNA damage response. The importance of DNA damage response in several cancers has been long recognized. DNA damage can occur due to different reason, through environmental factors, such as UV, but also through internal stressors, such as oxygen radicals or replication and recombination errors. Normally, DNA damage gets repaired by specific repair mechanisms, for example DNA damage by UV light gets repaired by nucleotide excision dimers (NER) [54]. In order to be able to complete the reparation of the damage, normal cellular responses lead to cell cycle arrest because of the activation of different cell cycle checkpoints. This allows time to repair the damage or if the damage is too severe it leads to the activation of senescence or cell death. In melanoma, as well as in several other cancers, the DNA damage response system is often altered in order to allow the cells to proliferate without control and accumulate more mutations. The occurrence of high levels of UV signature mutations in melanoma is not a rare event and suggests that the repair system of UV light induced mutations in melanoma must be defective. Until now it was not possible for researchers to identify a mutation in known DNA repair genes that explains the high levels of UV induced mutations in melanoma. A major unresolved question in

developing melanoma is how the UV induced DNA damage which leads to mutations can escape the DNA repair system [54].

Therefore our research focused on the understanding of the role of NCOA3 in melanoma, as our group suggests the involvement of NCOA3 in DNA damage repair as well as in the regulation of cell cycle checkpoints.

3. Aim of the thesis:

The goal of the research focusing on NCOA3 will be to better understand the role of NCOA3 in melanoma. Thereby the potential of NCOA3 as a novel biomarker for melanoma will be tested, as the hypothesis suggests. Furthermore, the effect of NCOA3 on cell proliferation and the formation of metastasis will be tested as some of the patient's data suggests.

As several recently conducted experiments suggest the involvement of NCOA3 in the DNA repair, special focus lies on the identification of the role of NCOA3 in cell cycle progression and the regulation of cell cycle checkpoints, as DNA repair and cell cycle are two tightly associated mechanisms. The aim is to find out which cell cycle checkpoint gets inactivated due to the overexpression of NCOA3 or activated due to the knockdown of NCOA3, by analyzing the involvement of several cell cycle checkpoint proteins by using quantitative real-time PCR and western blotting. This will allow to predict which cell death program is activated, namely apoptosis, senescence or cell cycle arrest.

All these experiments are conducted on cell lines, primary and metastatic melanoma cell lines, with reduced expression of the gene NCOA3 and are compared to the same cell lines with physiological expression of NCOA3, in order to see if there will be a significant difference in susceptibility which could be used as potential target for future melanoma treatments.

As it is already known that NCOA3 plays an important role in the development of breast cancer, it is believed that NCOA3 has also a major impact on melanoma. In some publications it is already suggested that NCOA3 may be used as a prognostic biomarker for melanoma patients, which can predict the survival chances of individual patients [40]. The experiments explained above should show that NCOA3 is an important biomarker in melanoma and may aid in developing new therapies due to its function in DNA damage response signaling associated with its role in the regulation of the cell cycle.

4. Material and Methods:

4.1. Lentiviral infection:

Cells were infected with a lentivirus containing shRNA targeting NCOA3 for the NCOA3 knockdown cell line or a Lentivirus targeting Luciferase as a control.
Cells were plated on a 10cm petri dish and grown until they were confluent. Media was aspirated and the cells were washed with PBS. 9 mL of media containing 8µg/ml of polybrene and inactivated FBS were added to the plate. 1mL of lentivirus containing the desired shRNA was added to the plate. The cells were incubated with the Lentivirus overnight and then the medium was replaced with medium containing the regular amount of serum. After 72hours, selection with puromycin was started.Puromycin was used for selection, to check if the cells got infected with the virus, as the puromycin N-acetyl-transferase (PAC) gene encoding the antibiotic puromycin was introduced into the plasmid for selection. Allow 2-3 days for puromycin selection, which is depended on the concentration and cell type.
For the infection shRNA targeting Luciferase was used to generate a control cell line, which means that nothing specific was targeted in the cell line as the cell line does not express the Luciferase gene. For the generation of a knockdown in NCOA3, an shRNA with the most efficient potential of knocking down NCOA3 was used, in this case shRNA 19703.

4.2. RNA extraction:

RNA extraction was performed in order to test the generated control cell line and the cell line with knockdown of the gene NCOA3 in the level of knockdown. The RNA extraction was performed using the RNeasy Mini Kit (Qiagen) following the manufacturer's instructions. The concentration and quality of RNA were determined by using the NanoDrop 2000 (ThermoFisher).

4.3. cDNA sysnthesis:

"Maxima First strand cDNA synthesis Kit for RT-qPCR" (ThermoFisher) was used for generating cDNA from high quality RNA.

Tbl. 1: Reagents and corresponding amounts for cDNA synthesis

Reagent	Amount
Reaction Buffer Mix	2 µl
Reverse Transcriptase Enzyme	1 µl
RNA	Volume corresponding to 500 ng of RNA
Nuclease-free water	Amount resulting in 10µl total Volume
	Total Volume = 10µl

Tbl. 2: Reaction steps used for cDNA synthesis

Temperature [ºC]	Time [min]
25	10
50	15
85	5

4.4. Realtime-PCR (TaqMan):

By using the TaqMan Gene expression Assays in accordance with the manufacturer's instructions and the 7500 Fast Real-Time PCR System (LifeTech) mRNAs were assayed. TaqMan probes for NCOA3, HPRT1, CCDN1, CCBN1, CCBN2 and CCAN1 were purchased from LifeTech.
The expression level of each gene was normalized to the human *HPRT1* gene before comparing. Runs were performed in triplicates in a 96-well optical plate.

Experiments were only proceeded with a knockdown of NCOA3 of 70% or higher.

Tbl. 3: Reagents and corresponding amounts used for TaqMan

Reagent	Amount
Fast Universal PCR Master Mix	17.8 µl
dH_2O	10.7 µl
cDNA (diluted 1:5)	5.5 µl
TaqMan probe	1.7 µl

Tbl. 4: Different steps of TaqMan procedure with corresponding temperatures and times

Temperature [ºC]	Time [s]	
95	20	
95	3	40 cycles
60	30	

4.5. Flow cytometry:

To determine the effect of NCOA3 on the cell cycle, flow cytometry was used. By means of Flow cytometry information on possible phase blocks and pathways leading to cell cycle arrest, senescence or apoptosis could be gathered.

4.5.1. Caspase 3/7:

Cells were counted in order to plate out 700 000 cells on starvation, meaning without the addition of serum to the media. They were incubated without serum overnight in order to synchronize the NCOA3 knockdown cell line with the control cell line. The cells were pelleted and washed with PBS and centrifuged. The pellet was resuspended in 1x Assay Buffer.

The User Guide “Muse Caspase-3/7 Kit User Guide” (Millipore) was used for further processing of the experiment[55].

4.5.2. Cell cycle analysis:

Cells were harvested, including the media containing the dead cells. 1mio cells were pelleted and washed with PBS and centrifuged again. The supernatant was aspirate until approximately 200 µL of PBS remained. To loosen the pellet 5ml of icecold 80% Ethanol were added dropwise while vortexing, in order to make the cells permeable, and incubated for at least 2 hours at -20°C. The cells were washed with 1x PBS and with staining buffer (1x PBS, 2% FBS mixture) to remove the ethanol. The cells were centrifuged for 3 minutes at 1300 rpm and the supernatant was aspirated. 1mio cells were resuspended in 500 µL PI/RNase Staining buffer (BD Pharmingen). The stained cells were incubated for 15 minutes at room temperature kept in darkness. By means of the Flow Cytometer (FACS) and the FlowJo (flow cytometer analysis program) the cells were analyzed.

4.6. Western blot:

Proteins were extracted by scratching the cells of 10cm culture dish by using the RIPA Lysis buffer (Santa Cruz Biotechnology), while keeping the culture dish on ice. The concentration of protein was quantified by performing a Bradford Assay using the BioRad Protein Assay Kit (Biorad) and a 96-well microplate reader.

Suitable separating- and stacking gels were prepared according to the size of the proteins of interest. In the case of PARP, a 10% separating gel and a 4% stacking gel were prepared. The surface of the separating gel was covered with n-Butanol during the polymerization process in order to prevent the gel from drying. The protein samples were prepared to a suitable final concentration. In the case of PARP, samples were prepared with a protein concentration of 10µg in 10µl and were mixed with the Laemmli sample buffer (Biorad). The samples were incubated at 65°C for 10 minutes and loaded on the gel. A SDS page was performed at 120V while keeping it on ice.
A semi dry transfer was performed at 25V for 25 minutes.
The membrane was blocked in 5% w/v nonfat dry milk, 1X TBS and 0.1% Tween-20 at room temperature for 30 minutes. The primary antibody was added at a concentration suitable for each antibody, PARP 1:200 (Santa Cruz Biotechnology) and betaActin

1:2000 (Cell Signaling) diluted in 5% w/v nonfat dry milk, 1X TBS, 0.1% Tween-20 and incubated over night at 4°C while gently shaking.
After the incubation of the primary antibody, the membrane was washed in 1X TBST 3 times for 5 minutes.
The membrane was incubated with the respective horseradish peroxidase-labeled secondary antibody diluted in 1XTBST at a concentration suitable for the antibody for 1 hour at room temperature.
After the incubation of the secondary antibody, the membrane was washed in 1X TBST 3 times for 10 minutes.
The binding was detected by using the ImmunoCruz Western Blotting Luminol Reagent (Santa Cruz Biotechnology) and a X-ray film developer.

4.7. Senescence:

Cells were plated on a cover slip in a 6-well plate and incubate overnight. The growth medium was aspirated. Cells were washed twice with 1ml of 1X PBS, then 1,5ml 1X Fixation Buffer were added and incubated at room temperature for 6-7minutes. The cells were rinsed with 1ml of 1X PBS 3 times. 1ml of Staining Mixture were added per well and incubate at 37°C without CO_2 until the cells were stained blue. After the staining, the cells were mounted on a microscope slide with citifluor and observed under the microscope.

Performance of analysis of senescence was done according to “ProductInformation” protocol from “Senescence Cells Histochemical Staining Kit” (Sigma-Aldrich)[56].

4.8. Colony formation:

A suitable number of cells, in case of C8161.9 and DO4 500 cells, were plated per triplicate in a 6-well plate and grown for an appropriate time period, in case of C8161.9 7 days and DO4 were grown for 10 days. After the formation of colonies, the cells were fixed and stained with crystal violet. The number colonies formed was counted by using ImageJ.

4.9. Testing of mutations in p53:

PCR:

Samples for PCR were prepared according to the table 5 below. Table 6 shows the steps of the PCR performed.

Tbl. 5: Reagents and corresponding amounts used for performing the PCR

Reagent	Amount
DNA	1 µl
Forward primer [10µM]	1 µl
Reverse primer [10µM]	1 µl
PCR SuperMix High Fidelity (LifeTechnologies)	19 µl

Tbl. 6: Different cycles of the PCR performed

1 cycle	35 cycles			1 cycle	
95°C	95°C	58°C	72°C	72°C	10°C
5 min	0:30 min	0:45 min	1:30 min	10 min	∞

Electrophoresis:

A 2% agarose gel was prepared. 5µl of the PCR samples were mixed with 1µl of an intercalating agent and loaded on the gel. An electrophoresis was performed for 30 minutes at 100V. The gel was analyzed on a GelDoc.

5. Results and Discussion:

5.1. Testing the cell lines for mutations in p53:

It has been shown that the expression of NCOA3 can be used to predict the outcome of melanoma in patients. Therefore cells with a high NCOA3 expression were clonally selected, as it gives them a proliferative advantage. Since p53 is an important cell cycle protein, it was necessary to determine the mutation status of TP53 in cell lines which were used for experiments in this study. p53 is not only involved in the regulation of apoptosis, but also in cell cycle regulation, including DNA repair. In the regulation of the cell cycle, p53 mediates a G1 cell cycle checkpoint in response to ionizing radiation (IR) damage. Human cancers which have a mutated p53, loose this G1 cell cycle checkpoint and fail to arrest cell cycle progression after IR damage[57]. Furthermore, p53 is involved in the DNA repair pathway as it helps to provide more time for DNA repair prior to the S phase entry.

By preventing cell cycle advance and DNA replication while chromosomal DNA is damaged and by inducing expression of DNA repair enzymes, p53 can reduce the rate at which mutations accumulate in cellular genomes. Conversely, cells that have lost p53 function may proceed to replicate damaged, still carrying unrepaired DNA. This can cause them to exhibit relatively mutable genomes that accumulate mutations at an abnormally high rate per cell generation.

If the used cell lines, DO4 and C8161.9, would carry a mutation in p53, none of the conducted experiments would be trustworthy, as mutations in p53 has an influence on DNA damage response, on cell cycle and on cell cycle arrest resulting in cell death programs. Therefore the cell lines were tested for mutations in p53 on different exons, namely exon 5, 6, 7 and 8 as it is known that these exons carry most of the p53 mutations[58].

As it can be seen in table 7 below, both of the cell lines, DO4 and C8161.9, show wildtype sequences in p53 in all exons, namely exons 5-8, which means that no

mutation was found in p53 indicating that the obtained results are not influenced by a mutation in p53.

Tbl. 7: Analysis of mutations in p53 in different cell lines, DO4 and C8161.9, on different exons, exons 5-8, which are the most dominant ones carrying mutations in p53. All the different exons testes in both cell lines show p53 wildtype.

Cell line	**Exon 5**	**Exon 6**	**Exon 7**	**Exon 8**
DO4	WT	WT	WT	WT
C8161.9	WT	WT	WT	WT

5.2. Generation of NCOA3 knockdown:

For the performance of the experiments, two melanoma cell lines were used, DO4, a primary melanoma cell line, and C8161.9 a highly metastatic melanoma cell line. In order to determine the role of NCOA3 in melanoma, a knockdown of NCOA3 in the different cell lines needed to be generated. Therefore, both cell lines were infected with a lentivirus containing shRNA targeting NCOA3 (anti-NCOA3 shRNA, called 19703). In order to generate a control cell line for comparison purposes, a lentivirus containing shRNA targeting Luciferase was used (the control group is therefore referred to as LUC).

RNA was extracted of the cell lines and further experiments were only conducted with a knockdown of NCOA3 above 70%.

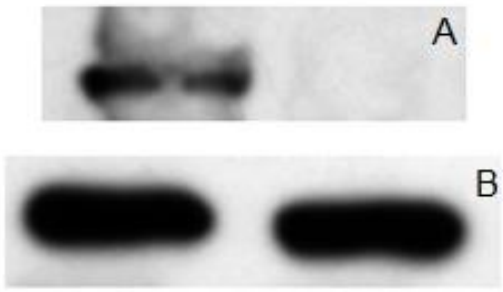


Fig. 4: (A) Western blot comparing the expression level of NCOA3 in DO4 LUC and DO4 19703 with (B) showing the corresponding expression levels of βActin as a loading control

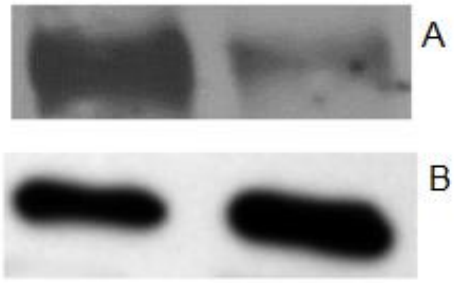


Fig. 5: (A) Western blot comparing the expression level of NCOA3 in C8161.9 LUC and C8161.9 19703 with (B) showing the corresponding expression levels of βActin as a loading control

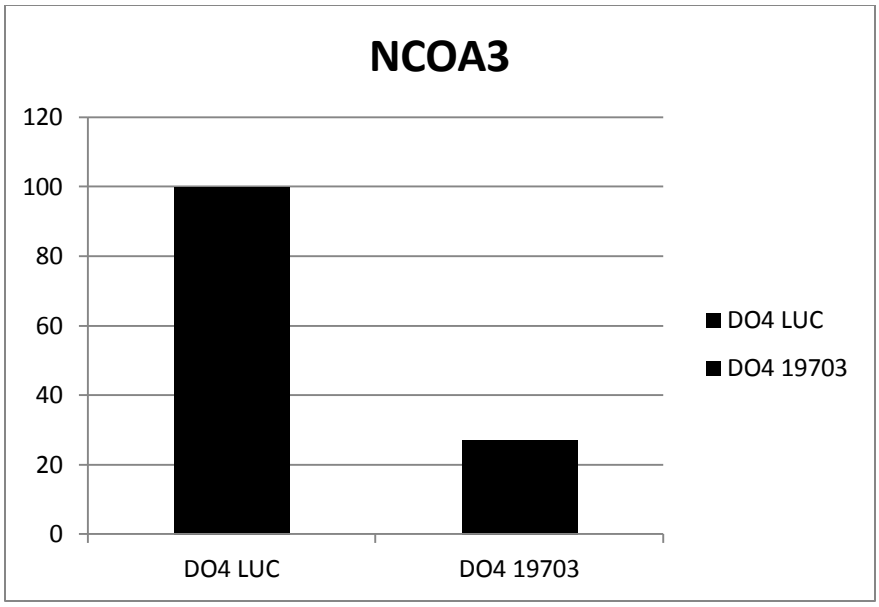


Fig. 4: Expression of NCOA3, analysed by qRT-PCR, in DO4 stably infected with anti-NCOA3 shRNA compared to vector encoding anti-luc shRNA.

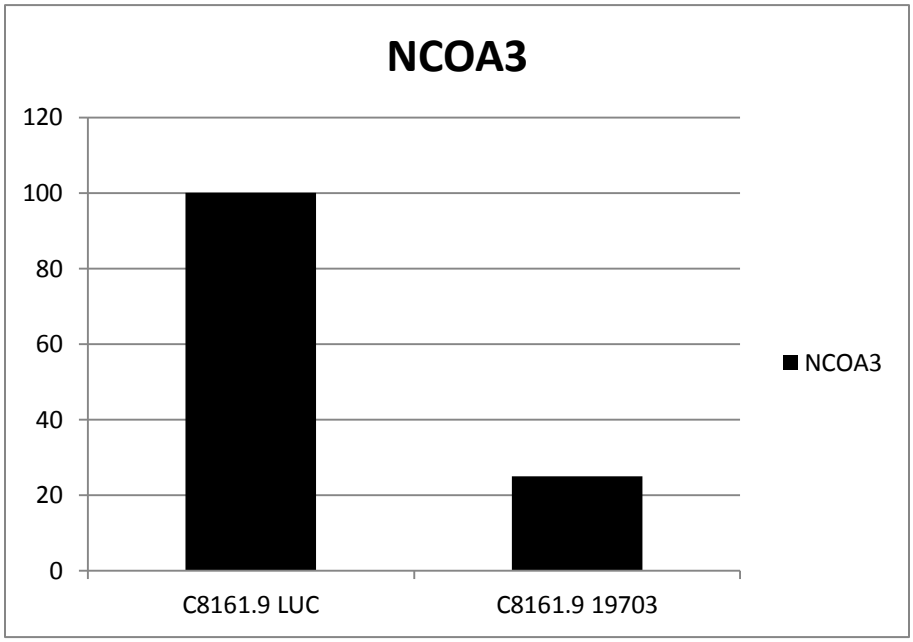


Fig. 5: Expression of NCOA3, analysed by qRT-PCR, in C8161.9 stably infected with anti-NCOA3 shRNA compared to vector encoding anti-luc shRNA..

Due to the changes in expression levels of NCOA3 in the cell lines, also changes in morphology occur, see Fig.8 below.

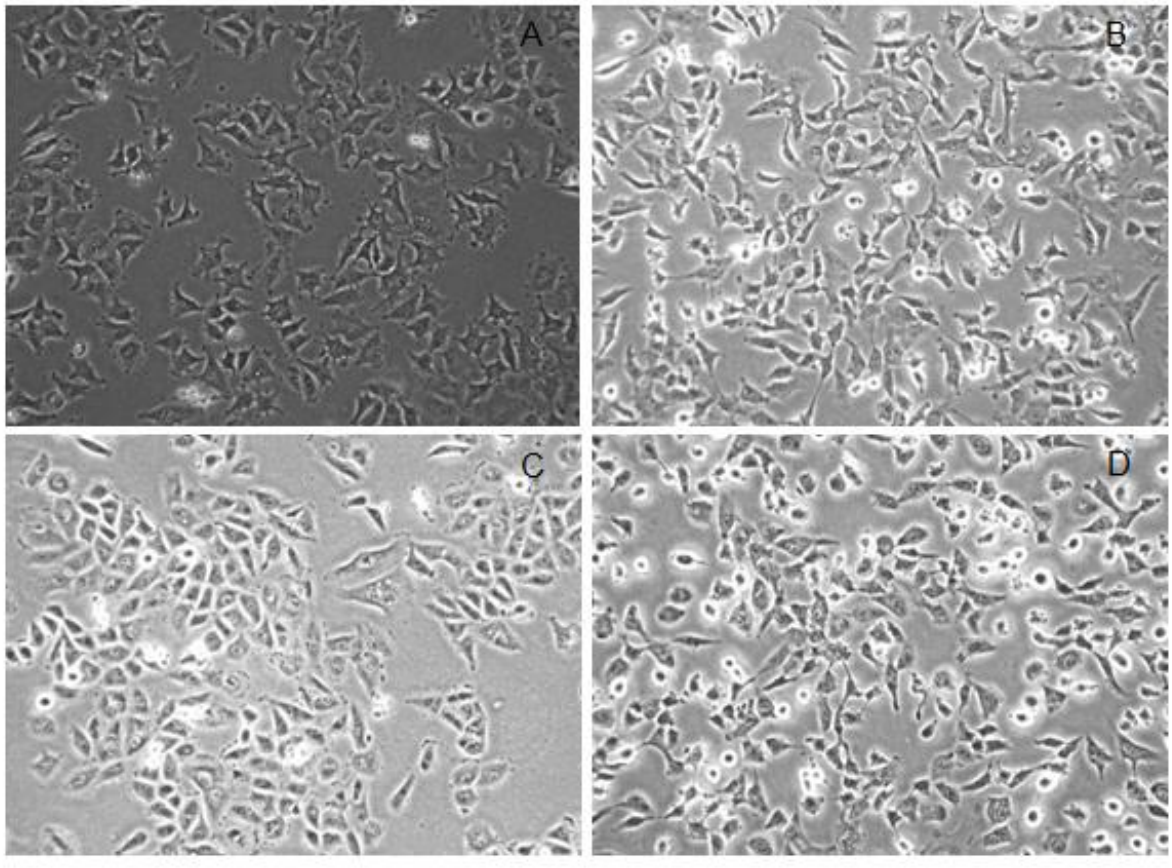


Fig. 6: The change of morphology can be seen in these pictures. (A) shows the cell morphology of DO4 LUC, while (C) shows the cell morphology of DO4 19703. It can be observed that the morphology changes from a star-shaped and larger form in DO4 LUC to a rounder and smaller shape in DO4 19703, when knocking down NCOA3. (B) shows the morphology of C8161.9 LUC and (D) shows the morphology of C8161.9 19703. Also in comparison of C8161.9 LUC to C8161.9 19703 a change in shape can be seen, although the change is not as clear as in DO4.

5.3. Colony formation:

To study the effect of NCOA3 on cell proliferation in melanoma, a colony formation assay was performed. A suitable number of cells of the control group, called LUC, and of the same cell line with reduced NCOA3, called 19703, was plated in a 6 well plate. The cells were grown for several days and the number of colonies formed of LUC was compared to the number of colonies formed by the cells with reduced NCOA3.

As Fig.9 below shows, a significant difference in the formation of colonies can be seen in the cell line DO4. The mean of the colonies formed by DO4 LUC was 117,3 ± 5,0, while the mean of colonies formed by DO4 19703 was 50 ± 6,7 (p=0,0022).

In Fig.10 below, it can be seen that the difference in the formation of colonies of C8161.9 LUC compared to C8161.9 19703 is not as significant as in DO4. The mean of the colony formation of C8161.9 LUC is 126,3 ± 2,2, while the mean of the colony formation of C8161.9 19703 is 96,6 ± 4,0 with a t-test showing that the results is highly significant (p=0,003). However, it was noticeable that the formed colonies differed significantly in size. While the mean of colony size in C8161.9 LUC was 52,1 ± 2,6, the mean size of the colonies formed by C8161.9 19703 was 27,2 ± 2,7 with a highly significant t-test (p=0,0025), shown in Fig.11.

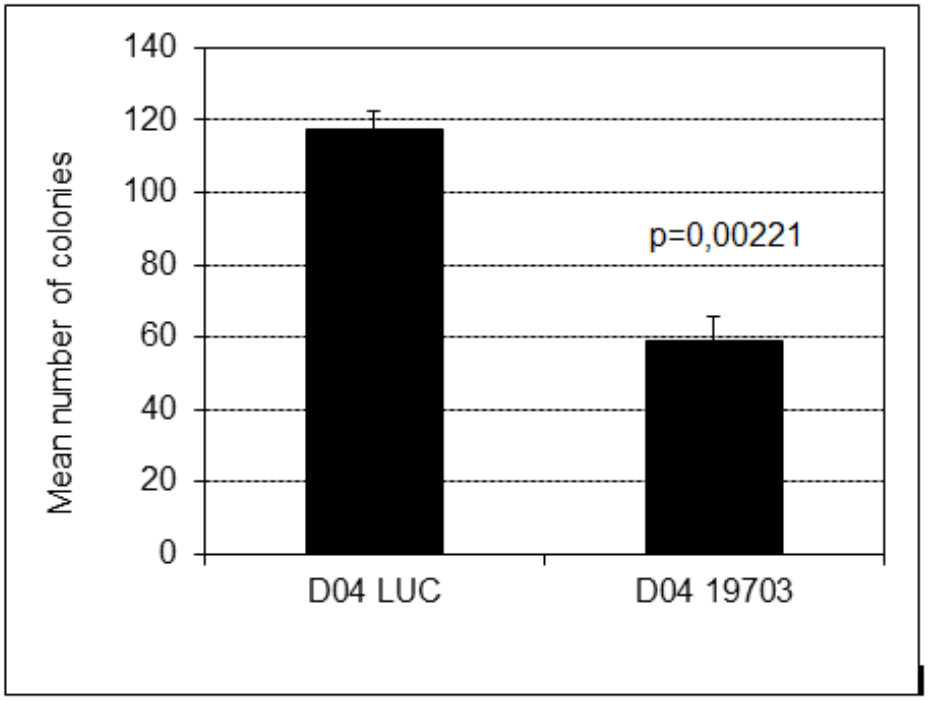


Fig. 7: Comparison of the mean number of colonies formed of DO4 LUC versus DO4 19703, showing a significant t-test (p=0,00221)

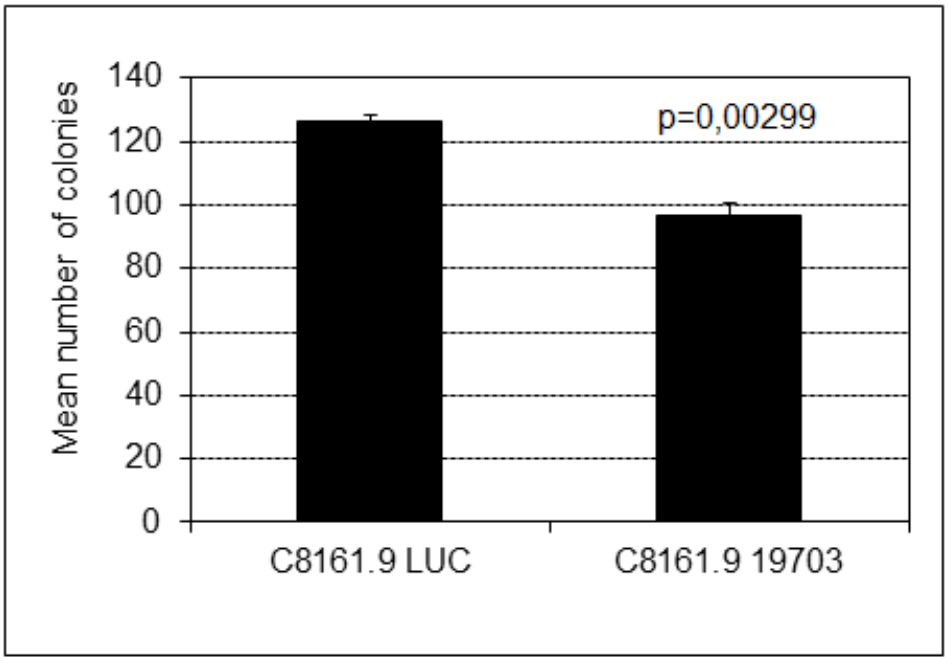


Fig. 8: Comparison of the mean number of colonies formed of C8161.9 LUC versus C8161.9 19703 with a significant t-test (p=0,0029)

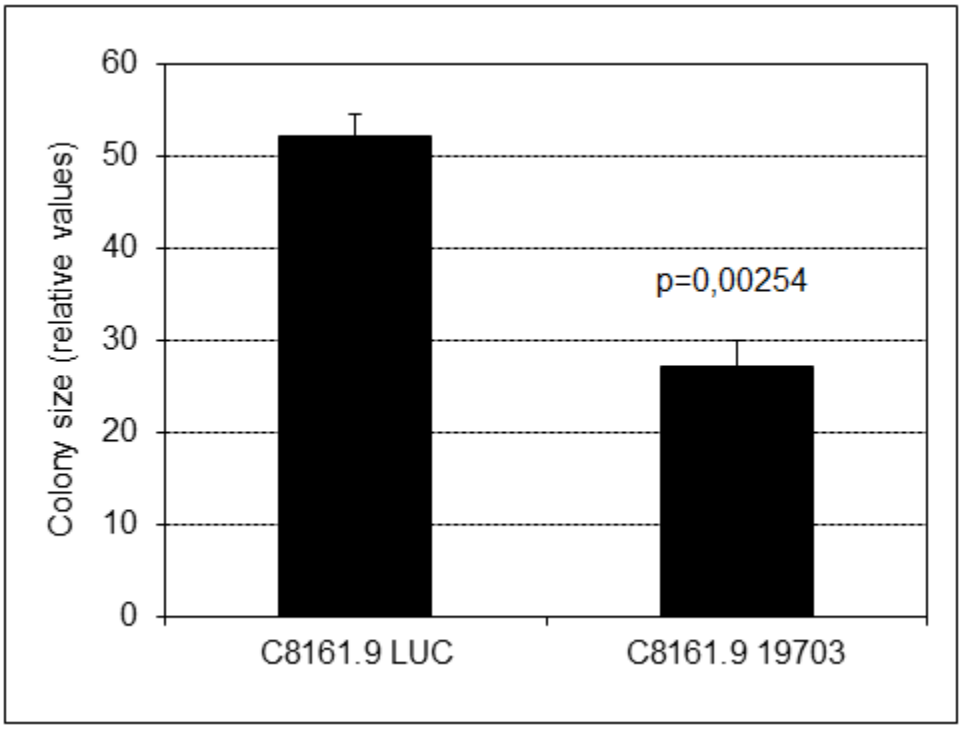


Fig. 9: Comparison of the colony size of C8161.9 LUC versus C8161.9 19703, showing a significant t-test (p=0,00254)

5.4. Cell cycle analysis:

Due to the identification of the role of NCOA3 in the formation of colonies generated by the colony formation assay, it was concluded that NCOA3 has an effect on cell proliferation. Therefore the role of NCOA3 in the regulation of the cell cycle in melanoma was investigated. By means of flow cytometry the cell cycle could be analyzed and possible phase blocks, as well as the activation of cell death programs could be identified.

Different cell lines, DO4 and C8161.9, were analyzed by flow cytometry. Both, the control cell line LUC, as well as the cell line with reduced expression of the gene NCOA3 were stained with PI/RNase Staining buffer (BD Pharming), analyzed with the Flow Cytometer and FlowJo, a flow cytometry analysis software, and compared to each other.

Fig.12 shows that there is an increase in subG1 phase in the cells with reduced expression of NCOA3, when compared to the control group, for both cell lines C8161.9 and DO4. SubG1 phase represents the apoptotic cells and indicates the activation of a cell death program due to the knockdown of NCOA3.

The Dean-Jett-Fox analysis model in Fig.13 analyses the distribution of percentages in the different phases of the cell cycle. While the percentage distribution in the analysis of DO4 LUC shows only a subG1-phase of about 1,83%, a G1-phase of about 56,6%, a S-phase of about 25,2% and a G2-phase of about 12,37%, the distribution in the analysis of DO4 19703 shows a subG1-phase of about 10%, a G1-phase of about 56%, a S-phase of about 14% and a G2 phase of about 17%. This results in an increase in subG1-phase of about 8%, indicating the activation of a cell death program in cells with reduced NCOA3 expression. An even more significant result can be seen in the comparison of C8161.9 LUC versus 19703 with an increase in subG1-phase of about 55%.

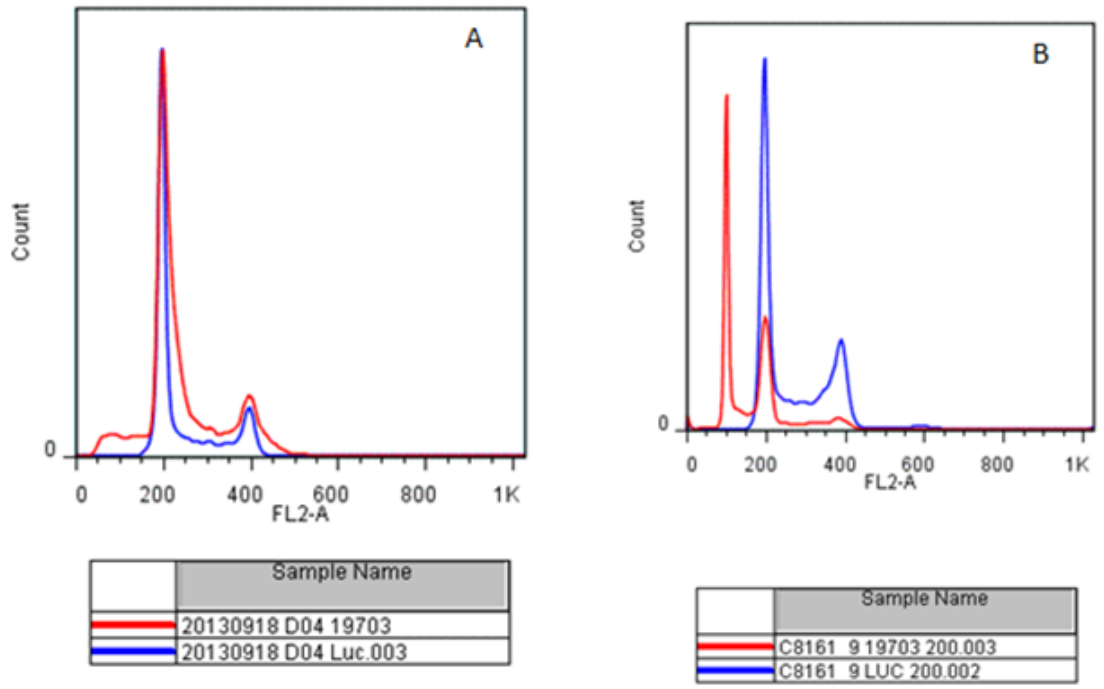


Fig. 10: (A) Cell cycle profile of DO4 LUC (blue line) versus DO4 19703 (red line) with an increase in subG1 phase in DO4 19703 and a majorly disorder cell cycle profile. (B) Cell cycle profile of C8161.9 LUC (blue line) versus C8161.9 19703 (red line) as well with an increase in subG1 phase in C8161.9 19703 and a majorly disorder cell cycle profile.

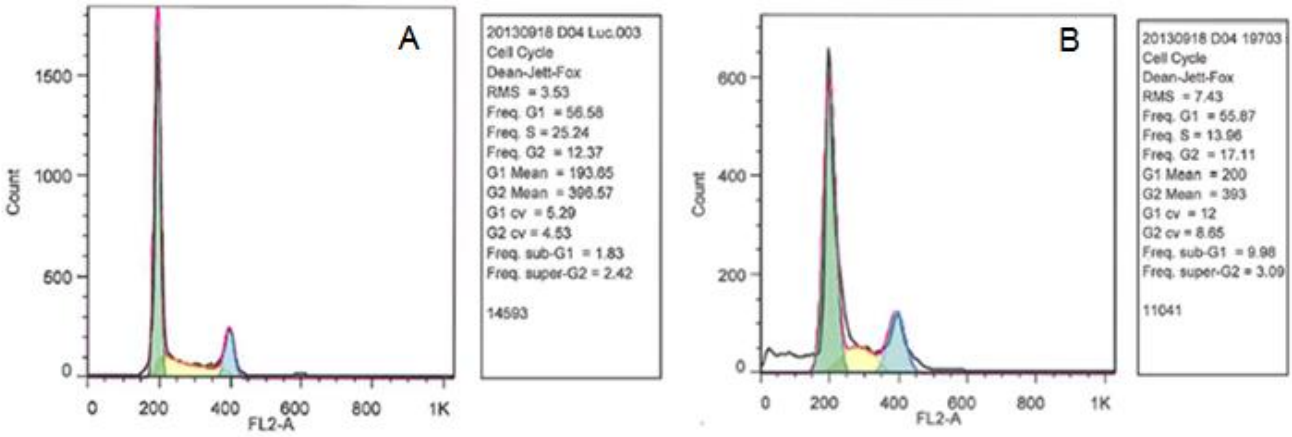


Fig. 11: (A) shows the Dean-Jett-Fox analysis model by FlowJo for DO4 LUC, where the subG1-phase is about 1,83%, the G1-phase about 56,6% and the S-phase about 25,2%. (B) shows the Dean-Jett-Fox analysis model for DO4 19703, where the subG1-phase is about 10%, the G1-phase about 56% and the S-phase 14%. This indicates an increase of about 8% in subG1-phase.

5.5. Activation of cell death programs:

As it could be seen that NCOA3 has an impact on the regulation of the cell cycle leading to a significant increase in subG1-phase in cells with reduced NCOA3 expression when compared to the control group, meaning to an increase in dead cells, the role of NCOA3 in the activation of different cell death programs was tested.

Apoptosis:

The mechanisms of apoptosis are highly complex, today two main apoptotic pathways are known, namely the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway. A third pathway existing involves T-cell mediated cytotoxicity and perforin-granzyme-dependent killing of cells which induces apoptosis via granzyme B or granzyme A. Although all three pathways get activated through different signals, all of them, except the pathway activated through granzyme A, converge in the same execution pathway with the activation of the executioner caspase 3 resulting in DNA fragmentation, degradation of cytoskeletal and nuclear proteins, the formation of apoptotic bodies and the final uptake by phagocytic cells. The pathway activated by granzyme A results in a caspase-independent pathway activation via single stranded DNA damage[59].

Therefore the first focus lies on the identification of the activation of apoptosis through the activation of the executioner caspase 3 and 7.

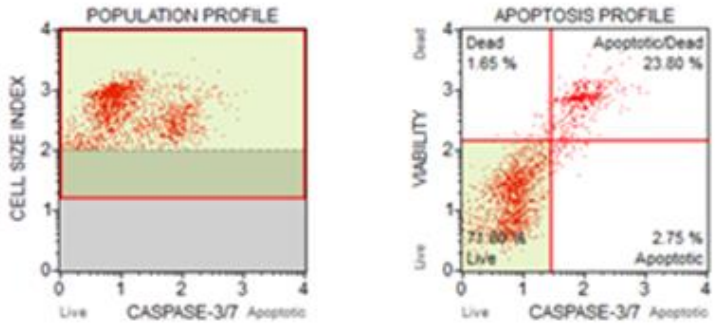
Analysis of the activation of apoptosis through the activation of caspase 3 and 7:

For the analysis of the activation of caspase 3, flow cytometry was used. MUSE Cell Analyzer, a flow cytometer of Millipore was used. The control cell line LUC and the cell line with reduced expression of NCOA3, 19703, for both cell lines DO4 and C8161.9 were treated according to the protocol “Muse Caspase-3/7 Kit User Guide” of Millipore, see Materials and Methods.

Fig.14 below shows the significant difference in the activation of the executioner caspase 3 and 7 in the cell line DO4. While in DO4 LUC still 71% of live cells are

present and only 26,55% of cells are apoptotic, in DO4 19703 only 53,75% of cell are living and 46,0% of cells are apoptotic which results in an increase of apoptotic cells of about 20%. When comparing the activation of caspases 3 and 7 in C8161.9 LUC and C8161.9 19703, no significant difference in the activation of executioner caspases can be seen, therefore the results are not shown. This could mean that the activation of apoptosis in C8161.9 results in the activation of a different apoptosis pathway, evading the activation of the executioner caspase 3.

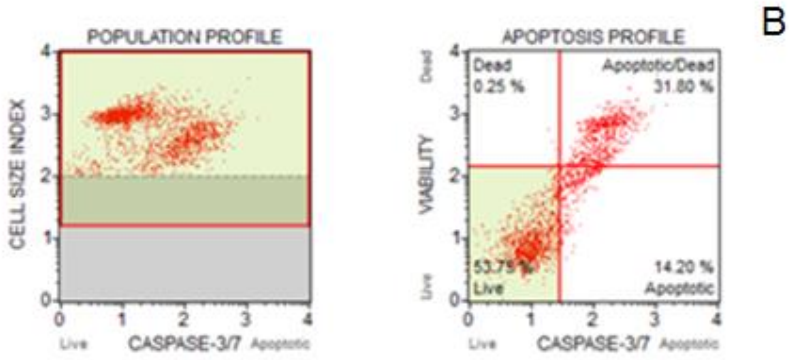
A



% Gated

Live (LL) :	71.80 %
Apoptotic (LR) :	2.75 %
Apoptotic/ Dead (UR) :	23.80 %
Dead (UL) :	1.65 %
Total Apoptotic :	26.55 %

B



% Gated

Live (LL) :	53.75 %
Apoptotic (LR) :	14.20 %
Apoptotic/ Dead (UR) :	31.80 %
Dead (UL) :	0.25 %
Total Apoptotic :	46.00 %

Fig. 12: (A) shows the activation of the executioner caspase 3 in DO4 LUC, while (B) shows the activation of caspase 3 in DO4 19703. In (A) 71,8% are live cells and only 26,55% are apoptotic cells, while in (B) only 53,75% are live cells and 46,0% are apoptotic cells.

PARP analysis for the activation of apoptosis:

For further analysis of the activation of apoptosis PARP-1 was used. PARP, poly (ADP-ribose) polymerase, is a nuclear DNA-binding zinc finger protein mainly involved in DNA repair due to environmental stress, modulation of chromatin structure and programmed cell death. The induction of caspases mediates PARP-1 cleavage into a 89kDa fragment and into a 24kDa fragment in human. As the cleavage occurs between Asp21 and Gly215, the fragments get separated at the amino-terminal DNA binding domain (24kDa) from the carboxyl-terminal catalytic domain (89kDa). Uncleaved PARP maintains cells viability, while cleavage of PARP serves as a marker for apoptosis[60,61].

For the analysis of the cleavage of PARP-1, the PARP-1 antibody from Santa Cruz Biotechnology was used and a Western blot analysis was performed. PARP-1 antibody from Santa Cruz Biotechnology shows the uncleaved PARP with a size of 116kDa as well as the cleaved PARP fragment with a size of 89kDa, which indicates the activation of apoptosis.

In Fig. 15 below, it can be seen that PARP gets cleaved into a 89kDa fragment in both cell lines, DO4 and C8161.9. When comparing the intensity of the bands of LUC versus 19703, the intensity of the band in cells with reduced expression of NCOA3, 19703, is higher than in the control cell line LUC, indicating the activation of apoptosis.

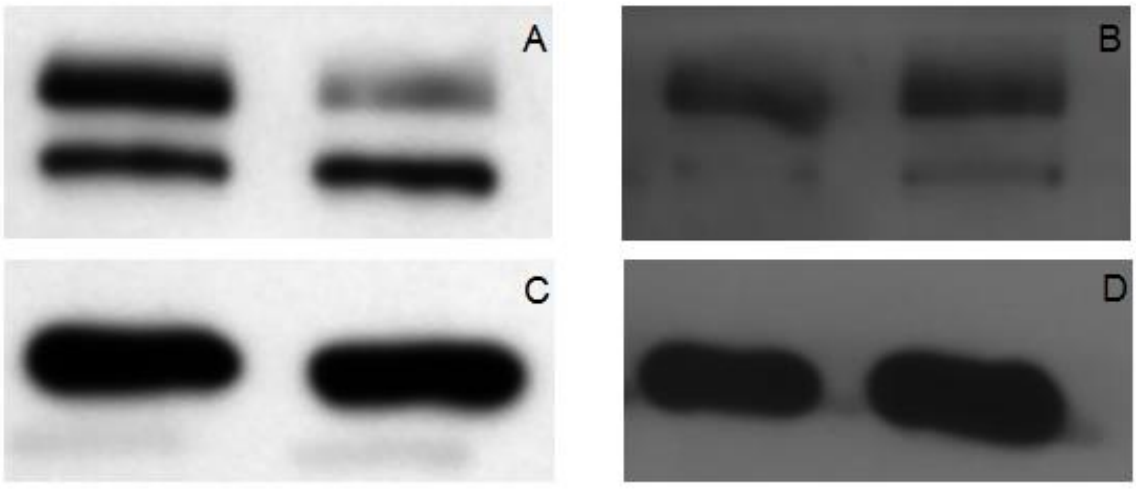


Fig. 13: Western blot analysis of the cleavage of PARP-1 in DO4 LUC versus 19703 and C8161.9 LUC versus 19703. (A) shows PARP-1 uncleaved with a size of 116kDa and PARP-1 cleaved with a size of 89kDa in DO4 LUC (left) and DO4 19703 (right). (C) shows the corresponding bands of DO4 LUC (left) DO4 19703 (right) of βActin, which was used as a loading control. (B) shows PARP-1 uncleaved and cleaved of C8161.9 LUC (left) versus C8161.9 19703 (right), while (D) show the corresponding βActin bands for C8161.9 LUC (left) and C8161.9 19703 (right).

5.5.1. Senescence:

Senescence is defined as the physiological program of terminal growth arrest, whereby cells move form an actively dividing to a non-dividing stage. Due to the loss of the ability to divide, changes in morphology, shape and physical appearance of the cells and in their gene expression pattern occur. Senescent cells may remain viable for a long time, although at the end of the process usually cell death occurs.

For the analysis of the activation of senescence in the cell lines, DO4 and C8161.9, with reduced expression of NCOA3, the Senescence Cells Histochemical Staining Kit from Sigma-Aldrich was used. The assay is based on the histochemical stain for β-galactosidase activity at pH 6. At this specific conditions, β-galactosidase activity is easily detectable in senescent cells, while undetectable in quiescent and immortal cells.

Fig. 16 and Fig. 17 show the expression of β-galactosidase in DO4 LUC versus 19703 and C8161.9 LUC versus 19703. While in Fig. 16, showing DO4 LUC versus 19703, no significant difference in the expression of β-galactosidase was visible, which would

indicate the activation of senescence, in C8161.9 LUC versus 19703, shown in Fig. 17, a significant increase of senescent cells in C8161.9 19703 can be seen when comparing C8161.9 19703 to the control group C8161.9 LUC.

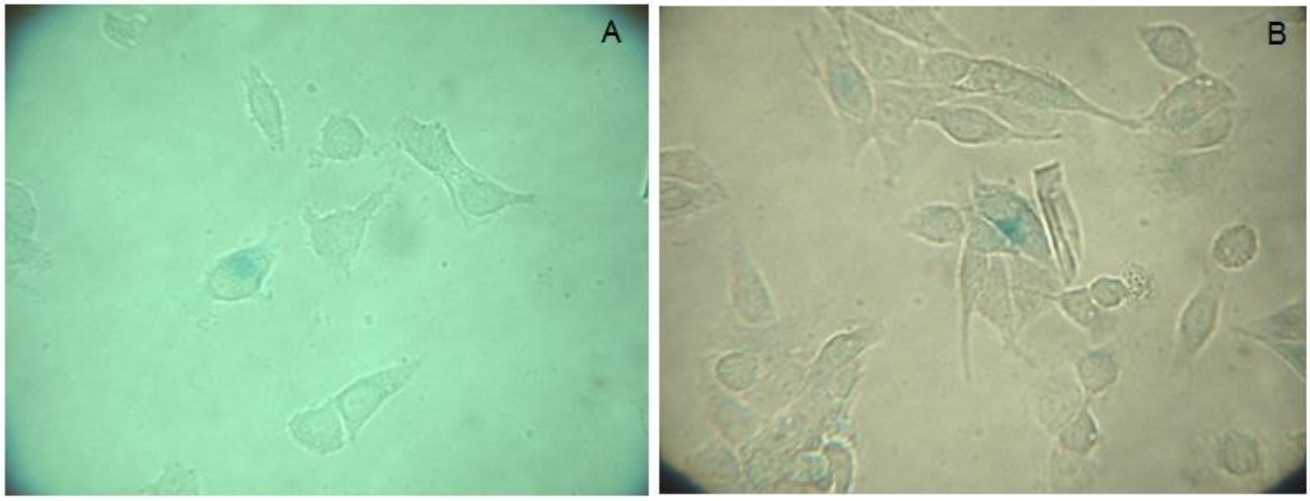


Fig. 14: (A) shows the expression of β-galactosidase in senescent DO4 LUC cells, while (B) shows the expression of β-galactosidase in senescent DO4 19703 cells.

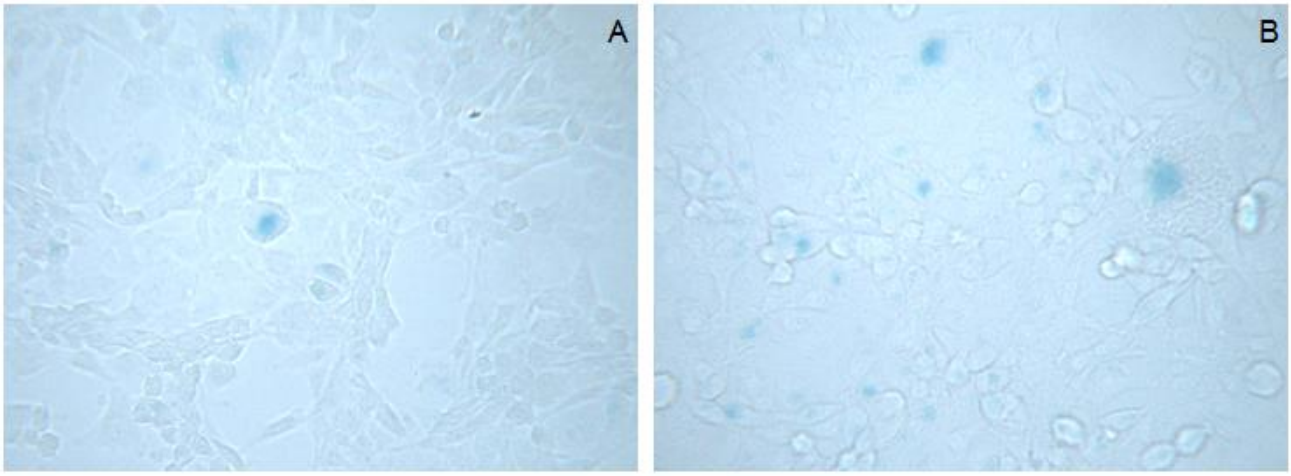


Fig. 15 (A) shows the expression of β-galactosidase in senescent C8161.9 LUC cells, while (B) shows the expression of β-galactosidase in senescent C8161.9 19703 cells.

5.6. Change in cyclin levels:

Cyclin-dependent kinases (CDKs) and the associated cyclins regulate the transition of cells in the cell cycle machinery from one phase into the next phase. Therefore specific steps of the cell cycle are controlled by changing the levels and availability of cyclins, during the progressioin of the cell cycle, which function by activating the catalytic unit of different CDKs.

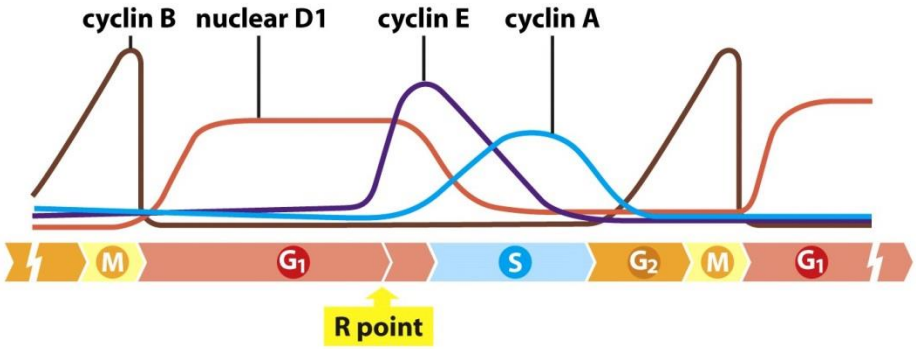


Fig. 16: Fluctuation of cyclin levels during the cell cycle.

In order to test the effect of NCOA3 on the expression levels of different cyclins in different phases of the cell cycle, quantitative real-time PCR was performed on both cell lines, DO4 LUC versus 19703 and C8161.9 LUC versus 19703.

Fig. 19 below shows the different expression levels of several cyclins in C8161.9 and DO4. As it can be seen, the expression levels of all cyclins tested decreases in cells with reduced expression of NCOA3 when comparing to the control group.
While the expression levels drop drastically in the comparison of C8161.9 LUC to 19703, the change in expression levels, when comparing DO4 LUC and DO4 19703, is not as dramatically. As the tested cyclins are active in different phases of the cell cycle, it is hard to conclude in which phase of the cell cycle NCOA3 is mostly active, as it has an influence on the expression level of all the cyclins tested. This conclusion matches with the obtained cell cycle profiles, which show a disorder in the whole cell cycle, seen in Fig.13.

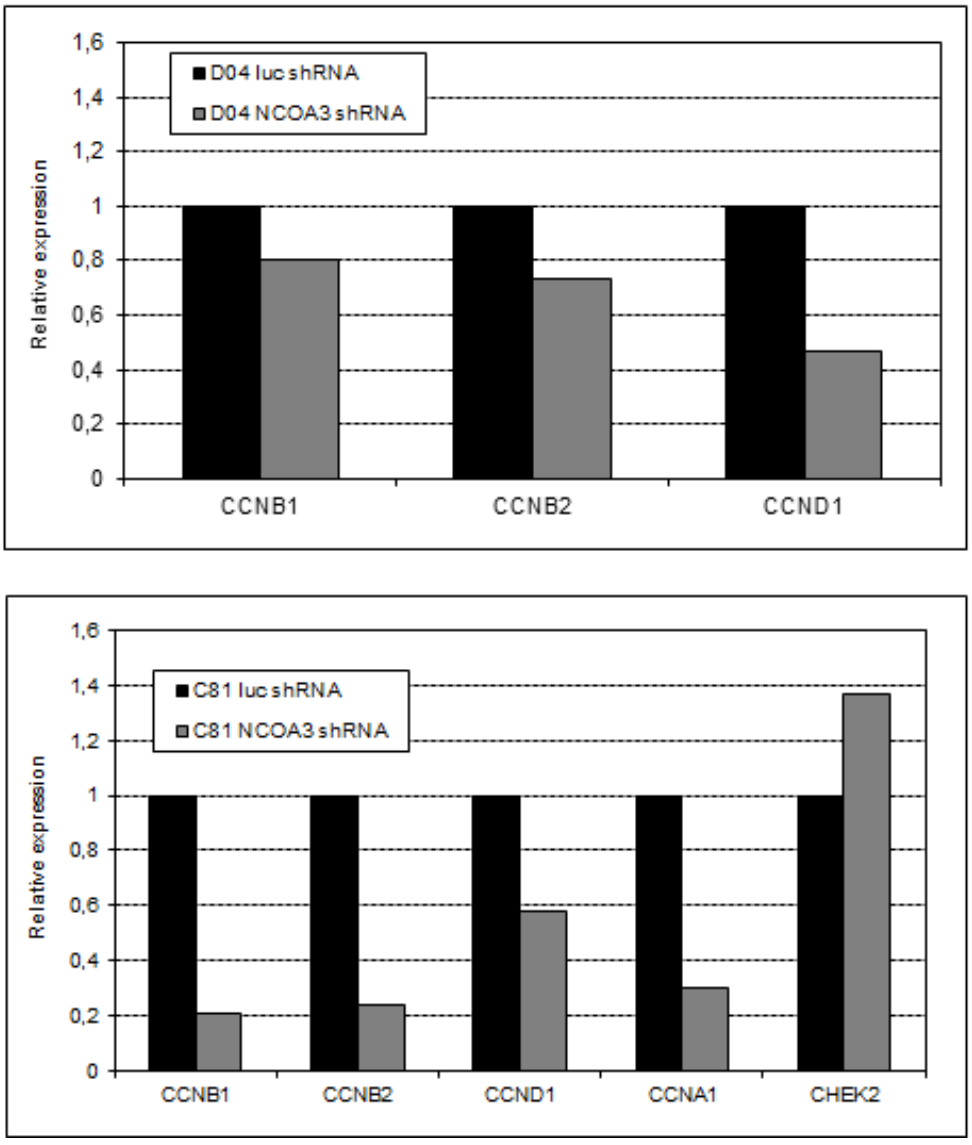


Fig. 17: Levels of expression of several cyclins in DO4 LUC versus 19703 and C8161.9 LUC versus 19703.

6. Conclusion:

NCOA3 is already known as an oncogene in breast cancer, which facilitates breast cancer progression by the deregulation of its pathway. NCOA3 is thought to act as a proto-oncogene in melanoma, although its role is still poorly understood.
This study focuses on understanding the role of NCOA3 in regulating the cell cycle machinery.

In the analysis of the cell cycle profile, cells with physiological expression of NCOA3 showed a continuous progression of the cell cycle machinery and evasion of cell death, while cells with reduced expression of NCOA3 showed cell cycle arrest and activation of apoptosis.
The activation of cell death programs does not only result in the activation of apoptosis, but also other programs such as senescence. While in the control group of C8161.9 only few cells were observed to be senescent, cells with reduced NCOA3 expression showed a significant increase in senescent cells.

The results obtained in analyzing the cell cycle profiles suggest that NCOA3 plays a major role in melanoma progression, resulting in uncontrolled proliferation in cells with high expression of NCOA3.
In order to allow uncontrolled proliferation in cells with physiological expression of NCOA3 several cell cycle checkpoints need to be inactivated.
Changes in the expression levels of several cyclins, such as cyclin D1, cyclin B1 and cyclin B2, which are involved in cell cycle checkpoint regulation and phase transition, further prove the involvement of NCOA3 in cell cycle progression. All the different cyclins analyzed in this thesis are downregulated in cells with suppressed expression of NCOA3. The data obtained shows that NCOA3 is involved in the regulation of different cell cycle checkpoints in different phases of the cell cycle machinery. Knockdown of NCOA3 in C8161.9 resulted in much lower levels of cyclins, activating different cell cycle checkpoints and resulting in cell cycle arrest, when compared to the control group.

Based on the data generated, it can be shown that NCOA3 can regulate melanoma cell proliferation by directly effecting the expression of cyclins leading to changes in cell cycle profile and to the activation of senescence and apoptosis. Therefore, it is suggested that NCOA3 acts as a proto-oncogene that promotes melanoma progression when NCOA3 is overexpressed.
NCOA3 can therefore be used as a prognostic marker, as the level of NCOA3 expression allows personalized predictions of the melanoma progression for each patient.

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8. Figure references:

Fig.1: http://www.biooncology.com/research-education/apoptosis/resisting (27.12.2013)

Fig.2: Hocker, T. L., Singh, M. K. & Tsao, H. Melanoma genetics and therapeutic approaches in the 21st century: moving from the benchside to the bedside. *J. Invest. Dermatol.* **128,** 2575–95 (2008)

Fig.3: Posch, C. & Ortiz-Urda, S. NRAS mutant melanoma--undrugable? *Oncotarget* **4,** 494–5 (2013)

Fig.18: Weinberg, R. A. *The Biology of Cancer*. (2013)