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In vitro anti-melanoma activity of the amphibian ribonuclease onconase (ONC):

comparison between monomeric and dimeric ONC species and correlation between ONC-elicited microRNAs upregulation and lower expression of specific onco-proteins

S.S.D. BIO/10

Coordinator: Prof.ssa Lucia De Franceschi

Tutor: Prof.ssa Marta Vittoria Menegazzi

Signature Marta Vittoria Menegazzi

Doctoral Student: Dott.ssa Rachele Campagnari

Signature Rachele Campagnari

SUMMARY

ABSTRACT

Melanoma, displaying a high mortality rate and an increasing incidence worldwide is considered the most aggressive forms of skin cancer. Several therapies have been approved during the last few years including target and immunotherapy but unfortunately, all strategies failed for the resistance acquired by tumor cells or for the severe side effects. In this context new therapeutical strategies urge to be discovered and the cytotoxic onconase (ONC), an RNase variant deriving from *Rana pipiens*' oocytes, may become a valid choice. This ribonuclease shows remarkable activity against different types of cancer in patients, reaching phase II and phase III clinical trials for non-small cell lung cancer and for malignant mesothelioma, respectively. Unfortunately, nowadays the most important limit for a therapeutic application of ONC is its renal toxicity even if reversible. Oligomeric species of this enzyme can be generated to overcome this problem, since enlarging ONC moiety dimension through dimerization should limit glomerular filtration and increase its circulating half-life. Interestingly, the dimeric form of ONC (ONC-D) displays an enzymatic activity similar to that of ONC.

In this thesis, different human cancer cell lines were incubated with increasing concentrations of ONC and ONC-D with the aim of comparing their biological activities. Besides two human cell lines from melanoma (A375 and MeWo), glioblastoma cell line (U251) and hepatocellular carcinoma cell line (HepG2) were also tested. In all cell lines, ONC-D exhibits slightly lower cytotoxic and cytostatic effects compared to the monomer. Therefore, the molecular mechanism of ONC and ONC-D antitumor activity has been deeply investigated in A375 and MeWo melanoma cells. Indeed, both ONC species demonstrated to inhibit the phosphorylation level of the proto-oncogene tyrosine-kinase Src (Src). Importantly, a strong inhibition of Tyr705 phosphorylation of the Signal Transducer and Activator of Transcription 3 (STAT3) was also registered with both ONC and ONC-D. This event could depend on the reduced Src activity because STAT3 is a target substrate of Src kinase. In addition, both Ser727 phosphorylation of STAT3 and the STAT3 total protein expression level were decreased. This suggests a complete downregulation of STAT3 transcriptional activity, which is highly activated in cancer, thus partially explaining the antitumor action of both ONC species.

Moreover, the number of apoptotic cells increased after ONC and ONC-D treatments. This result correlates with the lower expression level of the anti-apoptotic B cell lymphoma 2 (Bcl2) protein that is upregulated by STAT3.

Even if non-coding RNA species seem to be the preferential targets of ONC ribonucleolytic activity, the target specificity of this enzyme is still unclear. So, in this thesis we investigated the ONC ability to modulate the expression of several microRNAs (miRNAs) in the A375 and FO1 BRAF-mutated melanoma cell lines. RT-PCR, used to measure the expression levels of miRNAs, revealed that the

onco-suppressors miR-20a-3p, miR-29a-3p and miR-34a-5p were highly expressed in 48-h ONC-treated A375 cells. Moreover, immunoblots were carried out to study the expression level of some proteins that are codified by target genes of such overexpressed miRNAs.

Here it was also confirmed that ONC inhibits A375 cell proliferation, and its cytostatic effect was mechanistically explained by the sharp inhibition of proteins involved in cell cycle progression such as cyclins D1 and A2, as well as retinoblastoma protein and cyclin-dependent kinase 2. In addition, the expression of kinases ERK1/2 and Akt and of the hypoxia inducible factor-1α, proteins controlling pro-survival pathways, was inhibited by ONC. Finally, many crucial proteins involved in cell metabolism, migration, invasion, and metastatic potential were downregulated, as well. Since all mRNAs coding these proteins are predicted or validated targets of miR-20a-3p, miR-29a-3p and/or miR-34a-5p which are upregulated by ONC, we suggest that ONC anti-proliferative and anti-metastatic activities in A375 melanoma cells might depend on the upregulation of these onco-suppressor miRNAs.

In conclusion, the results obtained strongly suggest that ONC displays a pleiotropic anti-tumor effect against different human melanoma cell lines. Again, the possible oligomerization of this enzyme could break down the limit for ONC therapeutic application.

1. INTRODUCTION

1.1 Melanoma

Among skin cancers, melanoma is associated with the highest mortality rate as it is the most aggressive one[1]. It arises from the neoplastic transformation of melanocytes, the pigmented cells that are present in epidermis, eyes, and in the epithelium of nasal cavity, anus, vagina, and urinary tract. Under normal conditions, melanocytes produce melanin in response to solar ultraviolet radiation (UV) for skin protection. Conversely, the UV radiation, together with environmental exposure to toxic agents and the presence of genetic predisposition, are the main risk factors related to the onset of melanoma. In particular, fair skin individuals display a higher risk for melanoma because their epidermis presents lower amounts of melanin and consequently a higher quantity of UV radiation can penetrate, leading to sun-induced DNA damages. So, the melanocytes of people with fair skin phenotype are more prone to neoplastic transformation [2]. Concerning genetic alterations only 10% of melanomas are hereditary due to a mutation in the cyclin-dependent kinase inhibitor 2A/P16 (CDKN2A/P16), a tumor suppressor protein that plays an important role in cell cycle regulation. Moreover, in dysplastic nevi a large number of cells have been found to display driver mutations, in particular gain of function mutations that activate the mitogen-activated protein kinase (MAPK) pathway. Indeed, more than 50% of melanomas display mutations in the kinase domain of the BRAF oncogenic protein that lead to the constitutive activation of BRAF kinase, independently to the presence of extracellular stimuli and cause the over activation of the downstream MAPK/ERK pathway involved in cell proliferation and survival[3,4]. In 90% of epidemiological cases the aforementioned mutation is a substitution of the valine in the aminoacidic position 600 with a glutamic acid (BRAF V600E)[5]. The current World Health Organization (WHO) classification for melanoma subtypes is still based on the one proposed in the seventies by Clark[6]. By looking at the microscopic growth patterns in association with the age of the patient and the anatomic site of the primary tumour, Clark and colleagues distinguish four main types of melanoma: superficial spreading melanoma (SSM), lentigo maligna melanoma (LMM), nodular melanoma (NM), and acral lentiginous melanoma (ALM)[7]. Moreover, even if melanoma is still an unpredictable disease, its progression can be described by the same model (*Figure 1*). According to Clark, melanoma arises from the controlled proliferation of normal melanocytes that produce an initial lesion called benign nevus. If the melanocytes in a pre-existing nevus undergo abnormal growth, the initial lesion turns into pre-malignant lesion. Eventually melanocytes can proliferate horizontally in the epidermis (radial growth) giving rise to melanoma *in situ*. Once vertical growth begins the mesenchyme is invaded and

later malignant melanocytes can spread to lymph nodes, blood vessels and other organs (metastatic melanoma). More recently a new classification has been proposed by Bastian in the attempt to integrate clinic-pathological features with genetic alterations such as mutations in BRAF, NRAS and KIT genes[8]. Malignant melanoma is an important socio-economic problem considering that its incidence is constantly increasing worldwide: starting from 1960s, melanoma has become one of the most frequent cancers in fair-skinned individuals belonging to Caucasian population[9]. Notably, melanoma is now the fifth most common cancer in men and the sixth in women in the United States where the incidence has increased by 270% from 1973 to 2002. Melanoma, contrary to other cancer types, affects young and middle-aged population. The incidence is also sex-related, according to Markovic *et al.*[10]: women up to 40-year-old have a higher probability to develop melanoma than men of the same age, but then the situation completely changes as the probability becomes 3-times higher for men compared to women [10,11]. Prevention and therefore early detection of this malignancy is the key factor for a lower mortality as the poor prognosis is directly proportionate to the neoplastic stage at diagnosis. To support this concept, the drastic reduction in dermatological treatments during lockdown caused by 2019 coronavirus pandemic (COVID-19)[12], lead to fewer early recognition of new melanoma in 2020 in comparison to 2018-2019[13] and consequently resulted in increased morbidity and mortality worldwide[14]. Indeed, the cutaneous melanoma location is an advantage in cure compared to other cancers because early detection is possible through non-invasive approaches[15]. The European Society for Medical Oncology clinical practice guidelines for cutaneous melanoma highlight the importance of a detailed diagnosis (tumor location, stage, and genetic profile) for a proper therapy choice. The therapeutic options approved by the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) may be surgical resection, chemotherapy, radiotherapy, photodynamic therapy, immunotherapy, or targeted therapy[16]. Unfortunately, the invasion capability, the resistance to radiotherapy or chemotherapy and the adverse events (Aes) related to immune reactions are at the bases of its elevated mortality[17–19].

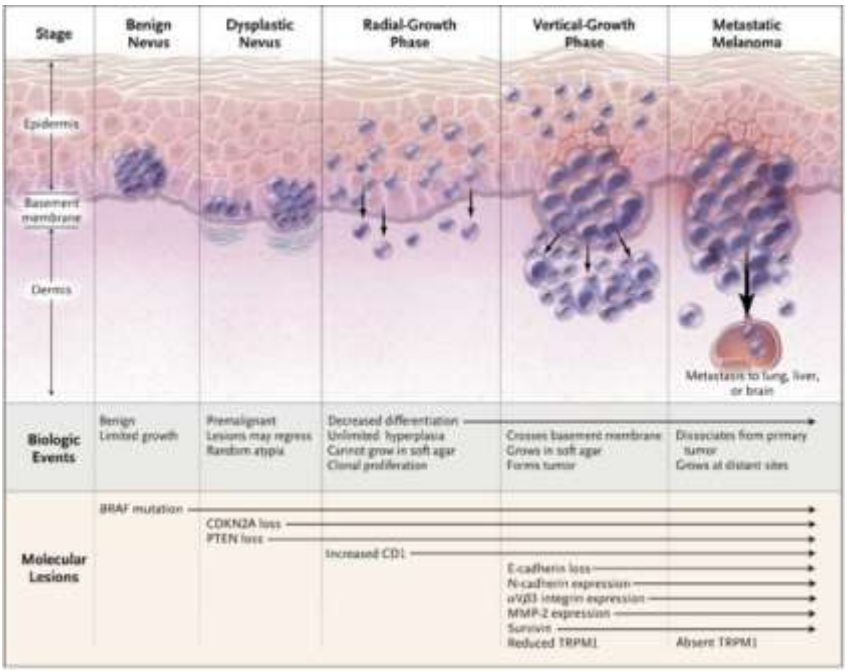


Figure 1. Schematic representation of melanoma progression according to the Clark's model[20].

1.2 Melanoma therapies

Surgery, radiotherapy, immunotherapy, targeted therapy, and chemotherapy are some of the treatments currently used in clinic for melanoma. After surgical removal of the primary melanoma, radiotherapy and/or chemotherapy may be used when cancer has an extensive spread to the lymph nodes or skin. Concerning immunotherapy, that was initially employed for melanoma treatment, interferon and interleukin cytokines, such as IFN-α and IL-2, which had already been approved by the FDA for a long time, were administered[21]. Unlikely, this approach did not show notable benefits for patients, due to the severe side effects such as capillary leak syndrome and to the low percentage (10%) of people experiencing a complete response[22–24].

More recently, immune checkpoint inhibitors have been used. They belong to a new class of cancer therapeutics that have the physiological purpose to regulate the activation of T cells[25]. Among them, the cytotoxic-T lymphocytes antigen 4 (CTLA-4) and the programmed cell death protein 1 (PD-1) are the most studied targets[25].However, better results were obtained combining ipilimumab and nivolumab (inhibitors of CTLA-4 and PD-1, respectively) in comparison to each drug administered alone. In particular, the combined treatment can reduce the size of the tumor and delay its growth, even if its causes far more side effects.

Targeted therapies: the proto-oncogene B-Raf (BRAF) and mitogen-activated kinase kinase (MEK) inhibitors revolutionized melanoma treatment in 2011, when these therapies were approved by FDA. Since BRAF is the most frequently mutated oncogene in melanoma, its inhibitors, such as dabrafenib and vemurafenib, have shown promising results in several clinical trials. Additionally, trametinib represents the first drug of the class of MEK inhibitors, to be approved by FDA as a single agent. A translational investigation led to the evidence of a possible synergistic relationship between MEK and BRAF inhibitors. In fact, many combinatorial approaches of these two types of inhibitors have shown very promising results, although presenting many side effects[26].

Dacarbazine (dimethyltriazeno-imidazol carboxamide) and temozolomide are the gold standard for melanoma chemotherapy. Dacarbazine (DTIC), approved by the FDA in 1975, is an alkylating agent. Similarly, to other chemotherapeutic drugs, it is not highly selective for cancer cells over healthy cells, and a high number of clinical trials have reported a modest anti-tumor efficacy. Nevertheless, dacarbazine remains one of the first-line treatments for metastatic melanoma[27]. Temozolomide displays the same molecular mechanisms of DTIC, but differently to DTIC, it can pass the brain blood barrier, reaching the central nervous system, one of the most common sites of melanoma metastasis[28].

The therapeutic strategies for melanoma have evolved significantly over the last decade shifting from cytotoxic chemotherapies like dacarbazine to targeted- and immune-therapies including immune

checkpoint inhibitors. These new drugs have extended the life of melanoma patients with an advanced disease. Nevertheless, a very effective and resolutory therapy is still not known so far because, unfortunately, tumor-resistance appears very fast after each treatment. For instance, the acquired resistance to BRAF inhibitors, commonly administered to BRAF-mutated melanoma patients, could be due to the re-activation of extracellular-signal regulated kinases (ERK)-1/2 by alternative pathways or to the induction of other pro-survival kinases, as phosphoinositide-3-kinase (PI3K) and protein kinase B (AKT)[29]. So, it is necessary to find new effective therapeutic tools.

1.3 Cancer Cell Death

Whenever a loss of balance between cell division and cell death is present, cancer may arise[30]. Similarly to normal cells, cancer cells may undergo either programmed cell death (apoptosis, autophagy, ferroptosis, necroptosis, pyroptosis) or necrosis. Interestingly, as they are tightly regulated some of these programmed cell deaths can be modulated by drug therapy[31]. As it has already been demonstrated ONC is able to trigger apoptosis in A375 melanoma parental cells as well as in A375 dabrafenib resistant cell subtype[32], we further investigated if apoptosis was triggered in other melanoma cells treated not only with ONC but also with its dimer or trimer.

Unlike necrosis, apoptosis is an active process that may occur both in physiological and pathological conditions. Apoptosis is a very complex process that, under physiological conditions, occurs once DNA damage is not repairable by the cell. During apoptosis, a family of cysteine-aspartate proteases, known as caspases, are activated by cleaving in response to stimuli[33]. In turn, caspases cleave a wide range of substrates, such as other downstream caspases and enzymes including Poly (ADP ribose) Polymerase 1 (PARP1). The latter is a 116 kDa, highly conserved poly-(ADP-ribosylating) enzyme. PARP1 is a DNA nick sensor that uses NAD^+ to form polymers of ADP-ribose to facilitate DNA repair. During apoptosis PARP1 on one hand is induced to synthesize poly (ADP ribose) and on the other hand is cleaved by activated caspases[34]. PARP1 is cleaved *in vivo* by active caspase-3 and active caspase-7, preferentially in correspondence of the DEVD motif. After cleavage, an 89 kDa fragment containing the catalytic domain and a 24 kDa fragment containing the DNA binding domain are generated[35]. It is important to point out that the exact cellular function of the cleaved products has not been yet clearly understood. However, PARP1 cleavage may prevent DNA repair and block energy depletion-induced necrosis in order to promote apoptosis[34]. Thus, it can be used as cellular biochemical marker for apoptosis together with activated caspases. PARP1 can also be considered a sensor of DNA damage and for this reason several studies have aimed to understand the PARP1-mediated NF-κB (nuclear factor-kappa B) activation. The pleiotropic function of NF-κB in melanoma lead to the development of several drugs that specifically target NF-κB[36]. In particular, this transcription factor induces many anti-apoptotic genes, such as Bcl-XL and the inhibitor-of-apoptosis protein 1 and 2. An interesting study demonstrated the link between PARP1 and NF-κB. As show in *figure 2*, once PARP1 recognize a DNA strand break its autoribosylation takes place and a complex containing IKKγ (IkB kinase γ), PIASγ (Protein Inhibitor of Activated STAT-γ) and ATM (Ataxia, Telangiectasia Mutated) proteins is formed. This complex is then able to activate IKKs and NF-κB[37]. Considering that the evasion from the apoptotic cell death is essential for tumor progression and uncontrolled proliferation, the majority of drugs used in melanoma therapy are pro-apoptotic drugs, such as cisplatin that is an activator of the apoptotic mitochondrial pathway[38]. Indeed, cell apoptosis

induction helps to reduce the tumor metastatic potential. Subsequently the Signal Transducer and Activator of Transcription 3 (STAT3) will be described as it can hamper apoptosis by inducing the expression of the anti-apoptotic protein Bcl2 [39].

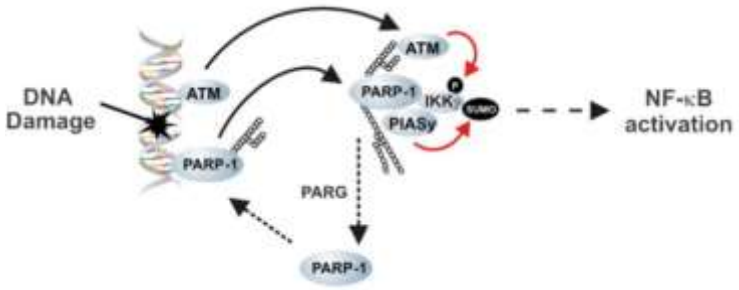


Figure 2. Schematic representation of the link between PARP1 and NF-kB[40].

1.4 STAT-3 signalling pathway in cancer

Signal Transducer and Activator of Transcription 3 (STAT3) is a transcription factor that mediates many extracellular signals, usually generated by the ligand binding to each cell surface receptor, and the transmission of these signals to the nucleus. Several studies strongly suggested that the constitutive activation of STAT3 is a common feature in human tumor cells. In fact, aberrant STAT3 activation signalling promotes initiation and progression of human cancers by inhibiting apoptosis, inducing cell proliferation, and activating angiogenesis, invasion, and metastasis. On the contrary, suppression of STAT3 activation results in apoptosis induction. For this reason, STAT3 is an important target in cancer therapy[41]. STAT3 can be activated by more than 40 different polypeptide ligands including many cytokines, growth factors, oncogenic proteins, as well as by numerous carcinogens. In normal cells, this signalling is highly regulated and the process is transient. The STAT3 is activated through the phosphorylation of its Tyr705 residue by several tyrosine kinase belonging to the Janus kinases (JAK) and Src kinases families. Therefore, Tyr705 phosphorylation converts STAT3 from an inactive form to an active form, allowing its dimerization, nuclear translocation and binding to the specific promoter sequences on its target genes [42]. Additionally, STAT3 transcriptional activity is further enhanced by Ser727 phosphorylation carried out by the Mitogen-Activated Protein-Kinases (MAPKs), the most important of which are ERK1/2.

1.5 ERK/MAPK signalling pathway and HIF-1α

The mitogen-activated protein kinase/extracellular signal-regulated kinase pathway (MAPK/ERK) plays a critical role in the regulation of several fundamental cellular processes such as cell proliferation, differentiation, and cellular response to different extrinsic stresses[43]. The ERK cascade is one of four distinctive MAPK pathways, together with c-Jun N-terminal kinase (JNK), p38 and bone marrow tyrosine kinase BMK cascades[44]. When a ligand binds to a transmembrane protein receptor tyrosine kinase (RKT), the pathway is activated[45]. Specifically, the ERK/MAPK cascade starts when the activation of small G proteins, such as Ras, transmit the signal from the cell membrane to Raf kinases which activates MAP3K also known as MEK, one of the possible kinases that phosphorylates ERK1/2[46]. ERK proteins can transmit different and sometimes conflicting signals in the same cells. The resulting signalling cascade culminates in the translocation of ERK into the nucleus, where ERK activates transcription factors driving gene expression[45] (*Figure 3*). In particular, in BRAF-mutated cells, such as A375 or FO1 melanoma cells, MAPK/ERK pathway activation can promote hypoxia inducible factor-1 alpha (HIF1-α) expression[47]. Together with the mammalian phosphoinositide 3-kinase/protein kinase B/rapamycin target (PI3K/Akt/mTOR) pathway, HIF-1α is a crucial regulator of tumor cell proliferation and glycolytic metabolism[48]. Considering that the activation of the MAPK/ERK pathway leads to sustained proliferation of tumor cells[49], blood supply may be insufficient, resulting in oxygen tension reduction below physiological levels. Hypoxic areas are common features of rapidly growing malignant tumors and their metastases and HIF-1 transcription factor complex, consisting of both HIF-1α and HIF-1β, is a major mediator of cellular responses to hypoxia[50]. HIF-1α is the oxygen-regulated subunit since its expression is stabilized under hypoxia[51]. Under normoxic conditions, hydroxylation of proline residues within the oxygen-dependent regulatory domain of the HIF-1α subunit facilitates binding of the von Hippel-Lindau protein, which in turn enables the HIF-1α ubiquitination and subsequently the transport in the proteasome for its degradation[52].

Under O_2 limiting conditions, proline hydroxylation is inhibited, thereby HIF-1α is stabilized, and can translocate to the nucleus and bind to the constitutively expressed HIF-1β subunit, forming the active HIF-1 complex[53]. HIF-1 activates a multitude of O_2-reactive genes, such as vascular endothelial growth factor (VEGF) and erythropoietin, which are involved in various normal cellular functions such as survival, apoptosis, glucose metabolism and angiogenesis[54,55]. The expression of HIF-1α is regulated by the MAPK/ERK and Akt/PI3K pathways[50]. Ras oncogene has been shown to regulate HIF-1α expression via the RAF/MEK/ERK pathway[56]. Under normoxic conditions, the activity of oxidative phosphorylation (OXPHOS) usually depends on NADH and $FADH_2$ produced in the citrate cycle[57]. The oxidative decarboxylation of pyruvate to acetyl-CoA, CO_2 and NADH

catalyzed by pyruvate dehydrogenase complex (PDC)[57] links glycolysis in the cytosol to the citrate cycle in the mitochondria. The aberrant expression of HIF-1α affects the disconnection of OXPHOS from energy generation and cells preferentially use glycolysis for fuel. Tumor cells of malignant melanoma can lead to metabolic reprogramming [58] and overexpress HIF-1α in an attempt of de novo vascularization (by VEGF upregulation) and to compensate O_2 deficiency[59].

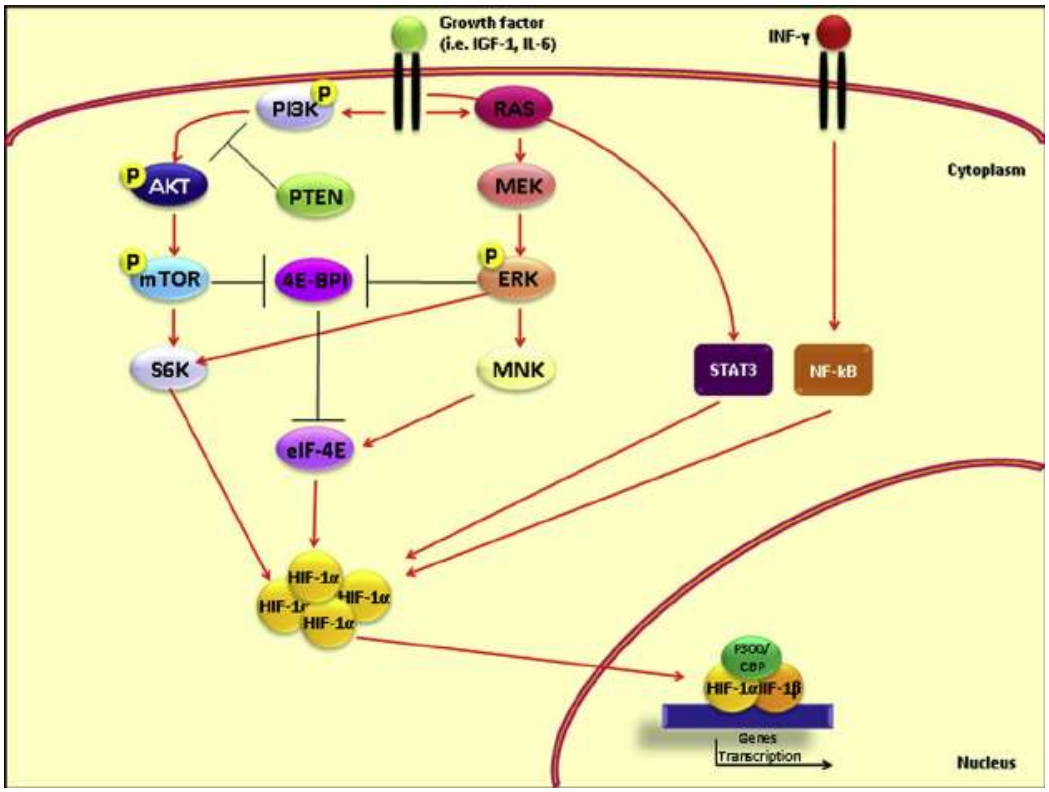


Figure 3. **A model of the MAPK / ERK pathway.**

After activation of the membrane receptor, the adapter proteins recruit RAS proteins to activate steps that lead to ERK activation. Subsequent phosphorylation steps amplify the signal, Raf → MEK → ERK, until ERK activates its cytoplasmic and / or nuclear targets. The PI3K-AKT pathway interacts with the MAPK / ERK node under normal conditions and in the cancer cells[60].

1.6 Cell cycle regulation

Cell cycle is a tightly regulated process. Such regulation relies on the activity of cyclins and cyclin-dependent kinases (CDKs), which form complexes and catalyse the progression through cell cycle[61]. Cell cycle can be divided into two main phases, mitosis (M), or duplication phase and interphase. The interphase includes 3 subphases (G1, S, G2), during which cells replicate their molecular constituents[62]. Eventually, G0 phase may take place if cells result to be quiescent i.e., they do not divide or do not prepare to divide[63]. As shown in *figure 4*, CDKs, are key factors in cell cycle regulation even if they are only transiently activated[64]. Once activated by cyclins, CDKs phosphorylate specific substrates that drive cell cycle and cell division events through the "checkpoints": before moving onto the next phase each previous process must be completed[62]. If the process is found to be incomplete or damaged at these checkpoints, the regulatory activity of cyclin-CDK is blocked to solve these problems[62,65]. For instance, the detection of DNA damage signals immediately leads to the inhibition of these CDKs inducing cell cycle arrest. CDK4, CDK6, CDK2 and CDK1, are the main CDKs sequentially involved in the progression of interphase, regulating the output and the entry of cell into the subphases[66].

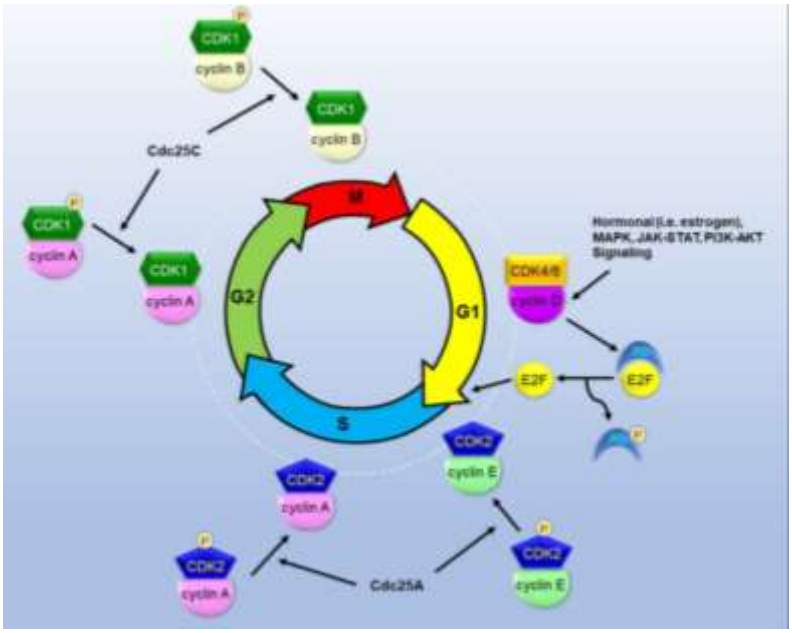


Figure 4. **The role of CDKs in the cell cycle cascade.**

CDKs bind to specialized cyclins to form active complexes that guide the progression of the cell cycle phase and the transition to subsequent phases. Growth and mitogen signals induce cyclin D and activate CDK4, thereby inactivating Rb and releasing E2F to trigger the progression to G1 phase[67].

During G1 phase, cyclin D increases in response to growth/mitogenic stimuli and complexes with CDK4/6. The CDK4/6-cyclin D complex phosphorylates the retinoblastoma sensitive protein (Rb), causing the dissociation of the transcription activator E2F from Rb[68]. Thus, activated E2F promotes cell cycle progression by mediating the transcription of cyclins E and A and other proteins, including ribonucleotide reductase (RNR), thymidylate synthase, dihydrofolate reductase and DNA polymerase, which are required for the subsequent DNA synthesis[67]. Thereafter, cyclin E binds to CDK2 to form the CDK2-cyclin E complex, which can initiate S phase. When cyclin A replaces cyclin E forming the CDK2-cyclin A complex, S phase progression is promoted. During the G2 phase, CDK1 replaces CDK2 to form the CDK1-cyclin A complex. Cdc25C is responsible for the dephosphorylation and activation of CDK1-cyclin A to facilitate the progression along the G2 phase[67]. The aforementioned Rb, when hypophosphorylated is considered to be a tumor suppressor controlling the G1 restriction checkpoint[66]. Conversely, the hyperphosphorylation of Rb (pRb) allows E2F release, which promotes the cells through the cycle[69].

1.7 Pro-metastatic proteins

Metastasis is a process regulated by several signalling pathways and modulated by the surrounding extracellular matrix. Local invasion, intravasation, survival in circulation, extravasation, and colonization are the stages that tumor cells undergo to spread from the primary tumor to distant organs through blood and lymphatic vessels[70].

Epithelial-mesenchymal transition (EMT) is a process that allows polarized epithelial cells to acquire a mesenchymal phenotype, enhancing their migratory and invasive capabilities[71]. EMT turns plays a crucial role in promoting tumor progression and metastasis[72]. Cadherins are proteins involved in cell adhesion, in the control of cell morphology, motility and intracellular signalling events[73]. A low expression of E-cadherin is at the basis of the EMT activation[74,75] and in this way the cell-cell contact turns to be based on N-cadherin[76].

A protein that binds to the cadherin/catenin complex is zonula occludens (ZO) protein-1[77]. ZO1 is a membrane-associated component of tight, adherent junctions found at cell-cell contact sites. ZO-1 was first described as a component of tight epithelial junctions together with claudins and occludins[78,79]. It is found in normal human skin where it is expressed mainly in the granular and in the transition layers of the epidermis and in the upper part of the spinous layer, as well as in the endothelial cells of the blood vessels of the dermis. In the granular layer and endothelium, ZO-1 is probably involved in the formation of tight junctions[80–82]. Instead, in malignant melanoma the upregulation of ZO-1 is associated with a more aggressive phenotype since it co-localizes and interacts with N-cadherin contributing to cell invasion and adhesion[83].

SIRT1 is a nicotinamide adenine dinucleotide (NAD^+ Protein Deacetylase) -dependent, belonging to the mammalian sirtuins family. SIRT1 is involved in many physiological processes, including cellular metabolism, senescence, and stress responses[84,85]. Over the past decades, several studies have shown that SIRT1 is involved in the initiation and progression of various cancers by altering cellular processes, such as cell proliferation, apoptosis, invasion, and metastasis[86–88]. However, the molecular mechanism of SIRT1 in melanoma progression and metastases remains poorly investigated. Only recently, Sun et al. reported that SIRT1 induces the EMT by accelerating the degradation of E-cadherin through autophagy, facilitating melanoma metastasis[89] (*Figure 5*).

SOX2 is a key regulatory gene that encodes for a transcription factor important in embryonic stem cell pluripotency[90,91]. Since its initial characterization in embryogenesis and development, SOX2 expression has been found in poorly differentiated neoplasms affecting a variety of organs[92,93]. It has recently been found that SOX2 is preferentially expressed in human primary and metastatic melanoma compared to non-malignant nevi[94]. Furthermore, in melanoma specimens SOX2

immunopositivity was related to dermal invasion, as assessed by the correlation with increased tumor thickness, a marker of poor prognosis[94].

The hepatocyte growth factor/scattering factor tyrosine kinase receptor c-Met plays a crucial role in the development of the invasive phenotype of neoplastic cells, thus representing an attractive candidate for targeted therapy in a variety of malignant tumors, including melanoma[95]. In fact, c-Met is a cell surface tyrosine-kinase receptor constitutively active in melanoma cells[95,96]. C-Met expression level was investigated together with the expression of the tyrosine-protein kinase receptor UFO (AXL), an oncoprotein involved in metastasis and resistance to various anti-cancer drugs[97], as well as the expression of Fos-related antigen 1 (Fra1) protein belonging to the transcription factor activator protein 1 (AP1) complex[98].

MAPK activation comprises the major signalling cascade that regulate differentiation-associated gene expression in the epidermis[99,100]. The cascade consists of the already mentioned upstream regulatory proteins (new protein kinase C and Ras), a MAPK module (MEKK1, MEK3 and p38 δ) and transcription factors AP1[134]. The activator protein transcription factors one (AP1) is one of the most interesting and important regulators of the epidermis growth[101]. An initial stimulus causes a cascade of sequential phosphorylation and activation of kinases leading to an increased level of transcription factor AP1. The AP1 binding to its DNA response element in the target genes leads to an increase in transcription[100,102]. AP1 complex includes members of the jun (c-jun, junB, junD) and fos (c-fos, FosB, Fra-1, Fra-2) protein families, which form jun/jun and jun/fos homo- or hetero-dimers[103,104]. AP1 proteins control keratinocyte proliferation[105,106], differentiation[107] and apoptosis[108] and are important in tumor progression[132,133,142]. Fos-like antigen-1 (Fra1) is frequently overexpressed in epithelial tumors where it can regulate the expression of several target genes[109–111]. Regulation of Fra1 occurs at both transcriptional and post-translational level[98]. Fra1 can affect many biological functions, such as tumor proliferation, differentiation, invasion, and apoptosis. Fra1 is overexpressed in breast, lung, colorectal, prostate, nasopharynx, thyroid, and other cancers including melanoma[111–113].

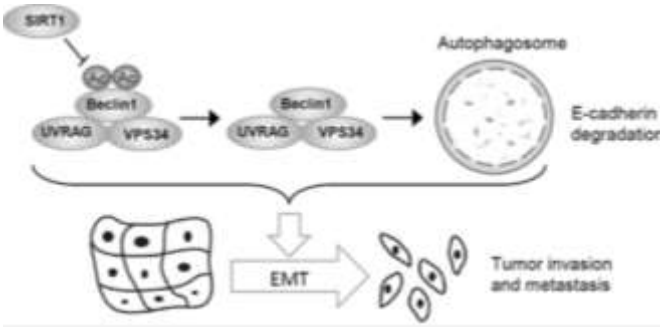


Figure 5. Schematic diagram of the mechanism of SIRT1-mediated autophagic degradation of E-cadherin stimulating EMT in melanoma cells[89].

1.8 Onconase

Onconase (ONC), also known as Ranpirnase or P30 protein, is a 12 kDa highly basic protein (pI 9.7) extracted from oocytes and early embryos of *Rana pipiens*. It is a monomeric member of the secretory pancreatic type ribonuclease A superfamily [114]. The ONC physiological functions have not been investigated. Perhaps, we suggest it can protect frog oocytes and embryos by the exogenous RNAs since ONC has been reported to be active against many pathogens. In contrast to its supposed protective effects in the extracellular fluids, it is well known that ONC becomes very dangerous if enters the cells where it is able to degrade intracellular RNAs. Thus, it is not surprising that this enzyme has shown antitumor properties against different types of cancer for which it reached phase II clinical trial for non-small cell lung cancer and phase III for malignant mesothelioma[115]. Thanks to its basic charge, it is better internalized in cancer cells which display more negatively charged residues on their plasma membranes than normal ones. Indeed, the presence of sulphate, phosphate and carboxylate groups of carbohydrates and lipids of the cancer cell membrane favours the establishment of Coulomb interactions with ONC[116]. Thus, ONC affects more selectively cancer cells than normal ones. It has been demonstrated how ONC enters the cells through AP-2/clathrin-mediated endocytosis to be routed to the receptor recycling compartment. Once in the early endosome, ONC neutralises the low endosomal pH so that an efficient translocation to the cytosol takes place[117].

As the other members of RNase family, it hydrolyses the phosphodiester bonds present in RNAs. However, the specificity of action against the different RNA types and the molecular mechanism involved in ONC-mediated cytotoxicity is still debated. It has been hypothesized that ONC, once in the cytosol, degrades tRNA leading to protein synthesis inhibition and therefore apoptosis induction[118], although other Authors reported that ONC antitumor activity is not ascribable to a generalized protein synthesis inhibition[119] (*Figure 6*). Moreover, Saxena et al. demonstrated that ONC selectively cleaves tRNA leading to an increased level of both tRNA turnover and synthesis[120]. More recently it has been discovered that, not only tRNAs, but also miRNA precursors are targets of ONC, some of which are known to induce resistance to PARP inhibitors, a class of molecules used in clinic to counteract several tumors including melanoma[121]. Furthermore, ONC displays other interesting properties, such as its extraordinary conformational stability (T_m = 87°C), and its resistance to proteases. The major contribute to its cytotoxicity is the ability to evade the endogenous ribonuclease inhibitor [121]. In fact, the cellular negatively charged RNase Inhibitor (RI), present either in the cytosol and in the nucleus of eukaryotic cells, usually acts as a barrier for other RNases, such as RNase A, RNase 1 and angiogenin (ANG), forming very tight complexes in order to hinder their enzymatic activity [122,123]. Notably, the Kd values of such interactions are comprised between the pico- and the femto-molar range[124]. Contrarily, amphibian ONC can evade RI as it lacks many of the flexible

regions and loops containing the key residues responsible for the RNase-RI interaction[125,126]. In fact, the Kd of ONC-RI complex is measurable only at salt concentrations lower than the physiological ones[127], and for this reason the intracellular ribonucleolytic activity of ONC is not affected or only partially reduced in the presence of RI.

ONC displays, both *in vivo* and *in vitro*, a synergic effect with several anti-cancer drugs, such as tamoxifen, cisplatin, vincristine, lovastatin[128] and doxorubicin[129]. In addition, it was recently demonstrated that A375 melanoma cell line treated for long time with the PARP inhibitor AZD2461 were more susceptible than the parental cells to ONC treatment suggesting that ONC can be an important tool to counteract melanoma following a previous chemotherapeutic treatment[130].

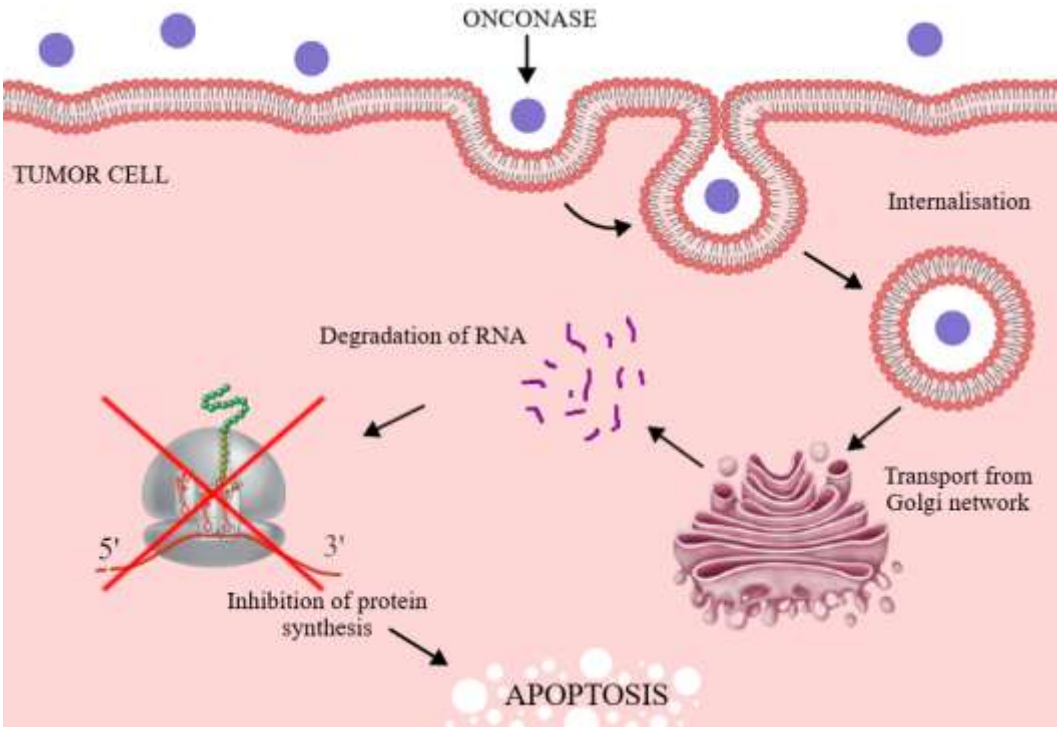


Figure 6. **Schematic representation of intracellular routing of ONC.**
Cytotoxicity of ONC is linked to its internalization in cancer cells and degradation of different types of RNA (miRNA, tRNA). It leads to inhibition of protein synthesis, cell cycle arrest, and/or apoptosis (programmed cell death).

1.9 Onconase oligomerization

The ONC small dimension allows its glomerular filtration and renal accumulation, so that the limit for ONC therapeutic use is its reversible nephrotoxicity[131]. The enlargement of ONC moiety dimension could help to limit its renal uptake and may represent a promising strategy to reduce side effects and increase the lifetime of the enzyme in the blood. It is known that some pt-RNases, such as RNase A and the natively dimeric bovine seminal ribonuclease (BS-RNase), can oligomerize [132,133] through the three dimensional domain swapping (3D-DS) mechanism[134] when they are lyophilized from 30-50% acetic acid (Hac) solutions[135] or after thermal incubation in various solvents, such as 40% aqueous ethanol (EtOH)[136]. These enzymes can be assembled in non-covalently linked dimers, trimers, and oligomers[124,137–139] by swapping both their N- and C-termini, so they can shape in different dimeric and multimeric RNase conformers [132,133,133,136,140,141]. Moreover, it had already been reported that RNase A oligomers display a cytostatic effect in acute myeloid leukemia cell lines and reduce tumor growth derived from human melanoma cells transplantation in nude mice [142]. Also ONC can form one dimer (ONC-D) through the same 3D-DS mechanism [143], and reconstitutes its active site and function upon dimerization[143] (*Figure 7*). Certainly, ONC can self-associate only through the swapping of its N-termini because the C-terminus is blocked by a disulfide involving Cys87 and the C-terminal residue Cys104. This constraint reduces its oligomerization tendency[144], but provides an increased enzyme stability[145]. Surprisingly, the recently resolved crystal structure of ONC-D has shown to be structurally quite different from the model that was previously proposed, although it was confirmed that it is N-swapped. Finally, it must be recalled that the ribonucleolytic activity of ONC is definitely lower that of RNase A, but it is necessary for its antitumor effect[146].

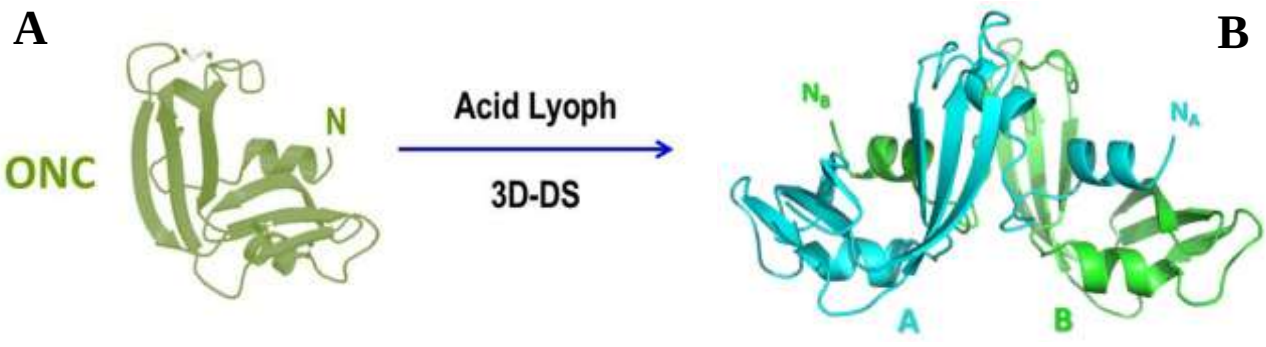


Figure 7. Structures and crystallographic structure. **A)** Amphibian onconase (ONC); **B)** Onconase dimer (ONC-D) crystallographic structure [147].

1.10 Micro RNAs

Micro RNA (miRNAs) are small non-coding RNAs (ncRNAs), about 20-24 nucleotides long, with regulatory functions. They were found for the first time in nematodes, and later observed also in other animals, plants and viruses[148]. MiRNAs are essential for mRNAs post-transcriptional regulation and they are implicated in many cellular processes and in protein expression as they control mRNAs degradation and/or translational repression of their target genes[149]. It must be pointed out that one single miRNA can bind to different targets thus affecting the expression of more than one protein but also a single mRNA may be the target of many different miRNAs[150]. Not surprisingly, miRNA dysregulation is linked to different human pathologies including cancer[151]. In fact, cumulative evidence showed that miRNAs are abnormally expressed in different tumor types. Thus, miRNAs displaying only inhibitory actions, may exhibit either tumor-suppressive or tumor-promotive effects depending on the function of their target genes. In cancer, miRNAs targeting tumor-suppressor genes are usually upregulated, in this way eliciting lower expression of such tumor-suppressors. At the same time, miRNAs that target oncogenes are downregulated, leading to an increase of oncogene expression and supporting malignant progression.

In order to become functional, miRNAs must undergo a complex maturation process consisting of two endonucleolytic cleavages carried out by multiprotein complexes. Such complexes, comprising RNase III enzymes Drosha in the nucleus and Dicer in the cytoplasm, are responsible for turning the primary miRNAs (pri-miRNAs) into precursor miRNAs (pre-miRNAs) first and subsequently into mature miRNAs[150,151]. Their biogenesis process is highly regulated and relies on the coordinated action of coactivator and corepressor RNA-binding proteins[150]. In addition, miRNAs expression is influenced by any change that may occur in the cellular environment, since a complex crosstalk between miRNAs and signal transduction effectors takes place in the cell, as, on one hand many signalling pathways regulate miRNAs biogenesis and on the other, miRNAs expression controls cell signalling downstream[151].

Although noncanonical binding sites exist, mature miRNAs usually bind the 3'untranslated region (3'UTR) of their target mRNAs to reach the inhibitory effect on translation [152].

Considering their importance in several cellular processes, miRNAs may have a key role in the development of new therapeutical strategies against different cancers.

2. AIMS OF THE THESIS

Therapy for malignant melanoma is far from being successful, therefore the main aim of this thesis is to find alternative therapeutical strategies to counteract melanoma progression and relapse. In this context, the antitumor effect of the cytotoxic ribonuclease ONC, was tested on several types of melanoma cell lines different for aggressiveness and/or for the presence or absence of specific mutations in their genome. Despite the promising *in vitro* results, nephrotoxicity is the main limit for a continuous therapeutical application of ONC *in vivo.* So, to decrease ONC filtration in kidneys and at the same time preserve its antitumor activity, enlarging the size of ONC exploiting its oligomerization tendency, could be a possible strategy. Therefore, in the laboratory of Professor Gotte, ONC-D has been produced by ONC lyophilization from 30-50% HAc solutions and its purification has been carried out. At the same time, ONC-D has been crystalized and its structure resolved for the first time in the laboratory of Professor Merlino, in Naples.
Our aim has been the study in parallel of ONC and ONC-D antitumor activity on several human cancer cell lines to better understand the influence of oligomerization on the antitumor activity of this enzyme. Therefore, we measured:

- Cell viability
- Cell proliferation rate
- Apoptosis
- Anchorage-independent growth capability.

Furthermore, the exploration of ONC molecular mechanism of action has been an important object of our study, as well. In detail both ONC species can inhibit the activation of signalling pathways crucial for cancer cell growth or death, such as STAT3 pathway.
Moreover, considering its ribonucleolytic activity, it is reasonable to think that ONC anti-melanoma effect could regulate the stability of some ncRNA species, which usually control protein expression in cell. The expression level of 16 miRNAs were analysed in A375 and FO1 cell line treated with ONC.
The following miRNAs were stabilized in ONC-treated samples:

- MiR-34a-5p: a tumor suppressor with a central role in cell proliferation and apoptosis
- MiR-20a-3p: studied in relation to malignant neoplasms in which it presents an altered expression

- MiR-29a-3p: generally considered to be a tumor suppressor miRNA

In the end, our attention has been focused on the expression level of proteins encoded by target genes of these upregulated miRNAs. Our investigations aimed at finding out which key proteins among those related to the overexpressed miRNAs were downregulated by ONC.

3. MATERIALS AND METHODS

3.1 Protein Production

Recombinant wide type-ONC monomer, dimer and trimer were produced from *E. coli* and purified using a Superdex 75 HR 10/300 column equilibrated with 0.1M Tris-acetate, 0.3M NaCl, pH 8.4 by Prof. Gotte, Neuroscience, Biomedicine and Movement Sciences Department, University of Verona.

3.2 Cell cultures and Mycoplasma detection

Human melanoma A375 and FO1 (CRL-12177) cell lines as well as Hepatocellular carcinoma HEPG2, glioblastoma U251 and Normal human epidermal melanocytes NHEM cell lines were grown in high glucose Dulbecco's modified Eagle Medium (DMEM, Gibco, BRL Invitrogen Corp., Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, BRL Invitrogen Corp., Carlsbad, CA, USA), and 1% antibiotic antimycotic solution (Gibco, BRL Invitrogen Corp., Carlsbad, CA, USA). Instead, Metastatic melanoma MeWo cells were cultured in Roswell Park Memorial Institute 1640 medium (RPMI, Gibco, BRL Invitrogen Corp., Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, BRL Invitrogen Corp., Carlsbad, CA, USA), and 1% antibiotic antimycotic solution (FBS; Gibco, BRL Invitrogen Corp., Carlsbad, CA, USA).

All cell types were cultured at 37°C in a humidified atmosphere with 5% CO_2 and were purchased from ATCC, Manassas, VA, USA.

Mycoplasmas represent a large group of microorganisms that often infect cell cultures interfering with cell physiology and metabolism[153]. Considering that experiment results may be altered by Mycoplasma infections, all cell lines were tested once a month to detect such infection according to the following protocol.

. To extract DNA, cell pellets were resuspended in 10 mM Tris-HCl, PH 8.8 and heated at 95°C for 10 min and then at 56°C for 5 minutes. Proteinase K (20 μg/μl) was added, and the samples were placed again at 56 °C for 10 minutes and at 95 °C for 10 minutes. Quantification was performed using Nanodrop UV-vis spectrophotometer (Thermo Scientific, Milan, Italy) and Polymerase Chain Reaction (PCR) was carried out as reported in *figure 8* using primers for specific mycoplasma

detection. PCR products were electrophoresed on 1% agarose gel at 100 V and the signal was detected by ChemiDoc (Bio-Rad, Hercules, CA, USA).

PRIMER FORWARD	5' - ACT CCT ACG GGA GGC AGC AGT A - 3'
PRIMER REVERSE	5' – TGC ACC ATC TGT CAC TCT GTT AAC CTC- 3'

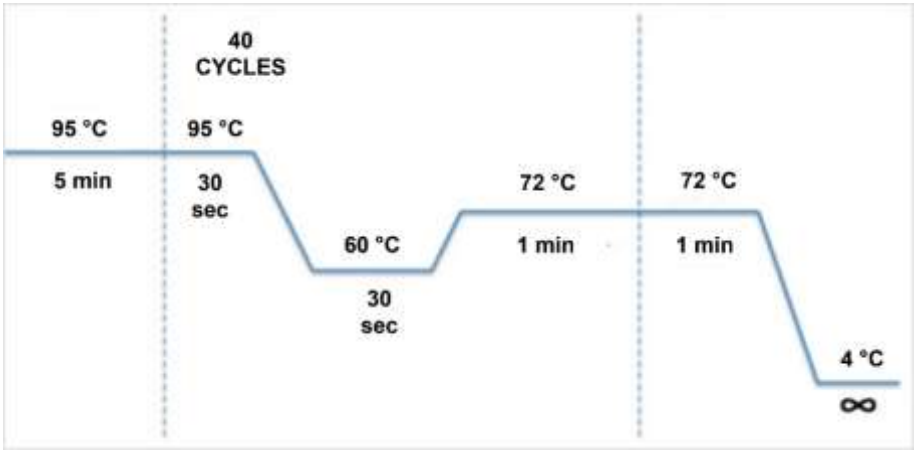


Figure 8. Primer used and schematic representation of PCR steps for mycoplasma detection.

The results of one of the PCR carried out with the primers specifically designed for Mycoplasma genome detection are shown in *Figure 9* Contrary to what happened in positive control samples in correspondence of 700 bp no bands appeared, suggesting that all cell lines were not infected by such microorganism.

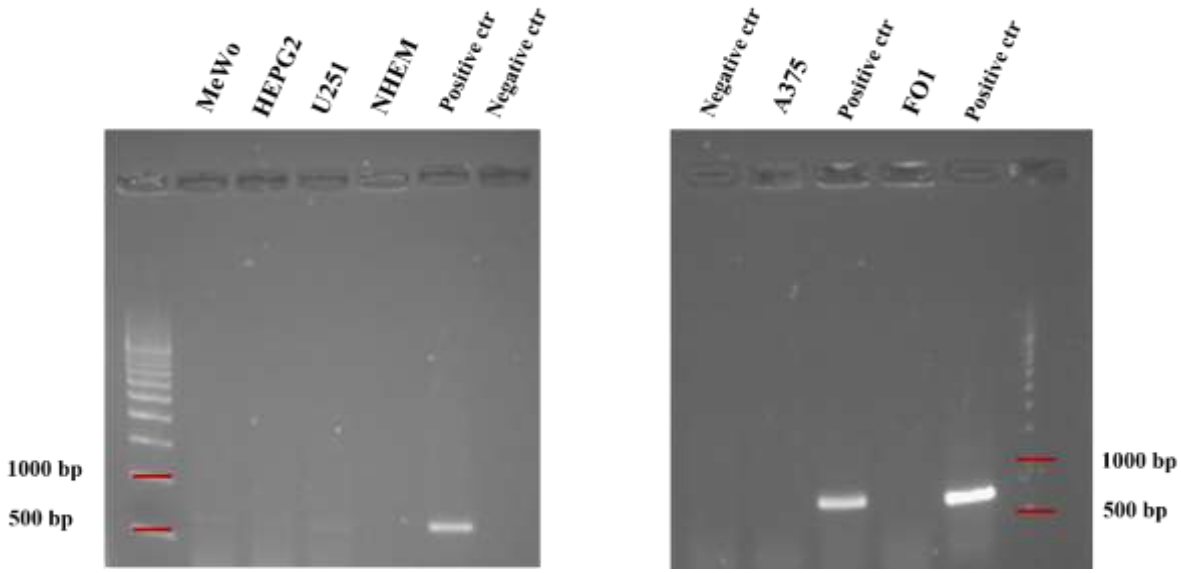


***Figure* 9.** Polymerase chain reaction (PCR) revealed that all cell lines used in this thesis (A375, MeWo, HEPG2, NHEM and FO1) are Mycoplasma free. Indeed, no bands are detected in correspondence of 700 bp while for the positive controls a strong signal is visible.

3.3 Cell viability assay (Sulforhodamine B assay)

Cells were seeded in 96-well plates at different densities (A375, FO1, HEPG2 and U251: 2.9×10^3 cells/well; MeWo and NHEM 5×10^3 cells/well). After 24 hours, cells were treated with different concentrations of ONC monomer, dimer or trimer or RNase A monomers or dimers for 24, 48 or 72 hours. Cells were fixed by adding 25 µL/well of 50% (W/V) trichloroacetic acid (TCA) directly into the culture medium. Plates were incubated at 4 °C for 1 hour, washed four times with dd-H_2O and dried at room temperature (RT). Staining was performed by adding 50 µL/well of 0.04% (W/V) sulforhodamine B sodium salt solution (SRB, Sigma-Aldrich, Milan, Italy). After 1 h incubation at RT, plates were rinsed with 1% acetic acid (HAc) and air-dried. SRB was solubilized in 10 mM Tris-base solution, at pH 10.5, and Abs_{540} measured in the Tecan NanoQuant Infinite M200-Pro plate reader (Tecan Group Ltd., Männedorf, Switzerland). Four to six replicates were performed for each condition.

3.4 5-Br-2′-deoxy-Uridine cell proliferation assay

Cell proliferation was assessed with a colorimetric immunoassay based on the measurement of 5-Br-2′-deoxy-Uridine (BrdU) incorporation during the DNA synthesis phase. MeWo or A375 cells were seeded in 96-well plates at the same concentrations used for SRB assays. 24 hours after seeding, cells were incubated with or without ONC monomer or dimer. Four hours before the end of the treatment BrdU (BrdU Elisa Kit; Merck, Darmstadt, Germany) was added to each well and incubated until completing the treatment. Then, the medium was aspirated, and cells were incubated with a fixing-denaturing solution for 30 min. Plates were washed three times and the anti-BrdU detector antibody was added for 90 min. After a 30 min incubation at RT with peroxidase goat anti-mouse IgG conjugate antibody, plates were washed three times. The tetramethylbenzidine chromogenic peroxidase substrate was added and plates left 5 min in the dark. Finally, a stop solution (1 M H_2SO_4) was added and the Abs_{450} measured using TECAN NanoQuant Infinite M200 Pro reader (Tecan Group Ltd.)

3.5 Flow cytometry experiments

2–5 × 10^5 A375 or MeWo cells were harvested after the desired treatment, pelleted by centrifugation (3 min, 300g, 4 °C) and resuspended in 100 µL of 1× Binding Buffer. Then, 2.5 µL of Annexin V-FITC (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA) were added to each sample. After 10 min incubation at RT, cells were washed and resuspended in 100 µL of 1× Binding Buffer. Before measuring fluorescence by MACS-Quant 10 analyzer (Miltenyi Biotec, Bergisch Gladbach, Germany), 20 µg mL^{-1} of propidium iodide were added to each well. Data analysis of four independent experiments was performed using FlowJo software version 10 (Tree Star, Ashland, OR, USA).

3.6 Soft agar colony formation assay

Before seeding cells in a 6-well plate, the bottom layer of each well was filled with 1% low gelling temperature agarose and 2× DMEM supplemented with 10% FBS and 1% Antibiotic Antimycotic Solution (Gibco, BRL Invitrogen Corp., Carlsbad, CA, USA) and air-dried for 30 min. A375 or MeWo cells (500,000 cell/well) were suspended in 0.6% agarose and 2× DMEM supplemented with 10% FBS and 1% Antibiotic Antimycotic Solution (Gibco, BRL Invitrogen Corp.) and placed over the 1% agarose layer. 0.2, 0.8µM ONC or ONC-D were added directly into each well, and 100 µL of fresh media were added twice a week. 14 days later cell colonies appeared, and they were stained with Crystal violet solution. The semi-solid cultures were performed in triplicate and images captured with the EVOS FL AutoCell Imaging System (Thermo Fisher Scientific, Carlsbad, CA, USA).

3.7 RNA extraction and reverse transcription

Cells were seeded in 12-well plates (150000 cells/well) and 1 µM ONC was added to the culture medium for 48 hours. At the end of the treatment, the culture medium was aspirated from the plate and 1 ml/well of Trizol Reagent (ThermoFisher Scientific, Carlsbad, CA, USA) was added. The content of each well was then transferred to 1.5 ml tubes and centrifuged for 5 minutes at 12000 g, 4°C. After adding 200 µl of chloroform, the samples were mixed by inversion, incubated for 3 minutes at RT and centrifuged for 15 minutes at 12000 g, 4° C.

The supernatant, containing the RNA, was placed into a new tube and 500 µl of 100% isopropanol were added. The samples were then incubated 10 minutes at room temperature and centrifuged for 10 minutes at 12000 g, 4° C. Isopropanol was removed and the pellet containing RNA was washed with 75% ethanol and centrifuged for 5 minutes at 7500 g. The supernatant was removed, and the air-dried pellet was resuspended in 30 µl of RNase free H_2O. RNA was quantified using Nanodrop UV-vis spectrophotometer (Thermo Scientific, Milan, Italy).

3.8 Real Time – PCR

After performing reverse-transcription, miRNA expression levels were determined by real-time polymerase chain reaction (RT-PCR). TaqMan Fast Advanced Master Mix and the specific probes for 16 selected miRNAs (Thermo-Fisher Scientific) were used. The expression levels were normalized on miR-191-5p. The real-time PCR was performed in the Bio-Rad CFX Connect Real-Time System using the TaqMan Advanced miRNA probes (Thermo-Fisher Scientific, Milan, Italy). Relative quantification was calculated by Pfaffl's formula[154]. Each measurement was carried out in triplicate in three different experiments. Differences in the relative expression levels were analysed using unpaired, two-tailed Student's t test.

3.9 Total protein extracts and sample preparation for western blot analysis

A375, MeWo or FO1 cells were seeded in 6 cm Petri dishes (190 300 cells/dish) and treated with or without 1 µM ONC monomer or dimer. After 48-72 h-treatment, cells were scraped using warm 1X sample buffer (2% SDS, 10% glycerol, 50 mM Tris-HCl, 1,75% β-mercaptoethanol, and bromophenol blue) and boiled at 99 °C for 10 min. Total protein extracts were kept at -80 °C until use.

3.10 Western blot analysis

Protein extracts were electrophoresed on a 7.5-10 % polyacrylamide SDS-PAGE and transferred to a polyvinylidene difluoride membrane (PVDF, ThermoFisher Scentific, Waltham, MA, USA). Membranes were blocked at RT for 1 h with TBST buffer (10 mM Tris-HCl pH 7.5, 100 mM NaCl, 0.1% Tween20) containing 5% bovine serum albumin (BSA, Serva Electrophoresis GmbH, Heidelberg, Germany). Then, they were incubated on a shaker, overnight at 4 °C, with a 5% BSA solution containing primary antibody against cleaved PARP (#5625, 1:1000), pTyr705 STAT3 (#9145, 1:1000), pRb (#8516, 1:2000), pThr202/Tyr204 ERK (#9101, 1:1000), LDHA (#3582, 1:1000), pSer473 Akt (#4060, 1:2000) (Cell Signaling Technology, Danvers, CO, USA); pTyr527 Src (GTX133473, 1:3000), Bcl2 (GTX100064, 1:4000), Akt (GTX121937, 1:3000), ALDO A (GTX101408, 1:3000), Cyclin D1 (GTX106624,1:3000), Cyclin A2 (GTX-103042, 1:3000), cMet (GTX100637), CREB (GTX112846, 1:3000), pThr160 Cdk2 (GTX-133862, 1:3000), p21 (GTX-629543, 1:3000), p27 (GTX100446, 1:3000), ENO-1 (GTX101803, 1:3000), ERK1/2 (GTX134462, 1:3000), G6PD (GTX101218, 1:3000), HIF1α (GTX127309, 1:3000), PDK1 (GTX 105999, 1:3000), PGM2 (GTX119168, 1:3000), pSer37 PKM2 (GTX133886, 1:3000), SOX2 (GTX101507, 1:3000), Src (GTX134412, 1:3000), uPAR (GTX100467, 1:3000), ZO-1 (GTX108592, 1:3000) (Genetex, Alton Pkwj Irvine, CA, USA); AXL (813196-1-AP, 1:2000) (Proteintech, Manchester, UK); SIRT1 (Sc-74465, 1:1000), pSer727 STAT3 (Sc-136193, 1:1000) (Santa Cruz Biotechnology, Dallas, TX, USA). Membranes were washed three times with TBST buffer for 30 min, and then incubated for 1 h with a horseradish peroxidase-conjugated secondary antibody (anti rabbit 1:6000, Genetex, Alton Pkwj Irvine, CA, USA or 1:400 anti-mouse, Cell Signaling Technology, Danvers, CO, USA). After this second incubation, membranes were washed other three times for 30 min with TBST buffer. Protein extracts were then normalized with a β-actin protein antibody (GTX-124214, 1:10000; Genetex). Immuno-detection was carried out with an ECL kit (GE-Healthcare, Little Chalfont, UK) and the chemiluminescence signals were detected with ChemiDoc (Bio-Rad, Hercules, CA, USA).

3.11 Gelatin zymography

A375 cells were cultured in a six-well plate in high glucose Dulbecco's Eagle's Medium (DMEM) supplemented with 10% FBS, 1% Antibiotic Antimycotic Solution (Gibco, BRL Invitrogen Corp., Carlsbad, CA, USA) and treated with or without 1μM ONC for 48 hours. The FBS containing medium was then removed and cells were washed twice with FBS-free medium. Cells were serum-starved for 24 hours, and the condition media was collected and concentrated 10X by using an Amicon Ultra-2 Centrifugal Filter Unit (Millipore, USA). The concentrated conditioned media were quantified using Bradford assay and 0.5 μg of each sample were added to 2 μl of non-reducing sample buffer (50 mM Tris HCl pH 6.8, 2% SDS, 10% glycerol, 0.1% bromophenol blue). The samples were then electrophoresed through a 7.5 % SDS-polyacrylamide gel containing gelatin (4 mg/ml) to separate proteins by molecular weight. Gel was run at 200 V, 350 mA for around 60 minutes at 4 °C. The gel was washed twice for 30 minutes with washing buffer (2.5 % Triton X-100, 50 mM Tris- HCl PH 7.5, 5 mM $CaCl_2$, 1 μM Zn Cl_2) in order to remove SDS.

After placing the gel in incubation buffer (1 % Triton X-100, 50 mM Tris- HCl PH 7.5, 5 mM $CaCl_2$, 1 μM Zn Cl_2) for 24 hours at 37 °C the gel was stained with staining solution (20% ethanol, 10% acetic acid, 0.5% Coomassie Brilliant Blue R-250, H_2O) for at least one hour. After rinsing the gel with water, it was incubated with destaining solution (methanol, acetic acid, H_2O) until white bands clearly appeared. The white bands correspond to the activity of gelatinases.

3.12 Glycolysis-cell based assay

To evaluate the glucose amount flowing into glycolytic pathway, L-lactate, the end-product of this metabolic process, was detected in ONC treated or non- treated cells. The commercial Glycolysis Cell-Based Assay kit (600450, Cayman Chemical, Michigan, USA) was used. A375 and FO1 cells were seeded in a 96-well plate (2.9×10^3 cells/well) and treated with or without ONC in FBS free medium for 48 hours. At the end of the treatment the plate was centrifuged at 1000 rpm for 5 minutes and 10 µl of the supernatant of each well was transferred into a new plate. The assay was carried out according to manufacturer instructions, the absorbance at 490nm (Abs490) was measured in the Tecan NanoQuant Infinite M200-Pro plate reader (Tecan Group Ltd., Männedorf, Switzerland). Four replicates were performed for each condition.

3.13 Statistics

All the results are reported as a mean value ± SD. p values were determined using unpaired, two-tailed Student's t test, (*, if $p < 0.05$, or **, if $p < 0.01$). For each type of experiment, a minimum of three independent biological replicates were performed.

4. RESULTS

4.1 Anti-Tumor Activity of RNase A Dimers on Human MeWo and A375 Melanoma Cell Lines

Before investigating the anti-melanoma activity of ONC, we analysed the possible cytotoxic effect of some dimeric forms of RNase A, which is not cytotoxic in its natural monomeric form. It is know that bovine seminal RNase (BS-RNase) together with other RNases display a cytotoxic activity on malignant cells only when they are in the dimeric form since in this form they can escape the inhibitory action of intracellular RI[155]. So, the antitumor activity of RNase A dimers was analysed after incubation of human MeWo and A375 melanoma cells with 25, 50, 100 μg/mL of N- or C-dimers obtained applying the two HAc- or EtOH-methods on RNase A monomer. Preliminary results (not shown) indicated the antitumor activity of the dimeric species suffers time latency, similarly to that exerted by ONC in A375 cell line[32,130]. Therefore, in *figure 10* only the results of the SRB cell viability assay performed after 72 h cells incubation were reported. The two RNase A monomer species (M_{-H}, or M_{-Et}) at high concentration (100 μg/mL) did not affect cell viability at all in both cell lines. Similarly, in MeWo cells, no significant reduction of cell viability was detected after 72 h incubation with 25 and 50 μg/mL C_{D-H} or C_{D-Et} species, but a significant decrease occurred at the highest concentration (100 μg/mL) of C_{D-Et}, but not of C_{D-H}. Conversely, both N_{D-H} and N_{D-Et} exerted a concentration-dependent reduction of MeWo cell viability (EC_{50}: 100 μg/mL). Indeed, in A375 cells both dimeric species, regardless to the method used for their oligomerization, and except for C_{D-H} and N_{D-H} at 25 μg/mL, were able to significantly decrease cell viability (*figure 10*).

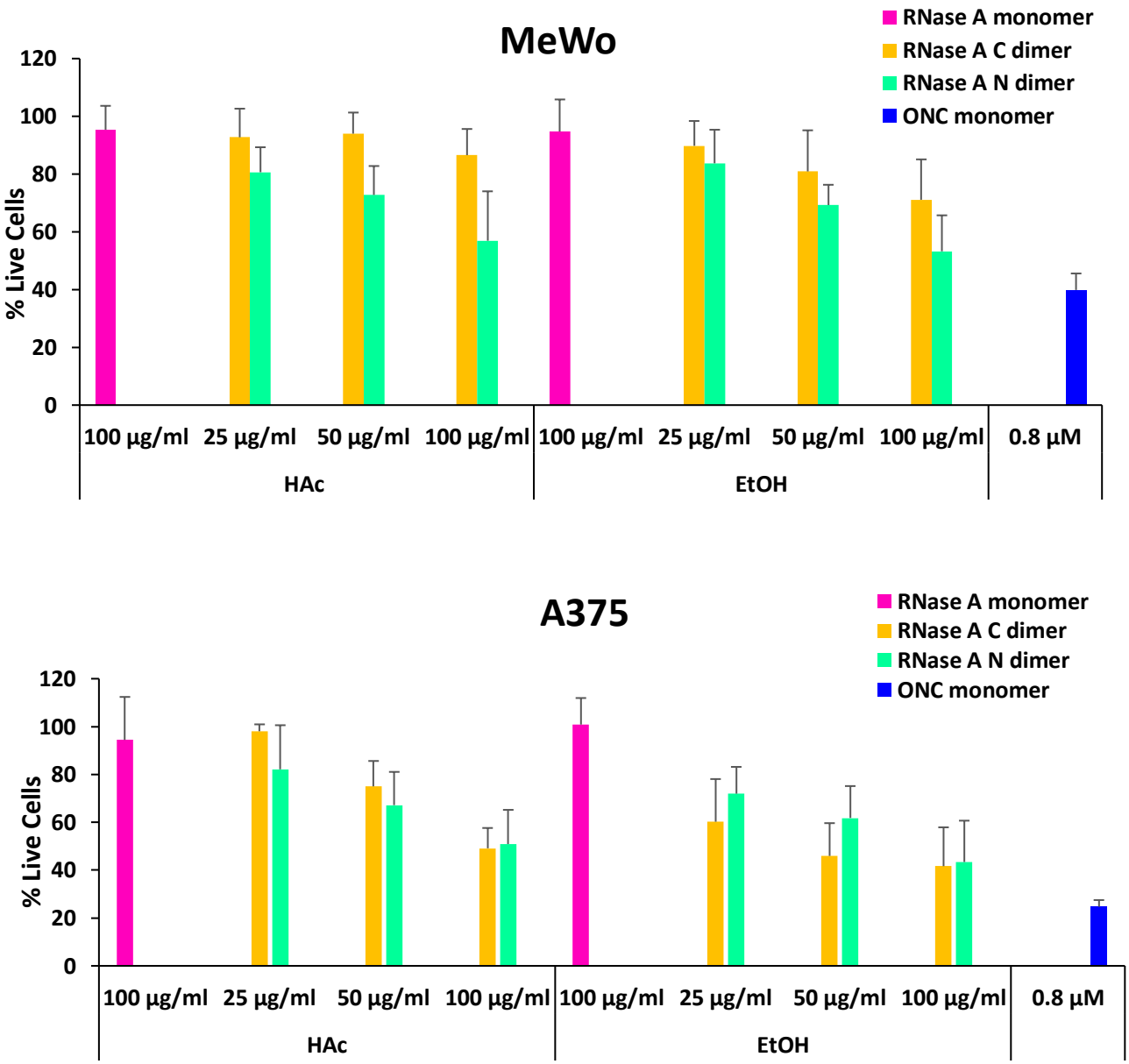


Figure 10. Effects of RNase A monomers or of N- or C-swapped dimers on MeWo and A375 human melanoma cell viability. SRB assays were performed after 72h treatments with the indicated concentrations of the different RNase A species. Blue bars show the effect of 0.8 µM of ONC on cell viability and it was used as positive control. All values reported are the mean ± S.D. of four to five independent experiments, each performed in six replicates. The statistically significant differences in cell viability induced by the dimeric species vs. each relative monomer are shown (* $p < 0.05$; ** $p < 0.01$).

4.2 ONC reduces cell viability in melanoma cells

It had already been demonstrated that ONC reduces cell viability of parental A375 melanoma cells and, at higher extent, on dabrafenib resistant and AZD2461-long treated A375 cells[32,130]. Moreover, ONC effect seems to be cancer cell-specific considering that no reduction in viability was detected in ONC-treated normal human epithelial melanocytes (NHEM). At this point we wondered whether ONC displayed a good cytotoxic/cytostatic activity also in FO1 cells, another BRAF mutated melanoma cell line. As shown in *figure 11 A*, ONC reduces cell viability of FO1 cells in a dose dependent manner, even if at lower extent when compared to A375 cells (EC_{50}: 0.5μM). Moreover, the immunoblot analysis was performed to understand whether ONC might induce apoptosis in FO1 cells. Indeed, the expression of the anti-apoptotic protein Bcl2 did not significantly change upon ONC treatment while Bcl-XL, another anti-apoptotic protein, slightly decreased. Notably, similarly to the results reported in Raineri et al. for A375 cells[32], the level of the well-known marker of apoptosis cleaved PARP 1 was significantly increased in treated cells compared to non-treated ones (*Figure 11 B*).

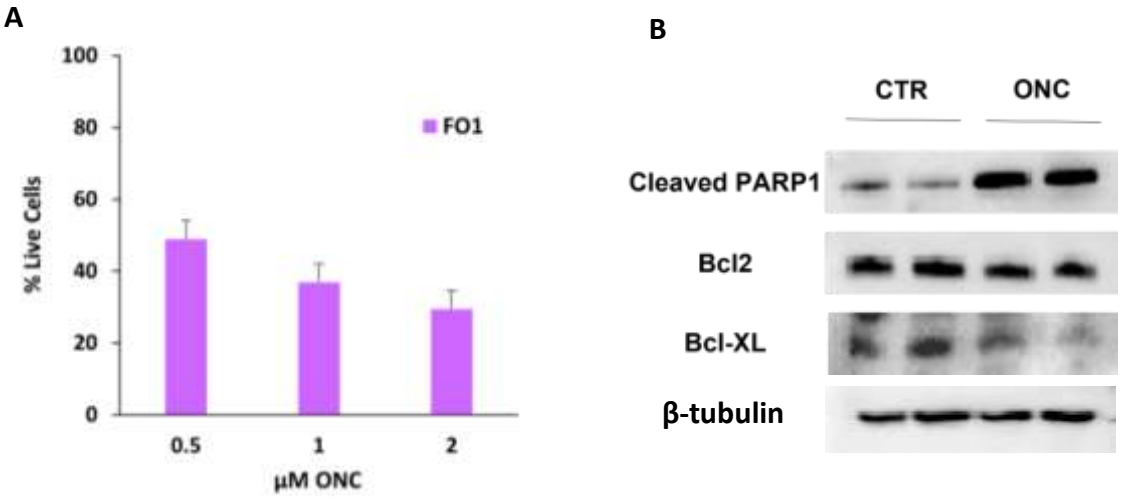


Figure 11. ONC effect on FO1 melanoma cell line.
A) Sulforhodamine B (SRB) assay performed on FO1 melanoma cells attesting ONC ability in reducing viability. All values reported are the average of at least four independent experiments, each performed in four replicates, ± S.D.
B) Representative immunoblots showing the expression levels of apoptosis-related proteins: ONC treatment significantly increases the expression of the apoptotic marker cleaved PARP1, the expression of the anti-apoptotic protein Bcl-XL is decreased while Bcl2, another anti-apoptotic protein is not significantly affected.

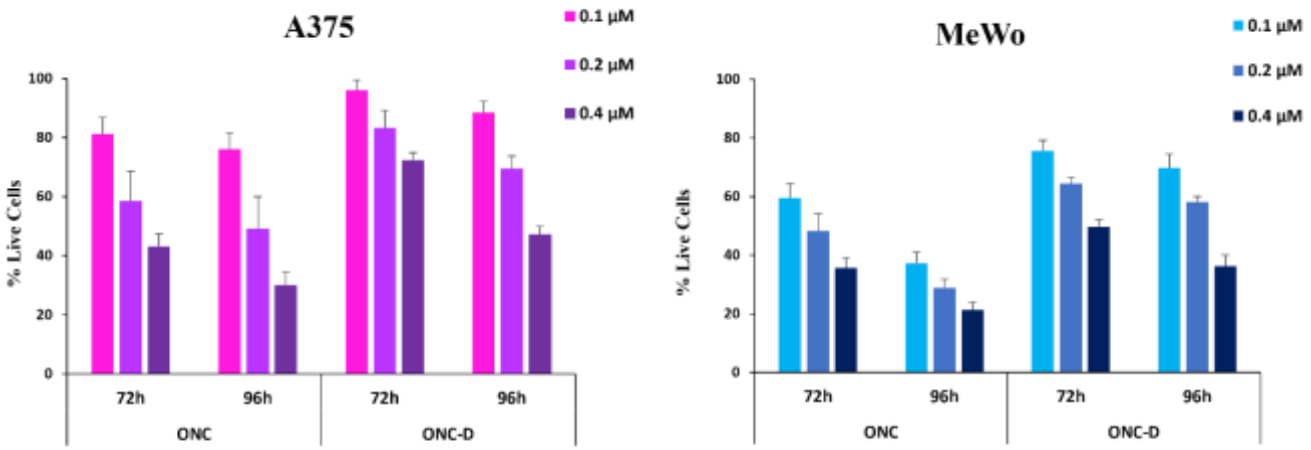
4.3 ONC-D reduces cell viability in melanoma cells

ONC dimerization leads to a structural variation that may affect the antitumor activity of such ribonuclease. Therefore, in order to understand whether ONC-D may reduce cell viability, SRB assay was performed on two melanoma cell lines. The BRAF mutated A375 melanoma cells and the wt-BRAF but p53 mutated MeWo cells were cultured in the presence or absence of increasing ONC or ONC-D concentrations for 24, 48, 72 and 96h. The results showed that cell viability was increasingly affected by both ONC and ONC-D in a time-dependent manner (*Figure 12 A*). Thus, we decided to perform further experiments only after 72h of treatment. As shown in *figure 12 B*, A375 cell viability was reduced in a concentration-dependent manner after 72 h-treatment with both ONC (red bars) and ONC-D (light red bars), but in the case of ONC-D the effect was less prominent. The effective concentration (EC) able to reduce A375 cell viability to 50% (EC_{50}) was 0.35 μM for ONC and of 0.8 μM for ONC-D. Notably, the monomer EC_{50} value is similar to those previously reported in Raineri et al.[32].

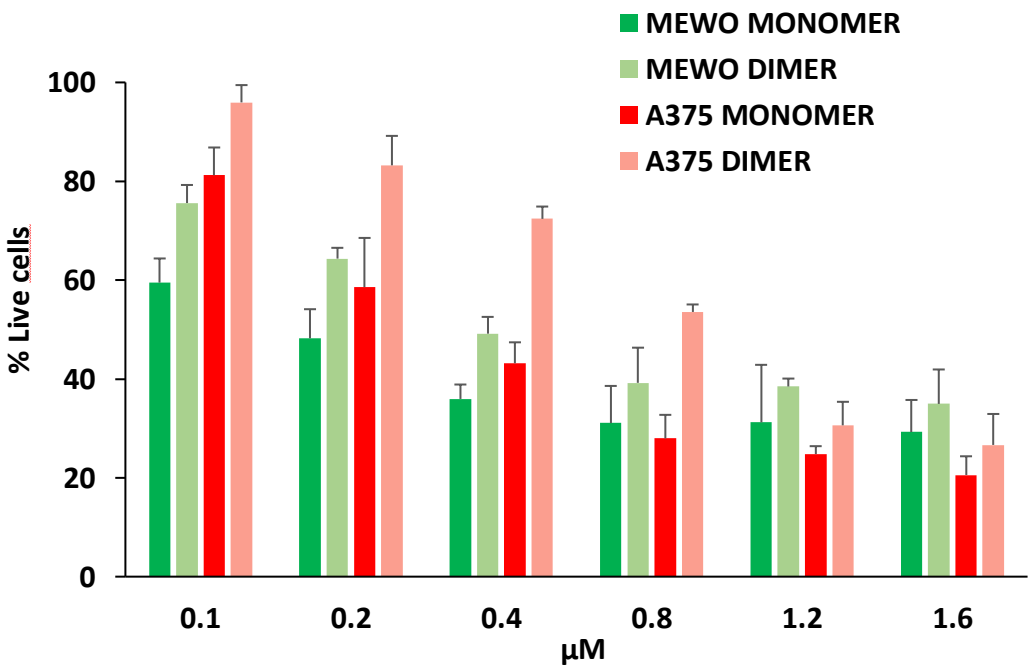
MeWo cells behaviour is similar: the viability is affected by both treatments even at lower concentrations compared to those used for A375 cells (*Figure 12 B*). Indeed, for MeWo cells the EC_{50} was 0.22 μM for ONC and 0.4 μM for ONC-D. Interestingly, the differences registered between ONC and ONC-D activities tend to disappear in both cells lines when their concentration increases.

The ability of both ONC forms to reduce MeWo cell viability achieve a plateau at concentrations higher than 0.8 μM. From these results emerges that ONC-D at low and medium concentrations was less active than ONC, but in any case, both ONC species were able to dose-dependently reduce cell viability of A375 and MeWo melanoma cells. Finally, NHEM cell viability was not significantly affected also by high ONC or ONC-D concentration (*Figure 12 C*). This behaviour could be explained by the melanoma cell features, that makes tumor cells more sensitive to ONC species than normal melanocytes. Indeed, tumor cells display a higher number of negatively charged sialic acid chains on their membranes[116], which facilitate ONC binding and its subsequent internalization in cells.

A


A375
0.1 µM
0.2 µM
0.4 µM
% Live Cells
100
80
60
40
20
0
72h
96h
72h
96h
ONC
ONC-D
MeWo
0.1 µM
0.2 µM
0.4 µM
% Live Cells
100
80
60
40
20
0
72h
96h
72h
96h
ONC
ONC-D

B


MEWO MONOMER
MEWO DIMER
A375 MONOMER
A375 DIMER
% Live cells
100
80
60
40
20
0
0.1
0.2
0.4
0.8
1.2
1.6
µM

C

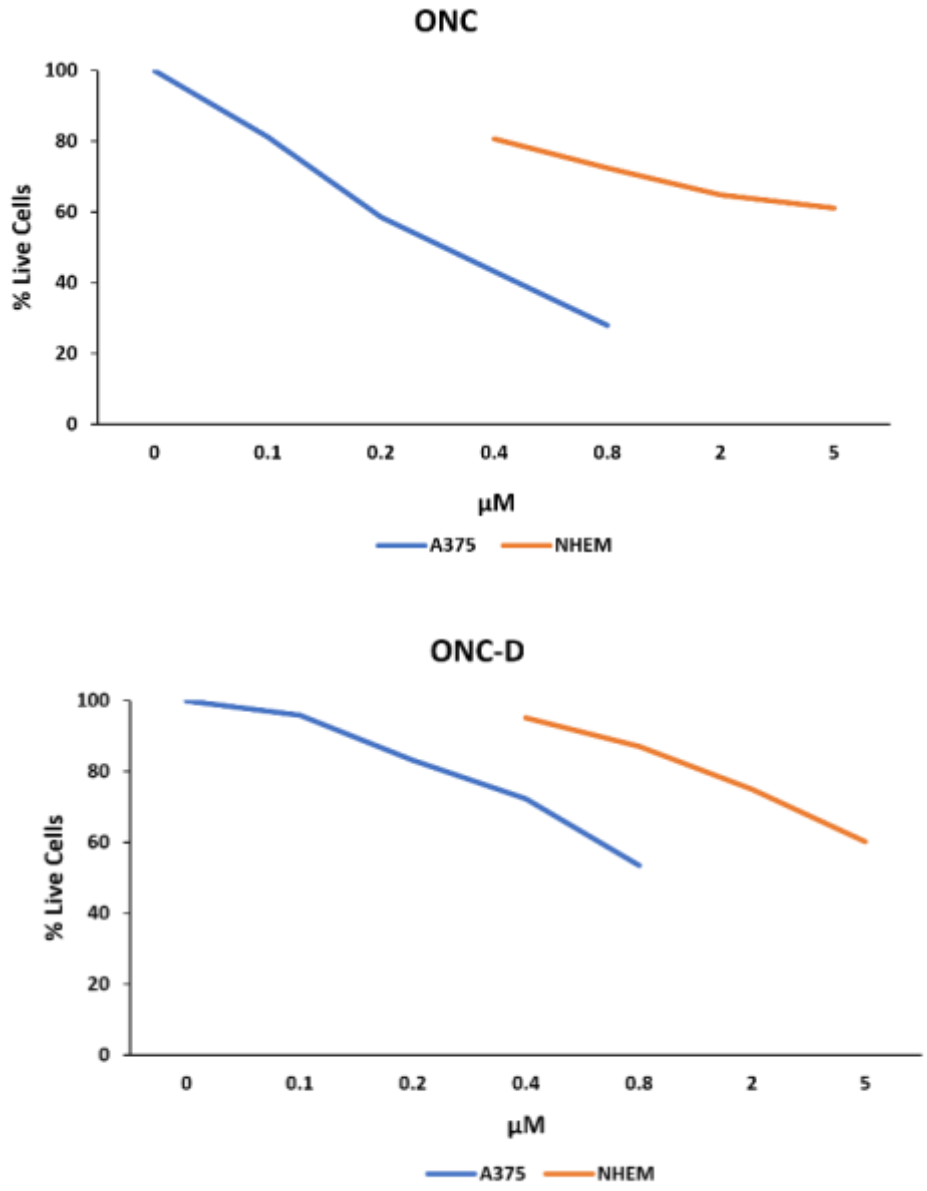


Figure 12. Cell viability (SRB) assay performed on A375 and MeWo cells treated with increasing concentrations of ONC or ONC-D.

A) ONC and ONC-D reduces cell viability of both A375 and MeWo cells in a dose and time dependent manner.

B) SRB performed after 72h ONC or ONC-D treatment. For each concentration, ONC and ONC-D comparison in MeWo cell line (0.1–0.4 µM), and in A375 cell line (0.1–0.8 µM) are statistically significant ($p < 0.02$). Instead, differences are not significant with higher ONC or ONC-D concentrations.

C) ONC and ONC-D treatments do not affect Normal Human Melanocytes cell viability at the same concentrations that were active in melanoma cell lines.

4.4 ONC can oligomerize into a trimer (ONC-T) that reduces cell viability in melanoma cells similarly to ONC

When the purified ONC after the oligomerizing procedure is chromatographed into a Superdex 75 HR 10/300 Increase SEC column three different peaks are obtained. The peak eluted at about 13.5 ml, corresponding to ONC, is preceded by a peak at 11.5 ml that is the ONC-D but also by a very little peak at 10.6 ml elution. This last peak could correspond to traces of a trimeric ONC specie (ONC-T) as the calculated molecular weight is about three times the ONC one. Considering the very low yield of ONC-T obtainable at every purification cycle we were able to perform only few experiments including SRB assay on A375 and MeWo melanoma cells. In both cell lines different concentrations of ONC-T after 72h-treatment strongly reduced cell viability in a dose dependent manner. Contrary to ONC-D, the trimeric form of ONC seemed to be at least as active as ONC. Indeed, the EC_{50} was 0.25 μM for ONC-T compared to 0.35 μM and 0.8 μM for ONC and ONC-D respectively in A375 cells (*Figure 13 A*). This trend was confirmed also in MeWo cells (*Figure 13 B*).

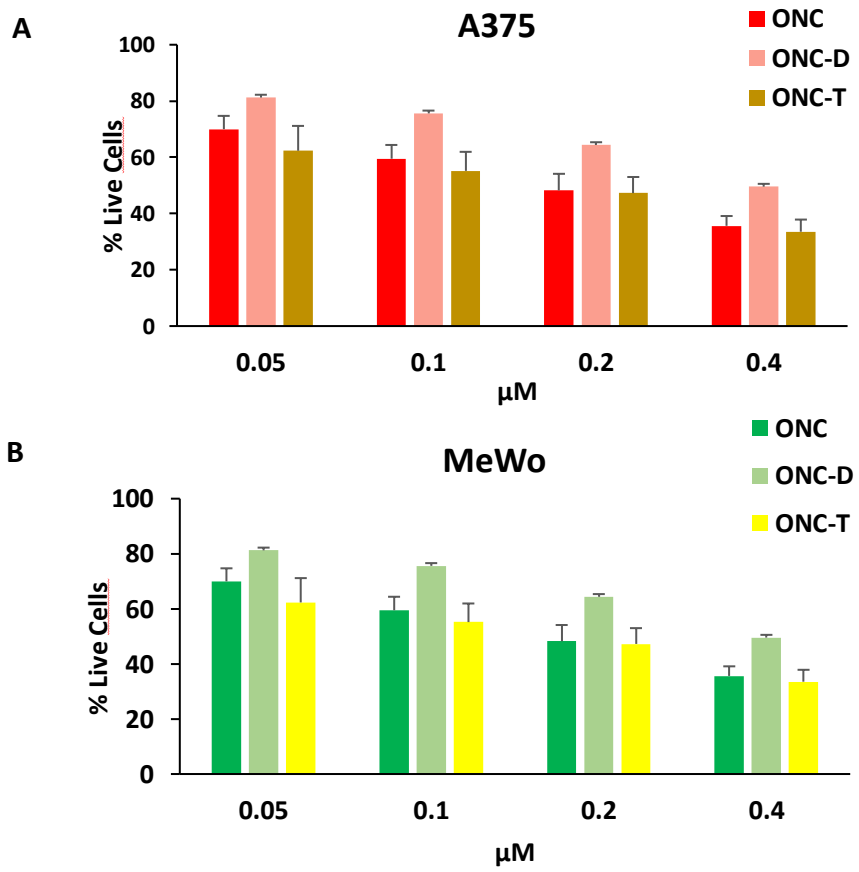


Figure 13. Sulforhodamine B (SRB) assay performed on A375 (**A**) and MeWo (**B**) cells attesting ONC-T ability in reducing viability similarly to ONC. All values reported are the average of at least four independent experiments, each performed in six replicates, ± S.D.

4.5 ONC species reduce cell viability in other cancer cell types

To validate the hypothesis that the two ONC species may be effective in reducing cell viability of human cancer cell types different from melanoma we performed SRB assays on hepatocellular carcinoma HepG2 and glioblastoma U251 cell lines. In detail ONC and ONC-D reduced cell viability in HepG2 and U251 cells respectively after 72h or 96h treatment (*Figure 14*). Even if the differences between the two ONC species were maintained in both cell lines, glioblastoma cells seemed to be more sensitive. Indeed, the calculated EC_{50} for HepG2 cells was 0.8 µM for ONC and higher than 0.8 µM for ONC-D (*Figure 14 A*) while for U251 cells EC_{50} was 0.1 µM for ONC and 0.4 µM for ONC-D (*Figure 14 B*). As shown in *figure 13 B* U251 cell were also treated with increasing concentration of ONC-T. Similarly, to what happened in A375 and MeWo cells, ONC-T showed to be as active as the monomer in U251 cell line with a EC_{50} of about 0.2 µM

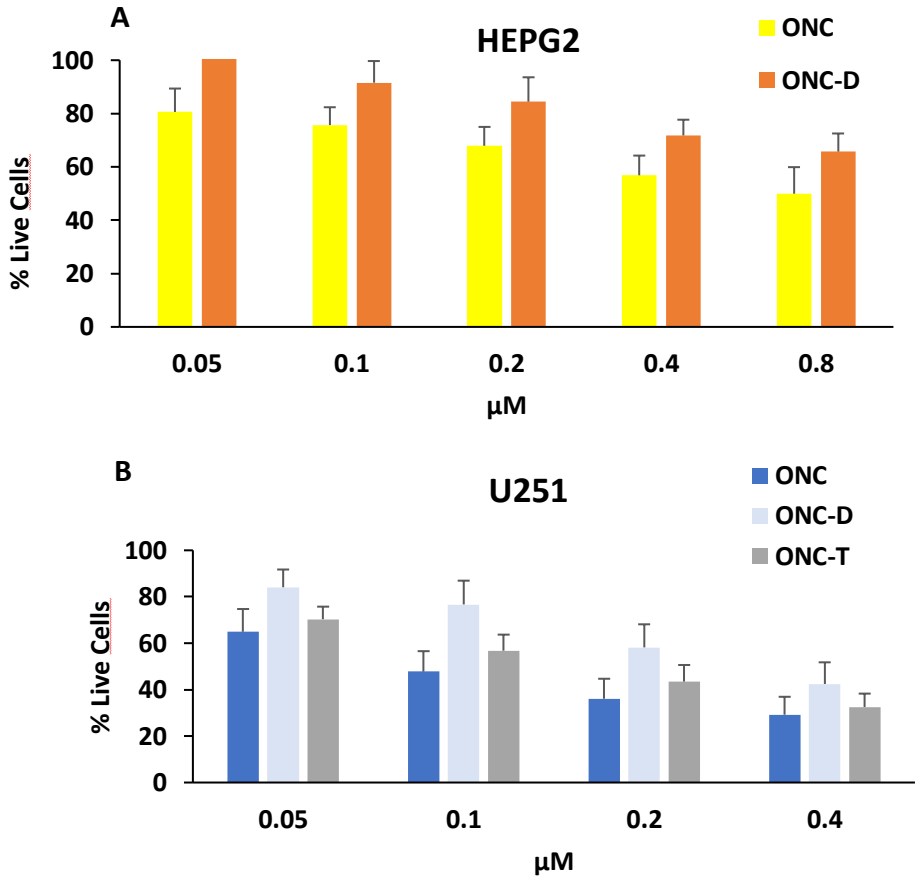


Figure 14. Sulforhodamine B (SRB) assay performed on cancer cell lines, other than melanoma, attesting ONC, ONC-D or ONC-T effect on viability is not melanoma specific. **A)** After 72h treatment with ONC or ONC-D the hepatocellular carcinoma HepG2 cell viability is reduced in a time dependent manner even if at lower extent when compared to A375 or MeWo cells. **B)** ONC, ONC-D and ONC-T exert a cytotoxic/cytostatic activity on U251 glioblastoma cell line after 96h treatment. All values reported are the average of at least four independent experiments, each performed in six replicates, ± S.D.

4.6 ONC, ONC-D and ONC-T reduces cell proliferation

Sulforhodamine B sodium salt assay showed a total loss in cell mass induced by the treatments, but it does not discriminate between increased cell death and decreased cell proliferation. Both processes, in fact lead to an overall loss in cell mass. To determine whether the ONC species affect cell proliferation rate, the measurement of 5-Br-2'-deoxy-Uridine (BrdU) incorporation during DNA synthesis was performed. After 72 hours of incubation with different concentrations of ONC, ONC-D or ONC-T, cells were treated with BrdU for 4 hours. A dose dependent reduction of BrdU incorporation was detected in A375 and MeWo cell lines treated with ONC species (*Figure 15).* However, for the poor yield of the trimeric form of ONC, it must be pointed out that only one experiment was performed with MeWo cells treated with ONC-T, for this reason no standard deviation can be measured in correspondence of that results in *figure 15*. These results demonstrated that the quantitative relationship existing between treatment types and concentrations used in the cell proliferation assays accurately reflects the results obtained under the same conditions with the SRB viability test. Furthermore, these suggest that a substantial part of cell viability reduction could depend on a cytostatic effect.

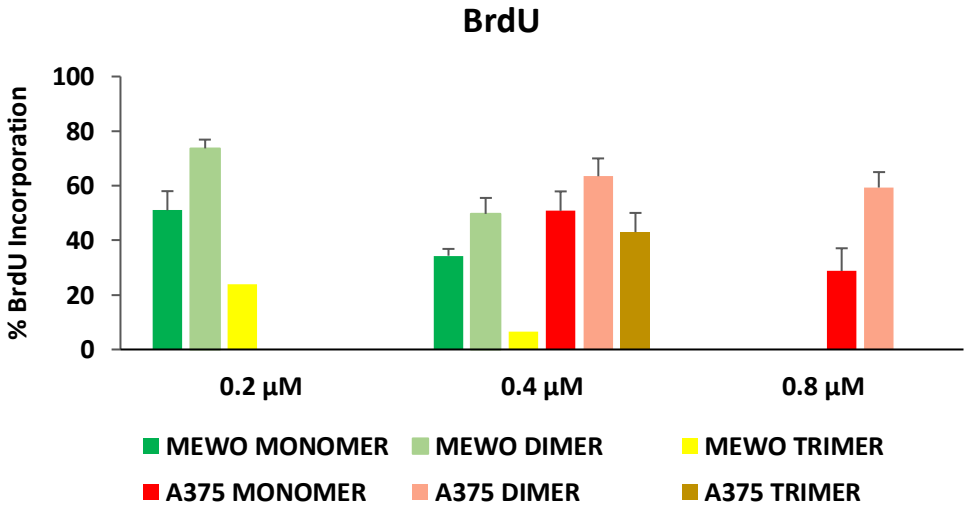


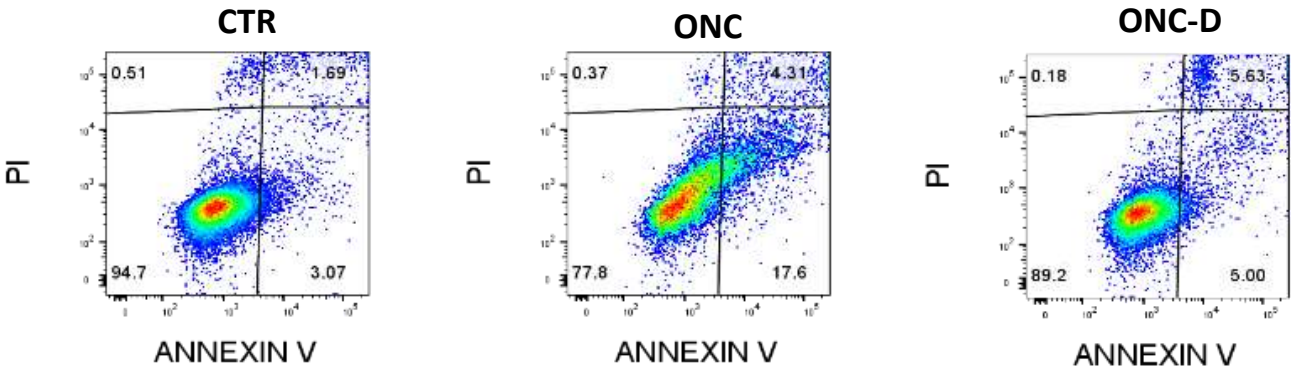
Figure 15. BrdU incorporation was carried out either in ONC, ONC-D or ONC-T treated cells. The comparison between ONC and ONC-D effects is statistically significant for each concentration ($p < 0.001$ for MeWo cells; $p < 0.01$ for A375 cells). All values reported, except for MeWo cells treated with ONC-T, are the average of at least four independent experiments, each performed in four replicates, ± S.D

4.7 ONC, ONC-D and ONC-T induces apoptosis only in A375 cells

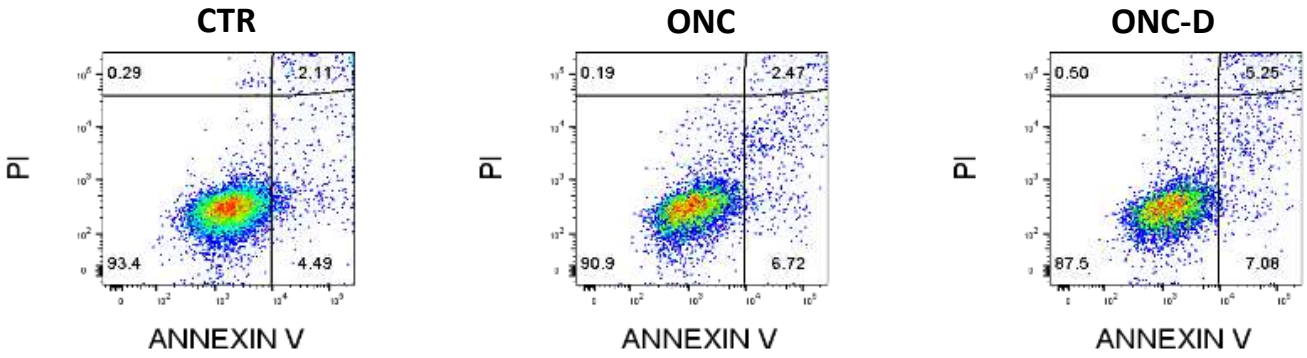
It had already been reported that, several A375 cells underwent apoptosis after ONC treatment[32], so we evaluated ONC or ONC-D ability to induce apoptosis in both A375 and MeWo cell lines. The expression of Poly (ADP-ribose) Polymerase (PARP) cleaved form, the anti-apoptotic protein Bcl2 by Immunoblot, and the cell positivity to Annexin V/Propidium Iodide (PI) in flow cytometry assay have been investigated (*Figure 16 A & B*). The percentage of Annexin V positive plus Annexin V/PI positive A375 cells after 72 h treatment with either ONC or ONC-D was about 22% and 11%, respectively, in comparison with a 5% value registered in not-treated samples. Data demonstrate that A375 cell line undergoes apoptotic cell death at a higher extent after ONC or ONC-D incubations than in the not treated control. Instead, no significant differences in the number of cells undergoing apoptosis occurs for either ONC/ONC-D treated or not-treated MeWo cells, as registered by flow cytometry and confirmed by cleaved-PARP immunoblotting (*Figure 16 A & B*). Due to the low yield of ONC-T only one flow cytometry experiment was performed. However, the preliminary result reported in *figure 16 C* shows that the percentage of Annexin V positive A375 cells after 72 h treatment with ONC-T was 51.9% in comparison with a 5% value registered in not-treated samples. Notably, for MeWo cells the 72h treatment with ONC-T did not lead to a remarkable increase in Annexin V positive cells (14.8%).

A

A375


CTR
ONC
ONC-D
PI
ANNEXIN V
0.51
1.69
94.7
3.07
0.37
4.31
77.8
17.6
0.18
5.63
89.2
5.00

MEWO


CTR
ONC
ONC-D
PI
ANNEXIN V
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2.11
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4.49
0.19
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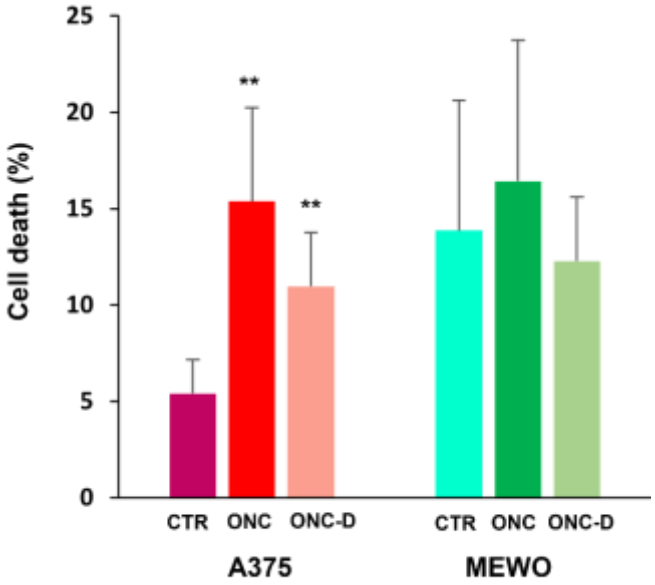
90.9
6.72
0.50
5.25
87.5
7.08


Cell death (%)
25
20
15
10
5
0
**
**
CTR
ONC
ONC-D
CTR
ONC
ONC-D
A375
MEWO

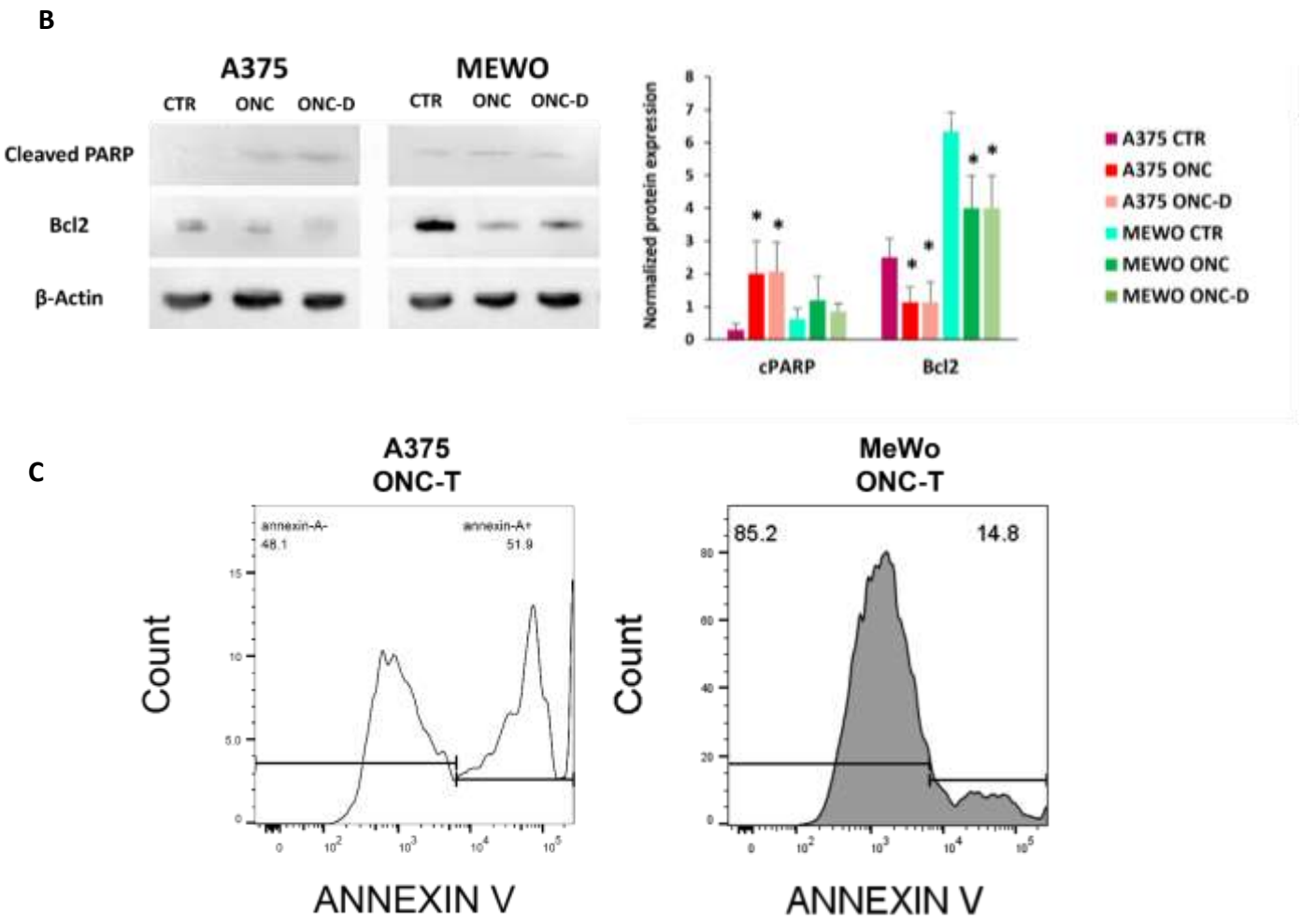


Figure 16 ONC, ONC-D or ONC-T induce apoptosis in A375 melanoma cells.
A) Representative flow cytometry experiment of cells labeled with Annexin V/PI: significant differences in Annexin V positive plus Annexin V/PI double-positive A375 cells incubated with 0.8μM ONC or ONC-D for 72h in comparison with not-treated control sample are shown; instead, no differences with the untreated control sample is visible in MeWo cells treated with ONC or ONC-D. The right panel shows the mean percentage (±S.D.) of apoptotic cell death after incubating cells with ONC or ONC-D, in comparison to the control. Values are calculated from four independent experiments; ** $p < 0.01$.
B) Left: representative immunoblot of the expression level of the apoptotic marker cleaved PARP, the antiapoptotic protein Bcl2 and β-actin in A375 and MeWo cells treated for 72h with 0.8 μM ONC or ONC-D. **Right:** quantification from three/four independent experiments. All comparisons were performed vs each control sample; * $p < 0.05$; **$p < 0.01$.
C) Preliminary flow cytometry experiment of A375 and MeWo cells treated with 0.8 μM ONC-T for 72h and labeled with Annexin V/PI. The percentage of Annexin V positive A375 cells in the treated sample is remarkably higher than the control (51.9% and 5% respectively). For MeWo cells the 72h treatment with ONC-T do not lead to a conspicuous increase in Annexin V positive cells (14.8%).

4.8 ONC and ONC-D affect Signal Transducer and Activator of Transcription 3 expression and activity

STAT3 is an important target in cancer therapy[150] as its activation regulates multiple gene functions during cancer, such as cell proliferation, apoptosis, metastasis, and angiogenesis. When different growth factors bind to their receptors the intracellular signalling can activate STAT3 [156]. In addition, Janus Kinases or the proto-oncogene tyrosine-kinase Src (Src) are able to induce STAT3 activation by the phosphorylation of its Tyr705 residue, allowing STAT3 nuclear translocation and binding to the promoter regions of many target genes[156].

Immunoblot results reported in *figure 17* displayed high level of STAT3 Tyr705 phosphorylation in not-treated A375 cells after 72 h culture. Whereas phosphoTyr705-STAT3 level was strongly reduced in A375 cells incubated for 72 h with either ONC or ONC-D. Differently, MeWo control cells, at the same time of culture, showed a lower phosphoTyr705-STAT3 level than A375 cells, suggesting that MeWo cells are less subject to stimuli capable of activating STAT3. In this cell line, ONC was still able to reduce STAT3 phosphorylation in tyrosine, whereas ONC-D appeared to be less active.

STAT3 can be phosphorylated also on serine 727 by several serine-kinases. ONC and ONC-D are able to decrease the phosphoSer727-STAT3 level in both melanoma cell lines, as well. Despite differences registered in their phosphorylation levels, STAT3 total protein is similarly expressed in both A375 and MeWo control cells. ONC and ONC-D can still reduce total STAT3 expression in both cell lines, although ONC-D in a less extent.

Finally, we tested by immunoblot the activation of tyrosine-kinase Src, the most important upstream kinase able to phosphorylate STAT3. In both A375 and MeWo cells, the expression level of the phosphorylated form of Src, as well as its total protein level, were well correlated with those of STAT3 Tyr705 and total STAT3, respectively (*Figure 17).* Notably, these results are comparable, as all the experiments were performed simultaneously with both cell lines.

In summary, these data suggest that the monomeric and the dimeric ONC can hinder STAT3 signalling at least by affecting Src expression and activity.

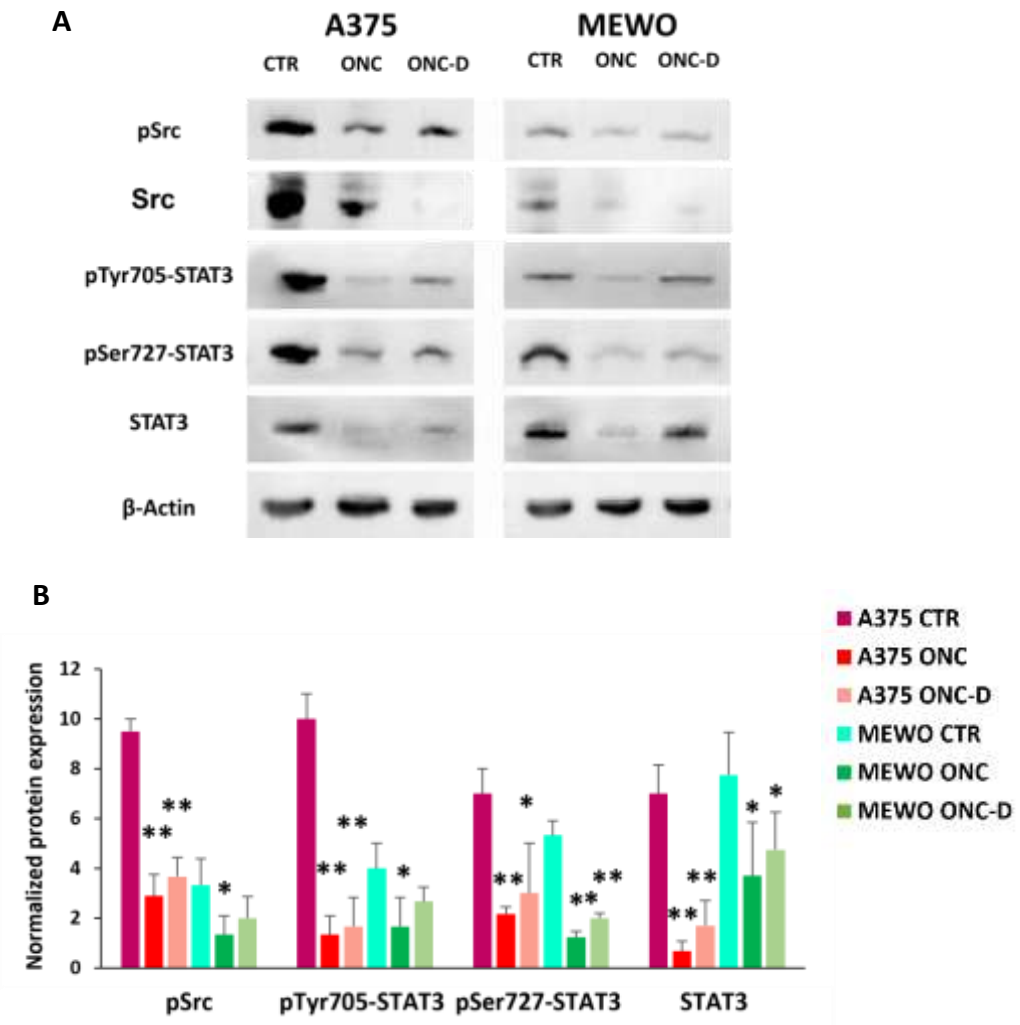


Figure 17 ONC, ONC-D affect STAT3 signalling pathway.

A) Representative immunoblot of the expression level of phospho-Tyr705 STAT3, STAT3, phospho-Ser727-STAT3, pSrc and β-actin in A375 and MeWo cells treated for 72h with 0.8 μM ONC or ONC-D.

B) Quantification from three/four independent experiments. All comparisons were performed vs each control sample; * $p < 0.05$; ** $p < 0.01$.

4.9 ONC and ONC-D effect on anchorage-independent cell growth

Tumor cells can grow in an anchorage-independent manner. The soft agar colony formation assay is a well-established method for characterizing this capability[157]. *Figure 18* shows a picture of A375 and MeWo colonies present in soft agar after 0.2 and 0.8 µM ONC or ONC-D incubations in comparison with untreated samples (CTR). ONC and ONC-D reduce at the same extent the colony formation capability of both cell types in a concentration-dependent manner.

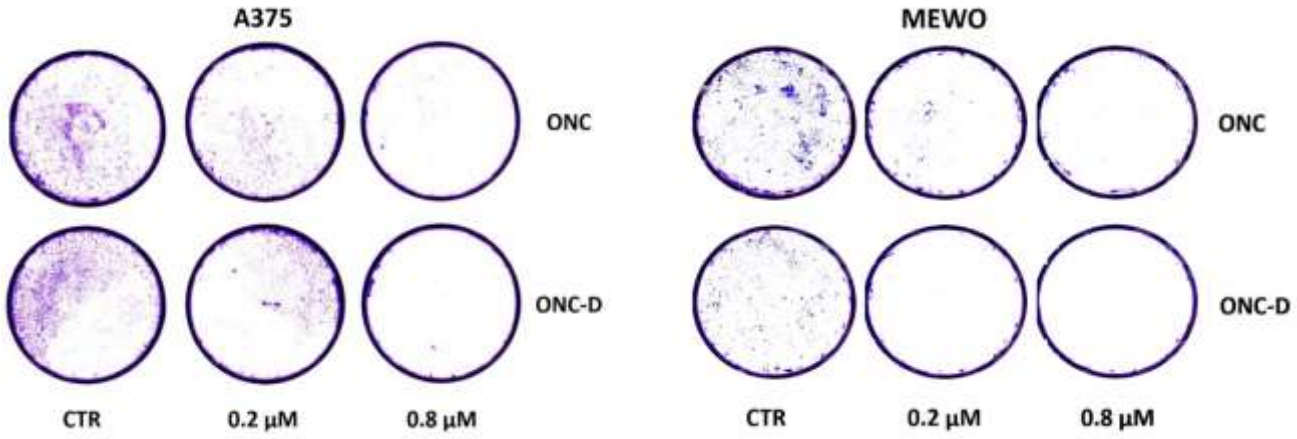


Figure 18. ONC and ONC-D affect anchorage-independent growth. A375 and MeWo cell lines were seeded on the upper soft agar layer in 6-well plate and treated with ONC or ONC-D. After 14 days, cell colonies were stained with a crystal violet solution and colony images detected with the EVOS FL Auto Cell Imaging System.

4.10 miRNAs are modulated by ONC in A375 and FO1, two BRAF-mutated melanoma cell lines

ONC is a ribonuclease that is known for damaging tRNA species and other non-coding RNAs. Nevertheless, its specific targets are still unknown. To deepen the knowledge of ONC mechanisms of action inside the cell, the monomer was chosen to perform the following experiments. In a first screening performed on A375 melanoma cells treated with or without 1µM ONC, the expression levels of 25 miRNAs that had been previously related to cancer have been measured by RT-PCR (not shown). In this case, 1µM ONC concentration was chosen according to the previous results of cell viability assay and considering that the cells were treated for 48 hours instead of 72 hours. The only two down-regulated miRNAs and many up-regulated miRNAs with at least 2.5-fold change expression were selected for further analysis. Then, the two downregulated miRNAs were not confirmed either in a second or a third experiment, therefore they have been excluded. From the other ones, 16 have been selected for their level of expression that were resulted rather comparable in a second and a third experiment. All of which were discovered to have onco-suppressor functions. Thus, in this thesis the effect of ONC on the expression of such 16 onco-suppressor miRNAs, was evaluated. In detail *figure 19 A* shows that miR-20a-3p, miR-29a-3p, and miR-34a-5p were significantly upregulated ($p < 0.01$), while other three miRNAs, miR-128-3p, miR-20a-5p and miR-941, were upregulated, but with lower p-values. The expression levels of the same up-regulated miRNAs (miR-20a-3p, miR-20-5p, miR-34a-5p, mirR-128-3p, miR-941, and miR-29a-3p) were also measured on FO1 melanoma cells treated with or without 1µM ONC. Data reported in *figure 19 B* show in FO1 cells a significant over-expression of miR-20a-3p, miR-34a-5p and miR-29a-3p, in agreement with the expression level of these miRNAs in A375 cells but with lower p-values ($p < 0.05$), except for miR-20a-3p that resulted highly expressed ($p < 0.01$) in both cell lines.

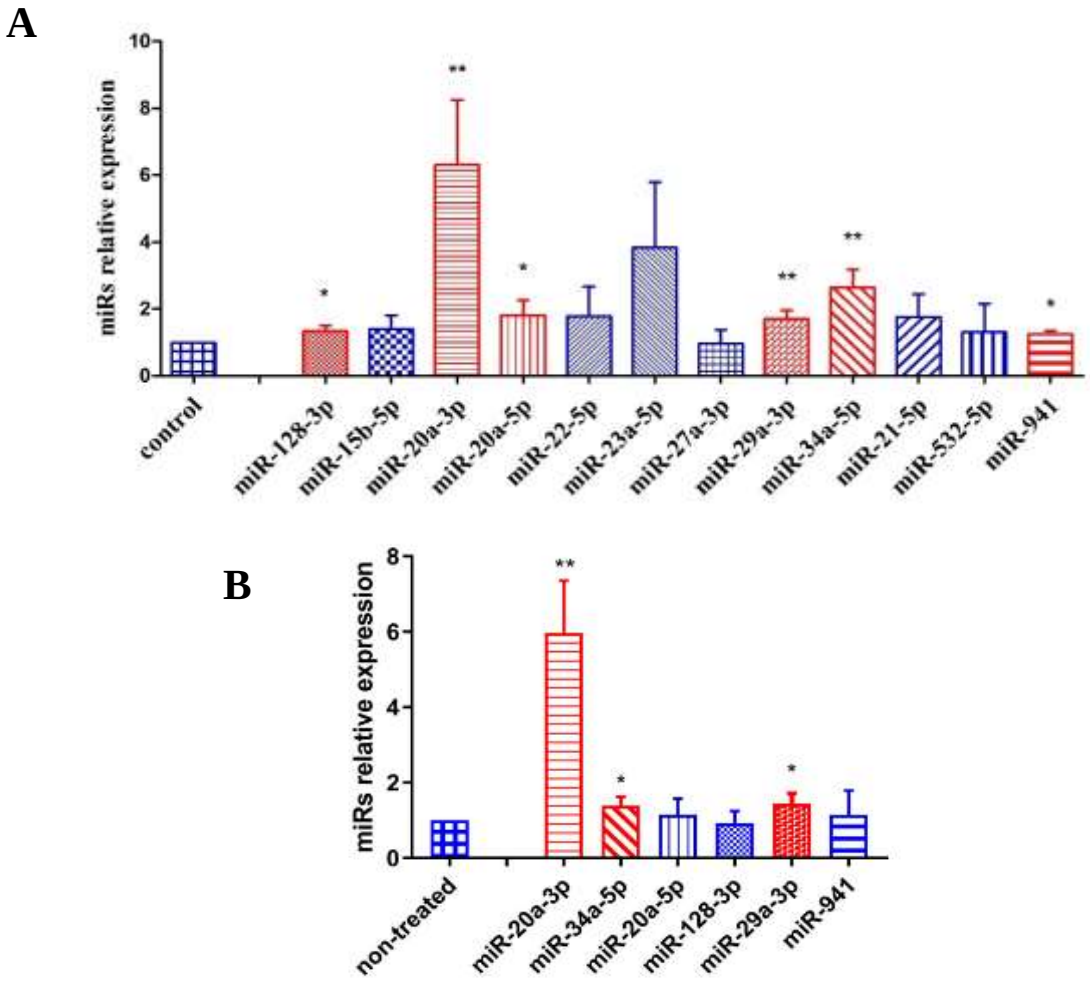


Figure 19. Relative expression of miRNAs after 48 h incubation of A375 (**A**) or FO1 (**B**) cells with ONC. Cells were cultured for 48 h with 1 µM ONC. Red bars refer to the onco-suppressor miRNAs that were upregulated by ONC at a statistically significant level; the blue ones refer instead to miRNAs whose expression level was not significantly different from the one relative to the untreated control. The mean values ± S.D. of miRNAs expression level measured by RT-PCR and deriving from three independent experiments are shown. All comparisons were performed vs. each control sample after normalization to miR-191 expression; * $p < 0.05$, ** $p < 0.01$.

4.11 Predicted mRNA-target interactions

In order to drive our investigation towards specific proteins that might be up or down regulated in ONC treated cells different databases were queried to determine the interactions occurring between the upregulated miRNAs and the mRNAs of proteins involved in cell cycle regulation, metabolism and signalling pathways usually altered in melanoma. In *figure 20* the genes that in literature have been predicted and/or validated to be targets of each miRNA of interest. This bioinformatic analysis was performed thanks to a collaboration with the Department of Neuroscience, Biomedicine and Movement Sciences, Biology and Genetics Section of the University of Verona.

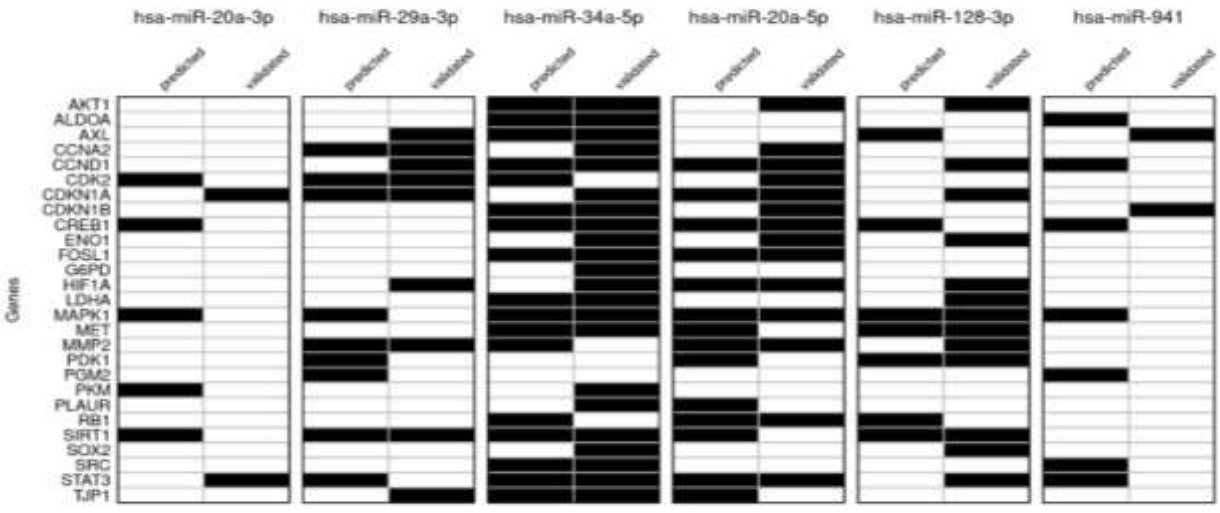


Figure 20. miRNA-target interaction: the table shows predicted and validated interactions between the miRNAs of interest and their targets. CDK2, CDKN1A, MAPK1, SIRT1, STAT3 are common targets of miR-20a-3p, miR-29a-3p, and miR-34a-5p.

4.12 ONC downregulates the expression level of key proteins involved in A375 and FO1 cell cycle progression

We had already described that ONC exerts a strong cytostatic effect in the A375 BRAF mutated cell line by reducing the BrdU incorporation into DNA in a time- and concentration-dependent manner[32]. Indeed, A375 cell proliferation was reduced of about 50–60% after 48 and 72 h incubation with 1 µM ONC. In this thesis, the mechanism of this cytostatic effect in the same melanoma cell line and in the BRAF mutated FO1 melanoma cells was investigated. The bioinformatic analysis, that was previously described, drove the investigation on the effect of ONC treatment on the expression of key proteins involved for instance in cell cycle progression. Immunoblot performed on A375 cells treated with 1 µM ONC for 48 or 72 h showed a robust reduction of both activated forms of RB (pRB) and Cdk2 (pCdk2) (*Figure 21 A*). Knowing that RB hyperphosphorylation is principally triggered by cyclin D1, and cyclin A2/Cdk2 binding is required for cells to enter S-phase, the expression level of both cyclins was analysed through immunoblot. A strong reduction of cyclins D1 and A2 was registered after 48 h from ONC administration, and this reduction was almost totally maintained for a further 24 h (*Figure 21 A*). These data suggest that ONC can hinder A375 cell cycle progression, by inhibiting cyclin D1 expression, which controls RB hyperphosphorylation and the G1/S transition point. In addition, cell cycle is further hampered by both Cdk2 phosphorylation and cyclin A2 expression reduction. The expression level of some of Cdks inhibitors such as P21/Cip1, P27/Kip1 and P16/Ink4A were investigated as well[158]. Their increase could be responsible for the pRB and pCdk2 low expression levels. Conversely, as shown in *figure 21 A,* immunoblot showed that the expression of P16 did not change at all, while the protein amount of both P21 and P27 was sharply lowered. To summarise, the ONC-elicited blockage of cell cycle progression cannot result from a P21, P27 or P16 different expression. Importantly, the same results were obtained by performing immunoblot analysis on FO1 cells treated with or without 1 µM ONC for 72 h (*Figure 21 B*).

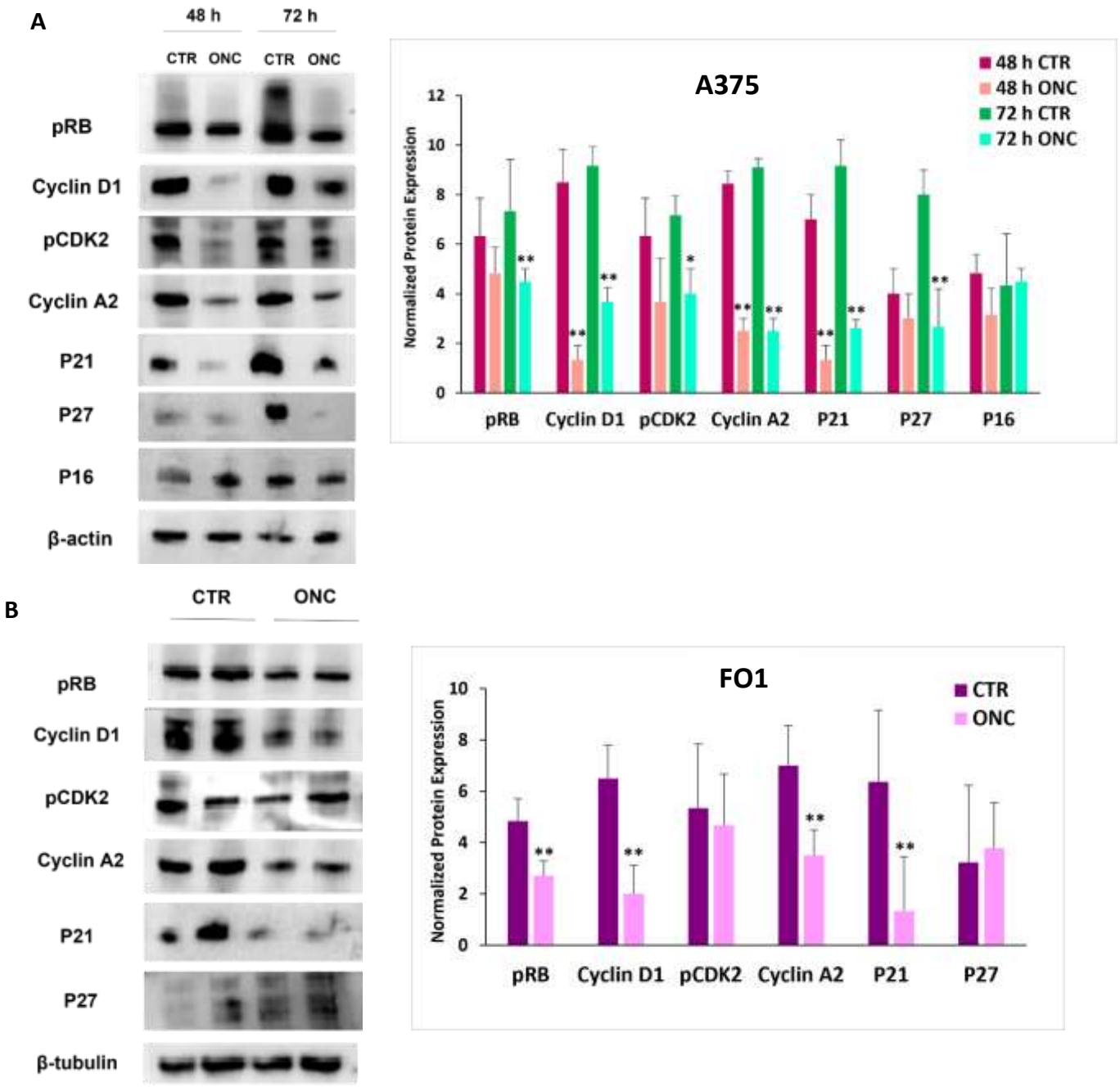


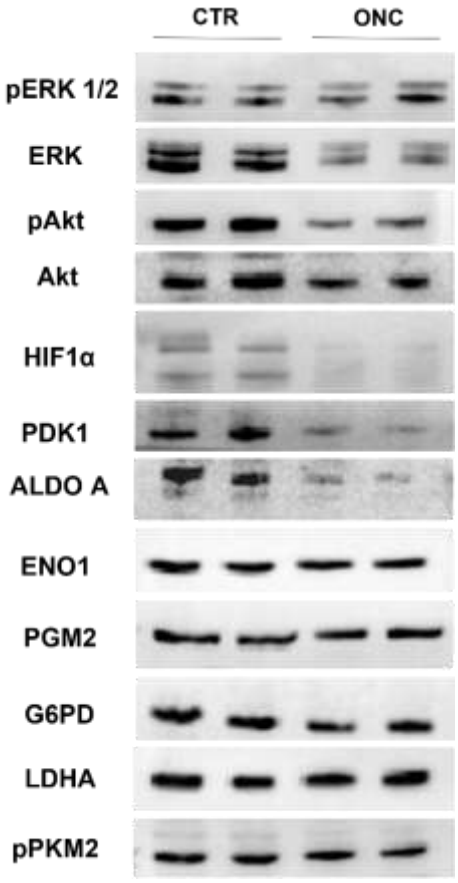
Figure 21. Cell cycle-related proteins expression is inhibited in A375 and FO1 melanoma cells treated with 1 µM ONC for 48 or 72 h. **A) left:** representative immunoblots showing the expression levels of cell cycle-related proteins in A375 cells; **right**: histograms reporting the mean values ± S.D. of protein expression level measured by densitometry and deriving from three to four independent experiments.
B) left: representative immunoblots showing the expression levels of cell cycle-related proteins in FO1 cells; **right**: histograms reporting the mean values ± S.D. of protein expression level measured by densitometry and deriving from three to four independent experiments.
All comparisons were performed vs. each control sample after normalization with β-actin

4.13 ONC differently affects the expression level of proteins involved in A375 and FO1 cell survival signalling and metabolism

When the MAPK/ERK pathway is activated in BRAF-mutated melanoma cells, as A375 cells are, it can promote HIF1α expression, leading to a high glycolytic rate[159]. HIF1α, as well as PI3K/Akt/mTOR pathways are crucial regulators of cancer cell proliferation and glycolytic metabolism[48]. Furthermore, pyruvate dehydrogenase kinase 1 (PDK-1) is able, upon its inhibitory phosphorylating effect on pyruvate dehydrogenase (PDH), to trigger the switch to glycolysis by hindering the oxidative metabolism of pyruvate[159]. *Figure 22 A* shows that both ERK1/2 and Akt total proteins expression levels together with pAkt expression level significantly decreased after 48h incubation with 1 μM ONC. Whereas the amount of the phosphorylated and active form of ERK1/2 did not change with the treatment.

HIF-1α was expressed in A375 cells after 48h culture at normal oxygen pressure. Remarkably, cell incubation with 1 μM ONC decreased almost totally the HIF1α protein level (*Figure 22 A*). At this point the expression level of some key enzymes were investigated to understand whether the metabolic phenotype of the A375 cell line was altered. The expression level of glucose-6-phosphate dehydrogenase (G6PD), phospho-glucomutase-2 (PGM2), enolase-1 (ENO1), lactate dehydrogenase A (LDHA), and the phosphorylated form of pyruvate kinase M-2 (pPKM2) did not vary upon 48h ONC incubation, so these enzymes were also exploited as housekeeping proteins for normalization. On the other hand, both aldolase A (ALDO A) and PDK1 significantly decreased their expression level (*Figure 22 A*). Importantly, similar results were obtained by performing immunoblot analysis on FO1 cells treated with or without 1 μM ONC for 72 h, except for PGM2 and LDHA that were decreased (*Figure 22 B*).

A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ONC
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ERK
pAkt
Akt
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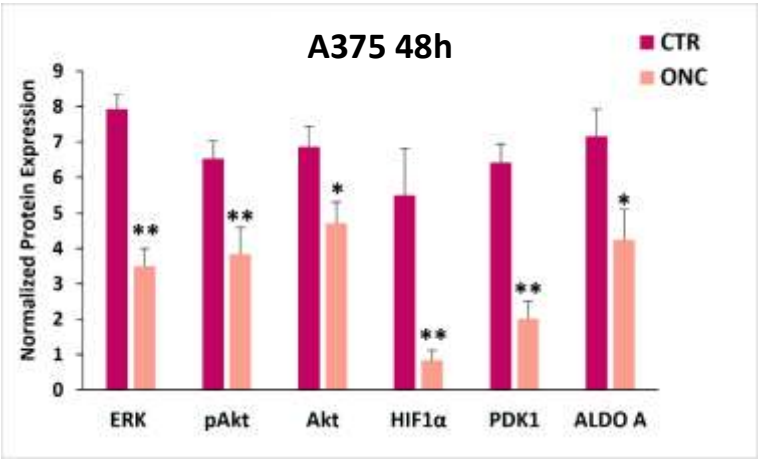
LDHA
pPKM2


A375 48h
CTR
ONC
Normalized Protein Expression
0
1
2
3
4
5
6
7
8
9
**
**
*
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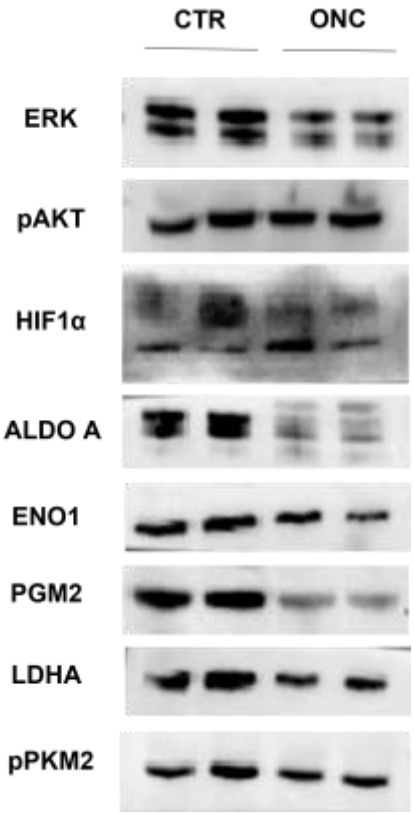
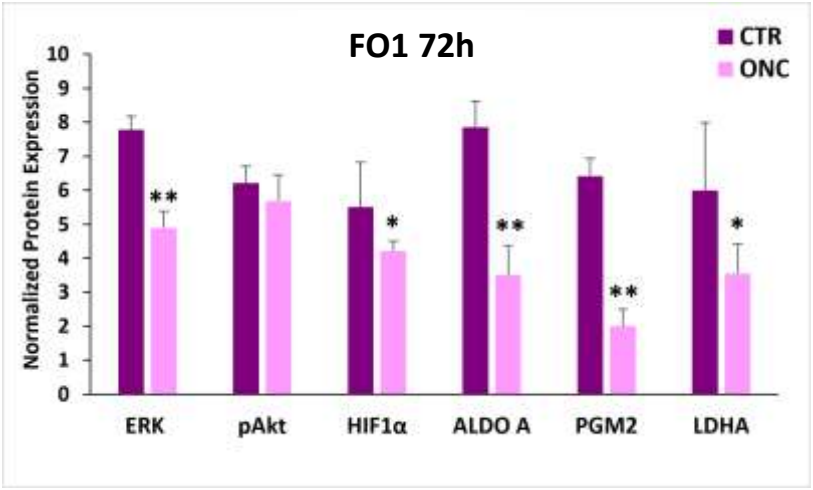
**
*
ERK
pAkt
Akt
HIF1α
PDK1
ALDO A

B


CTR
ONC
ERK
pAKT
HIF1α
ALDO A
ENO1
PGM2
LDHA
pPKM2


FO1 72h
CTR
ONC
Normalized Protein Expression
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**
*
ERK
pAkt
HIF1α
ALDO A
PGM2
LDHA

Figure 22. The expression level of proteins involved in cell proliferation signalling and metabolism is altered in A375 and FO1 melanoma cells cultured with 1 µM ONC for 48 h**.** **A) Left**: representative immunoblots showing the expression levels of extracellular signal-regulated kinase (ERK) and protein kinase B (Akt) and their active forms, and of many enzymes involved in metabolism in A375 cells; **right**: histograms reporting the mean values ± S.D. of the protein expression level measured by densitometry and deriving from three to four independent experiments. All comparisons were performed vs. each control sample after normalization with enolase-1 (ENO1) and phospho-glucomutase-2 (PGM2) expression; * $p < 0.05$, ** $p < 0.01$. **B) Left**: representative immunoblots showing the expression levels of the same proteins in FO1 cells; **right**: histograms reporting the mean values ± S.D. of the protein expression level measured by densitometry and deriving from three to four independent experiments.

To further understand whether ONC treatment altered the glycolytic rate of the cells, a glycolysis cell-based assay was carried out. This colorimetric method is based on L-lactate detection. L-lactate, the end product of glycolysis, is secreted by cultured cells and, when lactate dehydrogenase is added to the cell culture medium at the end of the treatment, it reacts with NAD^+ leading to pyruvate and NADH formation. Subsequently NADH directly reduces a tetrazolium salt, present in the reaction solution supplied by the manufacturer, to a coloured formazan. The quantity of formazan produced is proportional to the quantity of lactate in the culture medium and therefore it is an indirect measurement of glycolysis. In table 1 the result of this assay performed on A375 and FO1 cells is reported. Lactate release is not significantly reduced in ONC treated cells in both cell lines considering that the number of cells is reduced by the treatment as well (*see the last row*). These results are partially in line with immunoblot analysis results.

	A375			FO1		
	CTR	1 µM ONC	2 µM ONC	CTR	1 µM ONC	2 µM ONC
% Lactate	100	64.45	63.48	100	74.18	62.40
% Live Cells	100	71.48	71.21	100	79.15	66.76
% Lactate/ % Live Cells	1	0.90	0.89	1	0.94	0.93

Table 1: Result of the glycolysis cell-based assay performed on A375 and FO1 melanoma cells. Lactate release is not significantly reduced in ONC treated cells. These results were normalized on the cell number.

4.14 ONC treatment downregulates the expression of key proteins involved in A375 melanoma cells metastatic potential

As previously reported by us[32], ONC affects A375 cells capability to form colonies in an anchorage independent manner as well as migration capability, considering that the wound closure time after scratching is strongly decreased. Metalloproteinase (MMP)-2 activity is also impaired by ONC treatment[32]. In fact, in this thesis it was once again confirmed, by gelatin zymography, the strong inhibition of MMP2 activity in A375 cells administered with 1µM ONC (*Figure 23*).

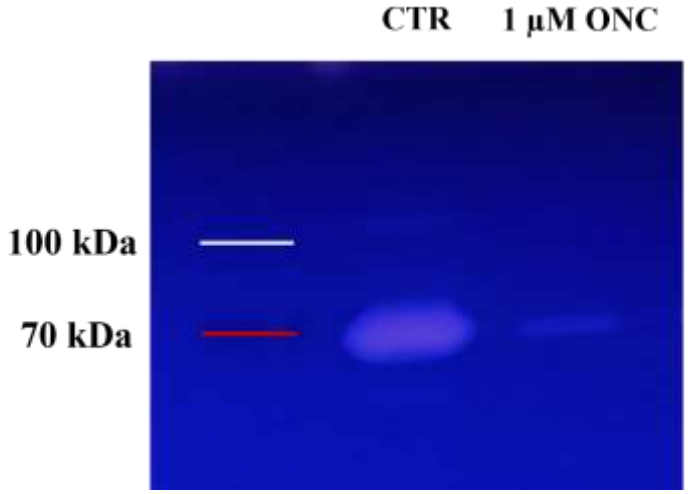


Figure 23. Gelatin zymography revealed that MMP2 activity is strongly reduced in A375 melanoma cells treated for 48 h with 1 µM ONC compared to non-treated cells.

Moreover, immunoblot analysis was carried out to study the expression level of key proteins involved in biological processes correlated to an increased metastatic potential such as ZO1, involved in the regulation of cell–cell contacts[83], or SIRT1 that induces EMT and facilitates melanoma metastasis[89,160]. In addition, SOX2 is an embryonic stem cell transcription factor associated with dermal invasion capability of melanoma cells[161], Urokinase plasminogen activator receptor (uPAR) is involved in melanoma and metastasis[162,163], while the cAMP response element-binding protein (CREB) is a transcription factor playing an important role in the acquisition of the metastatic phenotype of human melanoma cells[164]. The expression level of all the protein listed above (ZO1, SIRT1, SOX2, uPAR and CREB) were significant decreased in A375 cells treated with ONC for 48 h (*Figure 24 A*). Once again, similar results for the texted proteins ZO1, ZEB1, and CREB were

obtained by performing immunoblot analysis on FO1 cells treated with or without 1 µM ONC for 72 h (*Figure 24 B*).

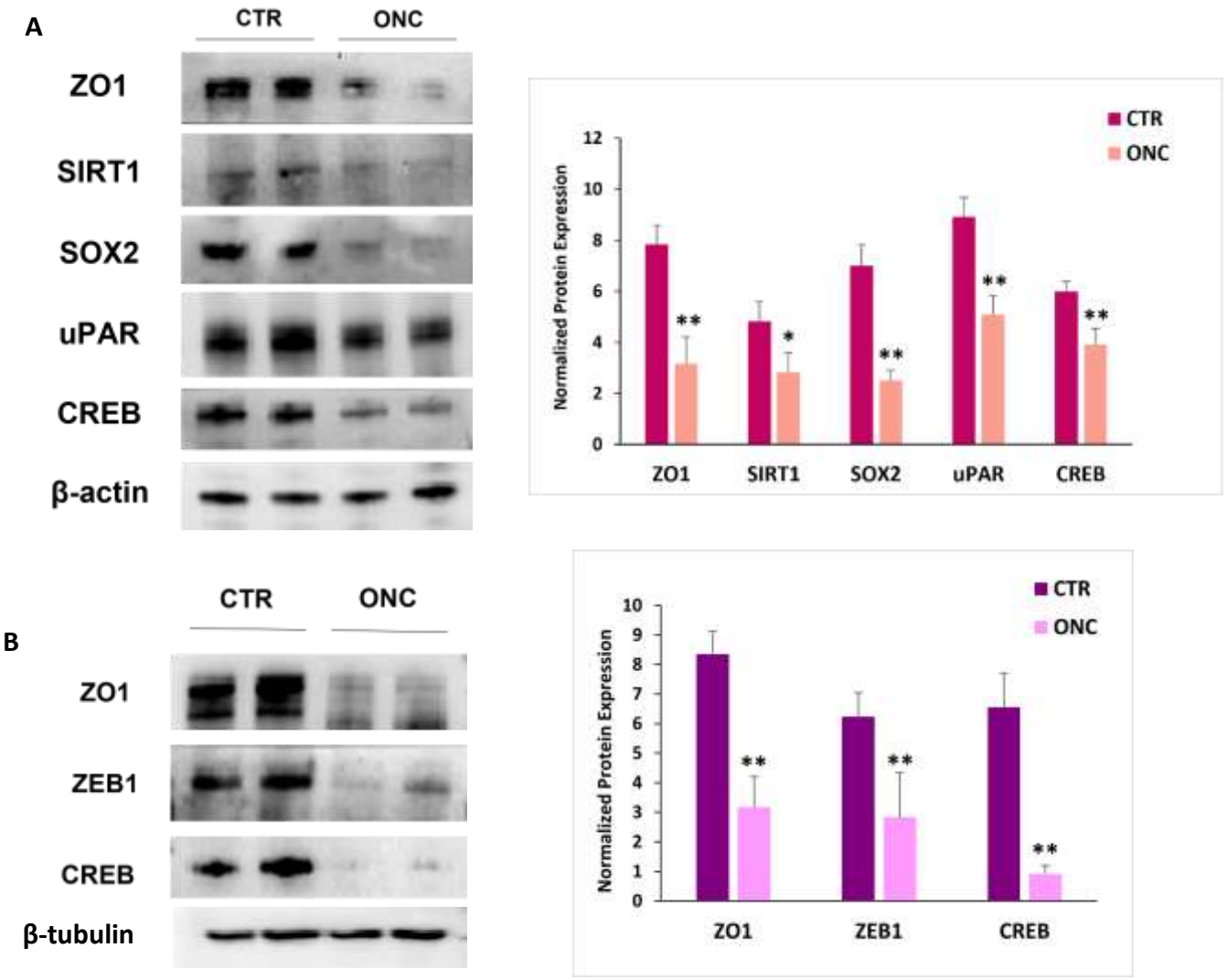
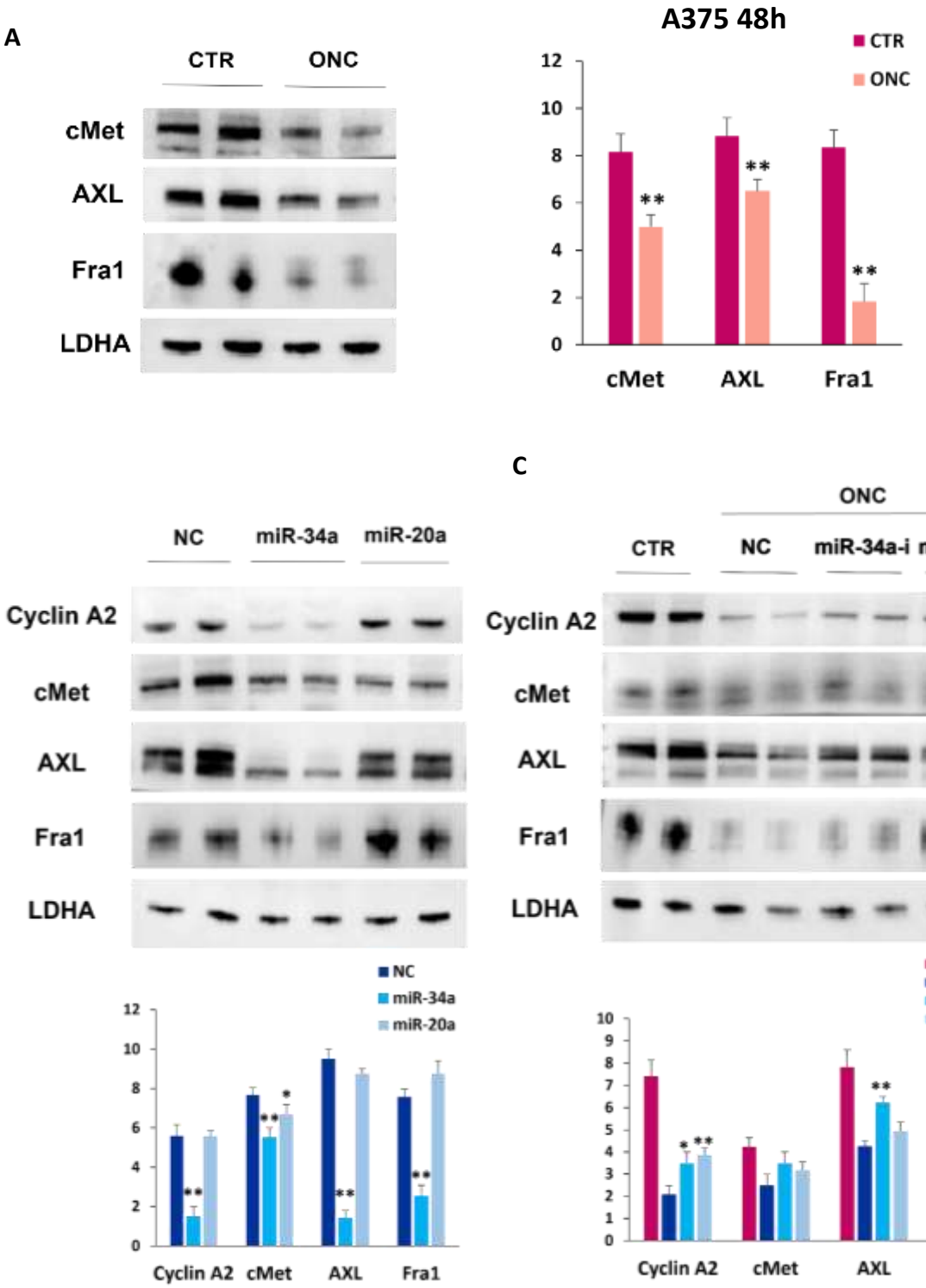


Figure 24. The expression levels of proteins involved in cell migration, invasion and tumor progression is inhibited in A375 and FO1 melanoma cells treated for 48 h with 1 µM ONC. **Left:** representative immunoblots showing the expression levels proteins involved in cell invasion in A375 **(A)** or FO1 **(B)** melanoma cells; **right:** histograms reporting the mean values ± S.D. of the protein expression level measured by densitometry and deriving from three to four independent experiments. All comparisons were performed vs. each control sample after normalization with β-actin expression; * $p < 0.05$, ** $p < 0.01$.

4.15 ONC-elicited downregulation of cMet and AXL tyrosine-kinase receptors and Fra1 transcription factor correlates with the upregulation of miR-34a-5p and miR-20a-3p expression in A375 cells

Importantly, Fra1 expression correlates with cell transformation to a more invasive phenotype[165]. The expression level of AXL and c-Met tyrosine-kinase receptors, as well as of Fra1 transcription factors, was lower in ONC-treated A375 cells (*Figure 25 A*). Taken together these immunoblot results, a possible correlation with the upregulation of some miRNAs was investigated. Indeed, miR-34a-5p and miR-20a-3p, the two most upregulated miRNAs in ONC treated A375 cells were chosen to find a possible link to cMet, AXL, Fra1 and cyclin A2 modulated expression. Thus, A375 cells were transfected with miR-34a-5p and miR-20a-3p mimics or negative control and protein expression was analysed through immunoblot after 72h. As shown in *figure 25 B*, overexpression of miR-34a-5p lead to a decreased expression level of all the analysed proteins. MiR-20a-3p overexpression partially downregulated cMet expression but showed no effect on AXL, Fra1 and cyclin A2 (*Figure 25 B*). To further verify if the downregulation of cMet, AXL, Fra1 and cyclin A2 protein expression elicited by ONC was related to the upregulation of these miRNA species, ONC treated A375 cells were transfected with miR-34a-5p or miR-20a-3p inhibitors, or with negative control. Seventy-two hours after the miR-inhibitor transfection and ONC treatment, immunoblot results showed that the miR-34a-5p inhibitor could partially revert the effect of ONC on cyclin A2, AXL, cMet and Fra1. These last results indicated that miR-34a-5p has a role in decreasing protein expression elicited by ONC (*Figure 25 C*). MiR-20a-3p inhibitor partially reverted the ONC effect on cyclin A2 and Fra1 expression level (*Figure 25 C*), thus indicating that miR-20a-3p might take part in the regulation of the last-mentioned proteins. Considering that for FO1 cells the results of the immunoblot analysis shown in *figure 25 D* did not shown a decrease in the expression, the possible correlation between miRNAs upregulation and protein expression was not investigated. Indeed, c-Met tyrosine-kinase receptor expression was surprisingly increased in FO1 ONC-treated cells for 72h while, AXL and FRA1 expression was not affected at all by the same treatment, contrary to what happened in A375 cells.


A
CTR
ONC
cMet
AXL
Fra1
LDHA
A375 48h
CTR
ONC
12
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cMet
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miR-34a
miR-20a
Cyclin A2
cMet
AXL
Fra1
LDHA
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miR-34a
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Cyclin A2
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Cyclin A2
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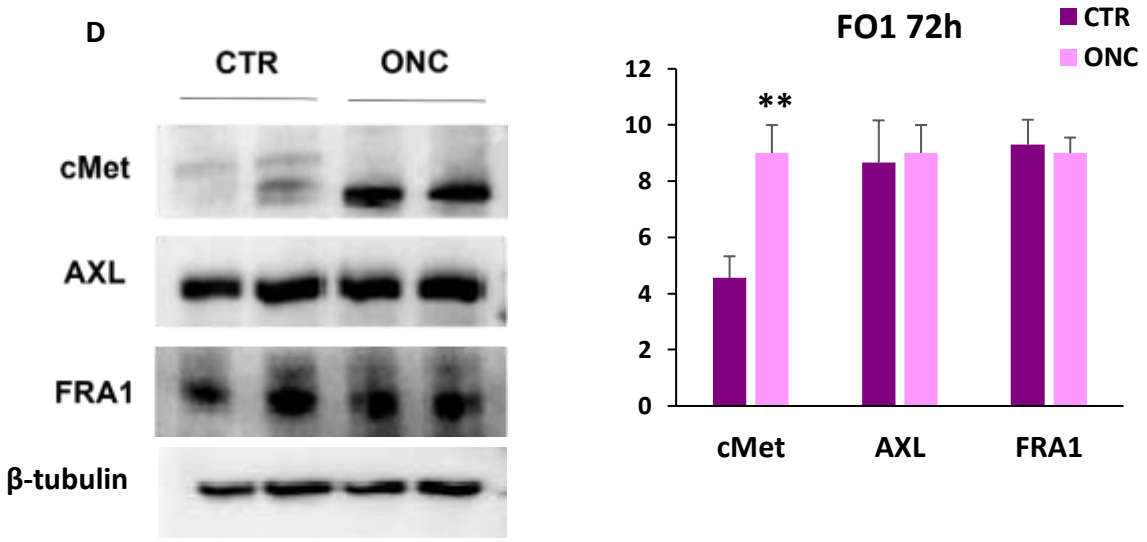

Figure 25. Representative immunoblots and densitometric data representing the correlation between mesenchymal– epithelial transition factor (cMet), tyrosine-protein kinase receptor UFO (AXL), Fos-related antigen 1 (Fra1) and cyclin A2 protein level and miR-34a-5p and miR-20a-3p in A375 cells. **A)** A375 melanoma cells were treated for 48 h with or without ONC; expression of cMet, AXL, and Fra1 proteins.

B) Effects of the overexpression (72 h) of miR-34a-5p and miR-20a-3p on cMet, AXL, Fra1 and cyclin A2 protein level in untreated cells.

C) Effects of miR-34a-5p and miR-20a-3p inhibitors on cMet, AXL, Fra1 and cyclin A2 protein levels on ONC-treated A375 cells transfected with 50 nM miRNAs inhibitors for 72 h.

D) Expression of the same miRNAs correlated proteins in FO1 cells. All the histograms of this figure report the relative mean values ± S.D. of protein expression level measured and deriving from three independent experiments. All comparisons were performed vs. each control sample after normalization with LDHA expression; * $p < 0.05$, ** $p < 0.01$.

5. DISCUSSION

For its worldwide increasing incidence, melanoma is becoming one of the major causes of mortality for malignancies of the Caucasian population[9]. Several treatments have been approved, going from surgical resection for non-invasive stages to chemotherapy for invasive phenotypes[16]. DTIC was the first drug to be approved for melanoma therapy. It is an alkylating agent that causes breaks in the DNA strand, but it correlates with a low response in patients due to many off target effects and acquired chemoresistance[166]. The characterization of the specific mutation V600E on BRAF kinase gene, which is harboured by the majority of melanoma patients and causes a constitutive activation of the MAPK pathway, led to the development of therapeutic tools based on drugs directed against a specific target[167]. Different BRAF inhibitors, such as dabrafenib and vemurafenib, have been approved in the last years, but, unfortunately, cellular responses to BRAF inhibitors are short-lived because patients in few months develop different mechanisms of resistance that involve the activation of alternative signalling pathways[168]. Immunotherapy is also employed in melanoma treatment, but the administration of interferon and interleukin cytokines, which had already been approved by the FDA for other types of cancer did not show notable benefits for patients[21]. Additionally, FDA approved ipilimumab in 2011, an anti-CTLA-4 monoclonal antibody that acts inducing the production of pro-inflammatory cytokines, favoring T-cells expansion and infiltration in the tumor [169,170] and nivolumab, a high affinity anti-PD-1 monoclonal antibody, that can inhibit the binding between PD-1 (Programmed cell death receptor protein 1) receptor and its ligands [171–173] increasing the immune response. Indeed, only 10% of people experiences a complete response after this kind of treatments, also due to severe side effects, like capillary leak syndrome[22,23].

For these reasons, new strategies for the treatment of advanced melanoma patients urge to be discovered.

BS-RNase and the oligomeric forms of RNase A, together with other secretory RNases, can become cytotoxic for their ability to digest intracellular RNA species when internalized in cells[155]. Indeed, we confirmed in A375 and MeWo melanoma cells that high concentrations of dimeric species of RNase A were able to decrease cell viability (*Figure 10*).

ONC is a small secretory ribonuclease, discovered in *Rana Pipiens* oocytes[116]. It is better internalized in cancer cells compared to non-malignant cells because of its structure and biochemical features. Remarkably, ONC can escape from the binding to the intracellular ribonuclease inhibitor contrarily to most of other secretory RNases[124,174] and, for this reason, it can be more active against tumors. Indeed, several studies had already proved that ONC exerts cytostatic and cytotoxic effects on many cancer cell lines. Cytostatic effect is related to the blockage of cells before the S-phase that finally

results in fewer DNA replicating cells. While its cytotoxicity was ascribed to apoptosis induction[175]. Interestingly, ONC displays a synergic effect with several anti-cancer drugs, such as tamoxifen, cisplatin, vincristine, lovastatin[128] and doxorubicin[129]. In addition, it was recently demonstrated by our research group that A375 melanoma cell line treated for long time with the PARP inhibitor AZD2461 were more susceptible than the parental cells to ONC cytotoxicity suggesting that ONC can be an important tool to counteract melanoma following a previous chemotherapeutic treatment [130]. ONC, is also able to restore anti-melanoma activity in dabrafenib-resistant A375 human melanoma cells affecting cell migration, invasion, and colony formation capability[32]. It has been previously reported that ONC can decrease the viability of two conjunctival melanoma cell lines, although its mechanism of action has not been clarifyed[176]. This ribonuclease was tested also *in vivo* revealing, for instance, that mice affected by Ml09 Madison carcinoma after ONC administration displayed 12-fold longer life compared to untreated control group[177]. Moreover, ONC has already reached phase II and III clinical trial for non-small cell lung cancer and mesothelioma, two types of tumor characterized by poor prognosis as well[115]. Considering that the major limitation for the therapeutical application of ONC is its renal accumulation and toxicity[131] and that the ONC dimeric form increased the apoptotic effect on pancreatic cancer cells[143], in this thesis the anti-melanoma effects of ONC dimer or trimer after in vitro oligomerization was explored. Therefore, it may represent a valuable horizon for the anticancer potential of this enzyme. Exactly as other pt-RNases, such as RNase A and the natively dimeric BS-RNase, ONC can oligomerize[48,49,52,56] through the three dimensional domain swapping (3D-DS) mechanism[134] when lyophilized from 30-50% acetic acid (HAc) solution[135]. Thanks to the collaboration with Professor Gotte and Professor Merlino, OND-D was produced and its crystallized structure of ONC-D was solved[147] revealing several structural variations compared to the already proposed model of ONC-D based on the structure of RNase A dimer[143]. As shown in *figure 26*, the comparison between the structures of ONC and ONC-D reveals that the positive charged residues Arg15 and Arg40 assume different positions in the protein conformation upon dimerization. Consequently, we wanted to investigate whether these differences could also lead to a change in their antitumor activity (*Figure 26 A*).

Actually, the different charge density and solvent exposure of some of their AA side chains noticed between ONC and ONC-D could have an impact on the interaction of these two species with cell membrane, RI or many RNA targets, influencing their antitumor activity (*Figure 26 B*).

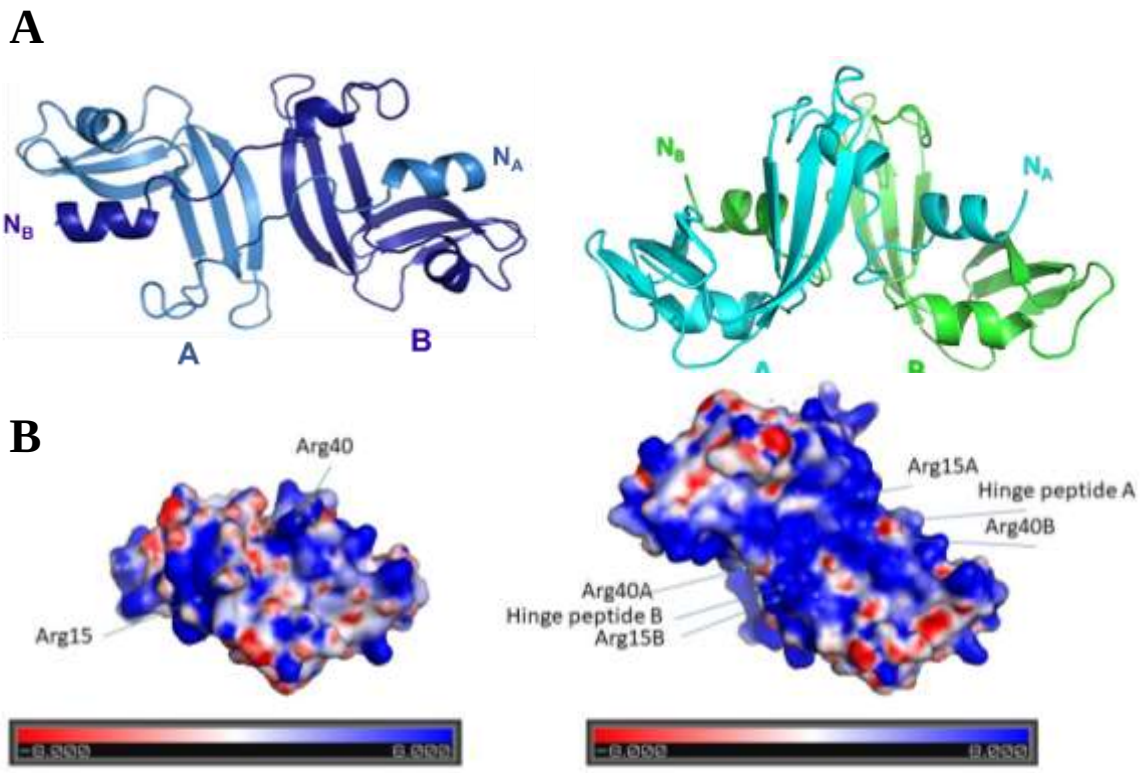

Figure 26. **A) Structural differences between ONC-D Model**[143] on the left side and **ONC-D crystallographic structure** [147] on the right.
B) Electrostatic surface potential of ONC species. ONC (left) and ONC-D (Crystal 1, right) positively charged, negatively charged, and uncharged atoms are colored in blue, red, and white, respectively[147].

So, to compare the antitumor activity of both ONC species, we mainly used A375 and MeWo cells: two different human melanoma cell lines. The A375 cell line derived from a primary melanoma and displays a common V600E-BRAF-mutated genotype, while MeWo cells derived from a lymph nodal melanoma metastasis and exhibit a wt-BRAF but mutated p53 genes. Additionally, ONC and ONC-D were tested in other human cancer cell lines: the hepatocellular carcinoma HepG2 (*Figure 14 A*) and the glioblastoma U251 cell lines (*Figure 14 B*) assuring us that the antitumor effect of both ONC species occurred in other cancer types, besides melanoma. Our data suggest that ONC-D is able to inhibit the viability of all the tested cancer cell lines, although with slightly less efficiency in comparison to ONC. In particular, in the two melanoma cell lines, the reduction of cell growth seems to be the prevalent effect, even if A375-treated cells showed an increase of apoptosis as well. Overall, the antitumor effect of both ONC species is dependent on the cell-type studied, as demonstrated by the differences discovered between A375 and MeWo cell lines that are following discussed. The

reduced efficiency in inhibiting the intracellular targets could be derived by the differences in the exposure at the surface of the arginine residues of ONC-D in comparison to ONC. To quantify the cytotoxic activity of both ONC species, a flow cytometry analysis was performed on the two melanoma cell lines. The results shown in *Figure 16 A* revealed that only in A375 cells, death was increased by ONC or ONC-D treatment. So, both ONC forms were able to trigger apoptosis in A375 cells but not in MeWo cells. These results were confirmed by immunoblot (*Figure 16 B*) where the expression level of cleaved PARP, a marker of apoptosis, was analyzed. Indeed, in A375 treated cells an increased level of cleaved PARP was registered, while in MeWo cells it did not change in comparison with not-treated control. Either ONC or ONC-D were able to decrease Bcl2 expression in both cell lines, highlighting that these RNases can inhibit the expression of an important target in cancer therapy. Moreover, Bcl2 protein level was higher in control MeWo cells than that expressed in A375 control sample. Although ONC species can decrease its level, the expression of this anti-apoptotic effector after the treatment remained higher in MeWo cell line in comparison with A375 cells. Hence, it may be suggested that the high expression level of Bcl2 could be involved in the stronger resistance to apoptosis displayed by the metastatic MeWo cell line.

We previously reported [32,130] that ONC can affect the NF-κB activity. In this thesis, we show that another pro-survival transcription factor has been affected by ONC. The hyperactivation of STAT3 pathway is a common characteristic of melanoma development[171,172]. For this reason, STAT3 is an important target in melanoma therapy[41]. Interestingly, in a mouse melanoma model, the silencing of STAT3 expression strongly inhibits tumor growth, thus suggesting that inhibiting STAT3 activation can reverse the malignant phenotype[178]. Different growth factors and cytokines after binding to their receptors can induce STAT3 activation. STAT3 can be switched on through the phosphorylation of Tyr705 residue by JAK or Src tyrosine kinases in addition to some tyrosine-kinase receptors. Following Tyr705 phosphorylation, STAT3 nuclear translocation and its binding to the promoter regions of many target genes occur[156]. STAT3 activation regulates multiple downstream genes involved in several cancer-promoting pathways, such as cell proliferation, apoptosis, metastasis, and angiogenesis. For instance, the anti-apoptotic Bcl2 is a gene target of STAT3 transcriptional activity. Considering our results, a reduction in the expression level of Bcl2 is in line with the inhibition of STAT3 activity elicited by ONC or ONC-D. ONC and ONC-D can firstly reduce STAT3 total protein level in both cell lines, while several cell type peculiarity emerged from investigating the expression level of phosho-Tyr705-STAT3 and phospho-Ser727-STAT3, the two active forms of STAT3. On one side, the level of the phosphorylated forms of STAT3 reflect its total amount in A375 cells. On the contrary, lower STAT3 molecules seem to be phosphorylated in Tyr705 in MeWo cells, suggesting that STAT3 could be a signalling pathway less active in this cell line. It should be

remembered that Ser727 phosphorylation of STAT3 was also inhibited. Data strongly suggest that ONC and in a less extent ONC-D exert a downregulation of STAT3 transcriptional activity, which is highly activated in cancer.

In this thesis we also demonstrated that both ONC and ONC-D species can inhibit the phosphorylation and the total (Figure 17) level of the proto-oncogene tyrosine-kinase Src. The phospho-Tyr705-STAT3 inhibition, indeed, could depend on the reduced Src activity because such STAT3 residue is a target of Src.

STAT3 can induce other important features of cancer cells, such as migration, invasion, and metastasis formation. It has been previously reported that ONC was able to reduce A375 colony formation[32], another hallmark of cancer cells. To this regard, to investigate whether ONC-D have an impact on the anchorage independent growth capability, we performed the soft agar colony formation assay by matching the two ONC species. Data showed that, compared the control cells, both ONC and ONC-D were able to strongly decrease the number of colonies in both cell lines and in a concentration dependent manner. Our data are very promising considering that the aberrant activation of STAT3 can increase metastatic potential of cancer cells.

Even though ONC-D maintains a substantial anticancer activity, some differences with ONC appeared both in the viability effects and in the ability to inhibit different molecular targets. In these cases, the ONC-D anticancer activity appears slightly reduced in comparison with its monomeric form. These differences can depend on the type of cells analyzed suggesting that intracellular targets of ONC species could be in a large number and specific for each cell-type. Interestingly ONC-T anticancer activity seem to be comparable to ONC one. Indeed, preliminary cytofluorimetric results showed a strong increase of apoptotic cell death in A375 cells treated with the trimeric form of ONC (*Figure 16*).

Subsequently, due to the limited availability of ONC-D, in first place we investigated important molecular targets for melanoma progression only for the monomer. The results obtained here with ONC are aimed to direct future experiments that will be carried out with ONC multimeric species. Thus, the molecular mechanism underlying the high cytostatic effect of ONC in A375 melanoma cells that had been previously described[32] has been investigated. Data suggest that ONC hinders cell cycle progression by inhibiting RB hyperphosphorylation and Cdk2 activity, as well as by reducing the expression level of cyclins D1 and A2, which are involved in the cell cycle phases G1/S and S/G2 checkpoints, not only in A375 cells but also in another BRAF mutated melanoma cell line FO1 (*Figure 21 B*). Considering that the specific intracellular RNA targets of ONC are still less known we tried to deeply investigate this aspect. ONC, as a ribonuclease that is internalized in the cells, has been reported not to be able to digest mRNA species but is able to digest preferentially ncRNAs.

Indeed, both tRNAs[120,179] and miRNAs precursors[180,181] can be targets of the ribonucleolytic activity of ONC. Considering that the most investigated proteins were downregulated by ONC, we hypothesized that ONC can upregulate some onco-suppressor miRNAs, that in turn may reduce cell cycle-related protein expression. This could occur either by ONC-increased miRNA precursors processing [182] or by ONC-elicited degradation of lncRNAs and circRNAs, which usually sponge miRNA species. Among the miRNAs whose expression has been measured in A375 cells, the tumor-suppressor miR-20a-3p, miR-29a-3p and miR-34a-5p were the most upregulated after 48 hours of ONC treatment (*Figure 19 A)*. The same miRNAs were also overexpressed in ONC-treated FO1 melanoma cells (*Figure 19 B*). Remarkably, cyclin D1 and cyclin A2 can be targets of miR-29a-3p and miR-34a-5p, while CDK2 could be a target of miR-20a-3p, miR-29a-3p and miR-34a-5p. The high expression level of these miRNAs can induce a decrease of cyclins and Rb/Cdk expression or activity, thus hampering cell cycle progression. P21, P27 and P16 are inhibitors of cyclin-dependent-kinases and, in turn, of cell cycle progression. Thus, an increase of their expression level could prevent Cdks activation. Unexpectedly, a sharp decrease of P21 and P27 expression level in A375 cells treated with ONC has been found. This result, although without explaining the interference in the cell cycle, are not surprising since both P21 and P27 could be a target of the some of the overexpressed miRNAs. Many studies have evaluated the ability of miRNAs to control cellular growth. For example, Shao et al. reported that miR-29a-3p downregulation promotes glioma cell proliferation[183]. Again, ectopic expression of miR-34a has been shown to induce cell cycle arrest[184]. Furthermore, transfection of miR-34a into uveal melanoma cells led to a significant decrease in cell growth and migration[185]. A recent study reported that A375 and A875 melanoma cell proliferation decreased after miR-34a overexpression and increased when miR-34a expression was suppressed[186].

The expression level of proteins controlling survival signalling pathways and metabolism has also been investigated in ONC-treated A375 cells and FO1 cells. Kupha et al. recorded a marked HIF-1α activity in melanoma cell lines under normoxic conditions[187]. Our immunoblot data confirm that not-treated A375 cells display high expression level of HIF1α, even if they were grown under normal oxygen pressure, whereas ONC administration nearly suppresses this expression. Recent studies have revealed that HIF-1α can be induced by several signalling pathways, such as the PI3K/Akt/mTOR, RAS/RAF/MEK/ERK, JAK/STAT and NF-κB pathways, controlling melanoma tumor growth, metabolism, motility, and avoidance of apoptosis[188].

Previously, it has been shown that ONC reduces the expression and activity of the transcription factors NF-κB[32,130] and STAT3[147] in A375 melanoma cells. In this study, we recorded the ability of ONC to lower Akt expression and phosphorylation levels, as well as the total protein expression of the kinases ERK (*Figure 22*). Therefore, the ONC-induced downregulation of these proteins involved

in signalling may fully contribute to the suppression of pro-survival pathways. The ONC-triggered downregulation HIF-1α led us to study other proteins involved in the metabolic state of A375 cells. The expression of many cytosolic enzymes belonging to glucose metabolism are not affected by ONC, except for ALDO A which is less expressed and may be a target of miR-34a-5p. It is known that the PDH mitochondrial enzyme controls the metabolic fate of pyruvate linking glycolysis with oxidative metabolism[159]. PDH activity is inhibited if the enzyme is phosphorylated by its PDK regulatory kinases. In tumor cells, PDK1 is induced by HIF-1α[189]. A lower expression level of PDK1 was shown, in agreement with the loss in HIF-1α expression. Thus, might result in increased PDH activity favoring in this way the oxidative metabolism of pyruvate. Both HIF-1α and PDK1, which protein expression were decreased, can be targets of miR-29a-3p and/or of miR-34a-5p.

Interestingly, miR-20a-3p was shown to be the most widely expressed miRNA after ONC treatment. In relation to cancer, its decrease has been associated with both breast cancer[190] and with the progression of pancreatic ductal adenocarcinoma[191]. MiR-20a-3p can target several genes, including MAPK1/ERK, STAT3 and CREB1. Thus, together with miR-34a-5p, miR-20a-3p may participate in ONC-induced growth inhibition and may counteract the metabolic phenotype of cancer. Furthermore, in silico analysis of predicted targets demonstrated that PDK1, HIF1α and ERK could be targets of miR-29a-3p, suggesting a possible role of miR-29a-3p as well in metabolism, in agreement with its role in insulin receptor signalling in the liver of diabetic rats[192].

Many oncoproteins are involved in the ability of melanoma cells to grow in anchorage-independent manner, as well as to increase motility and digest the extracellular matrix[193]. The increased expression of tumor-suppressor miRNAs that was found in A375 and FO1 cells treated with ONC could be a rationale for the previously shown inhibition of A375 metastatic potential[32]. For instance, miR-29a-3p can play a pivotal role, as inhibition of miR-29a-3p in melanoma cells improved colony-forming ability, and conversely, its transfection reduced the growth of A375 cells[194].

The trans-differentiation mechanisms, which allow reversible changes from epithelial to mesenchymal phenotypes and *vice versa* by reactivating embryonic transcriptional programs, are the main determinants of tumor stem cell (CSC) phenotype[195–197]. MiR-34a downregulates many CSC-related transcription factors, including SOX2 in head and neck squamous carcinoma cells[198]. Indeed, Sun et al. claim that miR-34a restoration significantly inhibited EMT and CSC phenotypes and functionally reduced both clonogenic and invasive capacity[198]. It was also found that miR-34a is involved in the regulation of osteosarcoma dedifferentiation by acting via SOX2 down-regulation[199]. SOX2, whose expression is reduced by ONC (*Figure 24*), is also involved in resistance to therapy with BRAF inhibitors in a Src- and STAT3-dependent manner[200]. We have previously reported a downregulation of both STAT3 phosphorylated forms and its upstream Src kinase in ONC-treated

A375 cells[147]. STAT3 and Src are validated targets of miR-34a. In addition, the restoration of miR-34a in triple negative breast cancer cell lines inhibited proliferation and invasion by targeting the proto-oncogene c-Src[201].

Histone deacetylase SIRT1 is downregulated by ONC (*Figure 24*) and has been reported to induce EMT and facilitate melanoma metastasis[89]. SIRT1 could be a target of miR-34a, as has been shown in a murine melanoma cell line[202]. In cervical cancer, miR-29a-3p has also been reported to target SIRT1[203].

Importantly, miR-34a inhibited cell migration and invasion also by silencing the expression of Metalloproteinase (MMP) 2, disrupting MMP2-mediated cell motility[204]. These results are in line with our previous data demonstrating the ability of ONC to decrease MMP2 activity and A375 cell motility[32].

Tumor progression is also characterized by increased expression of the uPA/uPAR system. Inhibition of uPAR expression with a specific uPAR antisense oligonucleotide inhibits cell invasion, angiogenesis, metastases and MMPs[162]. uPAR is downregulated by ONC (*Figure 24*) and could be a target of miR-34a-5p.

A key role of CREB in tumor growth and human melanoma metastasis has been demonstrated using a mutated dominant negative CREB gene within its DNA binding domain[164]. Cells transfected with a negative CREB gene showed a marked decrease in their ability to form colonies on agar, suggesting that CREB may be involved in the tumorigenicity and metastatic potential of human melanoma cells[164]. Previous data on the ability of ONC to decrease A375 colonies in soft agar[32] could also result from a decrease of CREB expression (*Figure 24*), possibly via miR-20a-3p and miR-34a-5p increased activity (*Figure 24*).

Finally, we discuss the role of cMet, AXL and Fra1, a cluster of proteins highly expressed in melanoma and involved in signalling. Both cMet and AXL belong to the class of cell surface receptors with tyrosine kinase (RTK) activity. RTKs can amplify the signal from growth factors and transduce it into the intracellular kinases, that is Src, ERKs, Akt, or the transcription factors such as STAT3, NF-κB and AP1, leading to an invasive cell growth program[96,205]. Indeed, many RTKs, including cMet and AXL, are overexpressed in malignancy showing oncogenic promotion of cancer progression and metastatic disease[96]. Indeed, high cMet expression was found in melanoma samples and it has been correlated with poor clinical outcome[96]. Additionally, increased cMet or AXL signalling can develop drug resistance, particularly to BRAF or MEK inhibitors[206]. Consequently, a combination therapy with BRAF/MEK inhibitors and a cMet or AXL inhibitor was considered for enrollment in a clinical trial[207]. Both cMet and AXL can be targets of miR-34a-5p, miR-128-3p and miR-20a-5p (*Figure 20*).

The RTK signalling can activate the AP1 transcription factor complex and Fra1 protein expression. Fra1 transcriptional activity, similarly to the other AP1 proteins, is involved in many stages of metastatic dissemination, including tissue invasion and colonization at distant sites[197]. A recent study has shown that Fra1 overexpression in melanocytes is sufficient to drive pro-tumor characteristics and dedifferentiation[197]. In fact, the silencing of FOSL1, the gene that codes for Fra1, decreased the expression of pERKs, as well as the proliferation rate in A375 and A2058 melanoma cells. This also induces a significant decrease in the soft-agar colony formation[165]. Cancer-associated downregulation of multiple tumor suppressor miRNAs contributes to the accumulation of Fra-1 in tumors[197]. Recent studies report that FOSL1 transcript is a target of miR-34a in many cell lines[165,208,209].

In *figure 25 A*, a decrease in ONC-induced expression of cMet, AXL and Fra1, in A375 cells after 48 hours from the ONC administration, was shown. This decrease is particularly evident for the expression level of Fra1. The data could explain the very potent effect of ONC in lowering the Fra1 protein level. In FO1 cells, contrary to what happened in A375 cells, AXL and Fra1 expression was not affected at all by the same treatment while c-Met tyrosine-kinase receptor expression was increased (*Figure 25 B*). Considering these results, the possible explanation of these contradictory data will be investigated in the future.

In conclusion, our data, summarized in *figure 27*, may help explaining the molecular mechanism underlying the suppressive effect of ONC exerted on some important oncoproteins in A375 melanoma cells.

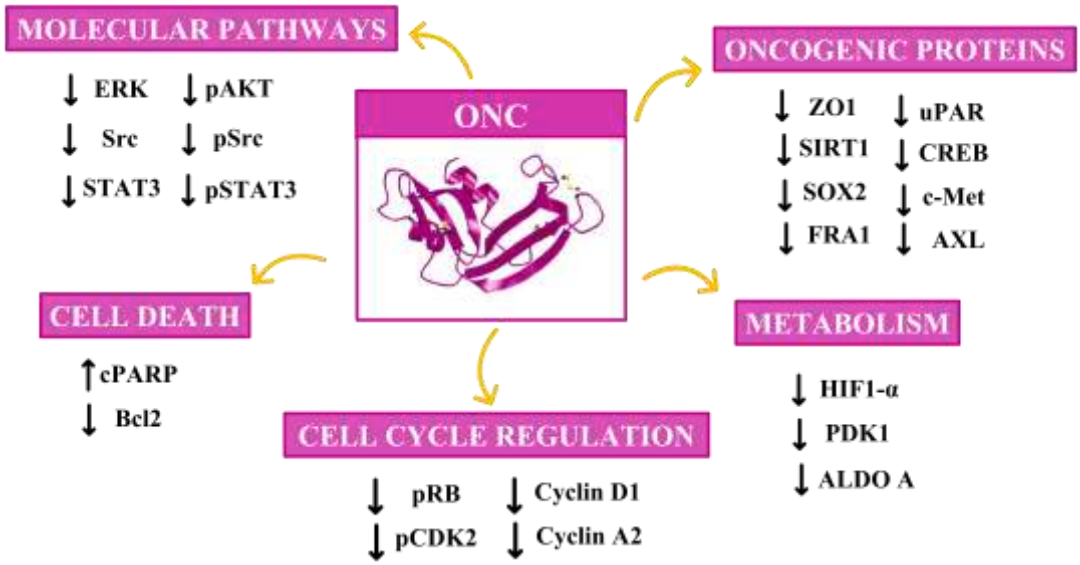

Figure 27. Schematic representation of ONC pleiotropic effect in A375 melanoma cells.

6. CONCLUSION

This study shed light on ONC cellular effects confirming the ribonuclease selectivity for cancer cells compared to normal ones. Contrarily to what happens for normal human melanocytes, ONC reduces cell viability of A375, MeWo and FO1 melanoma cell lines as well as U251 glioblastoma cells and HepG2 hepatocellular carcinoma cells in a time- and concentration-dependent manner. Despite its well-known antitumor activity, ONC use in clinic is limited by the induction of a reversible nephrotoxicity[131] and therefore exploiting ONC dimerization may be a good strategy to overcome this problem. Here it was compared ONC and ONC-D activity finding that the dimeric form displays a ribonucleolytic activity comparable to that of the monomeric enzyme and it reduces the viability of different cancer cell lines by targeting Src-kinase, STAT3 and Bcl2, although it displays an EC_{50} slightly lower than that of ONC. The slightly different antitumor effects registered within the two ONC species could be related to variations, caused by dimerization, in the relative position and/or conformation of some charged residues on the protein surface. However, this antitumor activity against more than one tumor cell line[143] is definitely promising and the possibility to produce ONC dimer or trimer through oligomerization may represent a valuable horizon for the anticancer potential of this enzyme. Despite the promising results obtained, the dimer is particularly difficult to study considering its low yield.

Considering that the specific ONC targets are still unknown, the modulation of the expression of several onco-suppressor miRNAs in the A375 and FO1 BRAF-mutated melanoma cell lines was studied. ONC is able to upregulate the expression level of miR-20a-3p, miR-29a-3p, and miR-34a-5p, together with a downregulation of many miRNAs-associated onco-proteins. These results could explain the pleiotropic anti-melanoma activity induced by ONC treatment involving cell cycle progression, survival signalling pathway, metabolism, and metastatic potential. Moreover, this data may lead to the hypothesis that ONC ribonucleolytic activity may be directed towards long-non-coding-RNA or circular-RNAs responsible for miRNAs stability[210]. Hopefully, this study may represent a first step towards the development of a new therapeutic strategy in which ONC must be considered an important tool against the onset of tumor metastasis.

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8. REFERENCES

(1) Liu, Y.; Sheikh, M. S. Melanoma: Molecular Pathogenesis and Therapeutic Management. *Mol. Cell. Pharmacol.* **2014**, *6* (3), 228.

(2) Mattia, G.; Puglisi, R.; Ascione, B.; Malorni, W.; Carè, A.; Matarrese, P. Cell Death-Based Treatments of Melanoma:Conventional Treatments and New Therapeutic Strategies Review-Article. *Cell Death Dis.* **2018**, *9* (2). https://doi.org/10.1038/s41419-017-0059-7.

(3) Goldstein, A. M.; Chan, M.; Harland, M.; Hayward, N. K.; Demenais, F.; Bishop, D. T.; Azizi, E.; Bergman, W.; Bianchi-Scarra, G.; Bruno, W.; Calista, D.; Albright, L. A. C.; Chaudru, V.; Chompret, A.; Cuellar, F.; Elder, D. E.; Ghiorzo, P.; Gillanders, E. M.; Gruis, N. A.; Hansson, J.; Hogg, D.; Holland, E. A.; Kanetsky, P. A.; Kefford, R. F.; Landi, M. T.; Lang, J.; Leachman, S. A.; MacKie, R. M.; Magnusson, V.; Mann, G. J.; Bishop, J. N.; Palmer, J. M.; Puig, S.; Puig-Butille, J. A.; Stark, M.; Tsao, H.; Tucker, M. A.; Whitaker, L.; Yakobson, E.; Group, T. L. M. S. Features Associated with Germline CDKN2A Mutations: A GenoMEL Study of Melanoma-prone Families from Three Continents. *J. Med. Genet.* **2007**, *44* (2), 99–106. https://doi.org/10.1136/jmg.2006.043802.

(4) Elder, D. E. Melanoma Progression. *Pathology (Phila.)* **2016**, *48* (2), 147–154. https://doi.org/10.1016/j.pathol.2015.12.002.

(5) Tentori, L.; Lacal, P. M.; Graziani, G. Challenging Resistance Mechanisms to Therapies for Metastatic Melanoma. *Trends Pharmacol. Sci.* **2013**, *34* (12), 656–666. https://doi.org/10.1016/j.tips.2013.10.003.

(6) Balch, C. M.; Gershenwald, J. E.; Soong, S. J.; Thompson, J. F.; Atkins, M. B.; Byrd, D. R.; Buzaid, A. C.; Cochran, A. J.; Coit, D. G.; Ding, S.; Eggermont, A. M.; Flaherty, K. T.; Gimotty, P. A.; Kirkwood, J. M.; McMasters, K. M.; Mihm, M. C.; Morton, D. L.; Ross, M. I.; Sober, A. J.; Sondak, V. K. Final Version of 2009 AJCC Melanoma Staging and Classification. *J. Clin. Oncol.* **2009**, *27* (36), 6199–6206. https://doi.org/10.1200/JCO.2009.23.4799.

(7) Takata, M.; Murata, H.; Saida, T. Molecular Pathogenesis of Malignant Melanoma: A Different Perspective from the Studies of Melanocytic Nevus and Acral Melanoma. *Pigment Cell Melanoma Res.* **2010**, *23* (1), 64–71. https://doi.org/10.1111/j.1755-148X.2009.00645.x.

(8) Bastian, B. C. *The Molecular Pathology of Melanoma: An Integrated Taxonomy of Melanocytic Neoplasia*; 2014; Vol. 9. https://doi.org/10.1146/annurev-pathol-012513-104658.

(9) Caini, S.; Gandini, S.; Sera, F.; Raimondi, S.; Fargnoli, M. C.; Boniol, M.; Armstrong, B. K. Meta-Analysis of Risk Factors for Cutaneous Melanoma According to Anatomical Site and Clinico-Pathological Variant. *Eur. J. Cancer* **2009**, *45* (17), 3054–3063. https://doi.org/10.1016/j.ejca.2009.05.009.

(10) Markovic, S. N.; Erickson, L. A.; Rao, R. D.; McWilliams, R. R.; Kottschade, L. A.; Creagan, E. T.; Weenig, R. H.; Hand, J. L.; Pittelkow, M. R.; Pockaj, B. A.; Bardia, A.; Vachon, C. M.; Schild, S. E.; Laman, S. D.; Maples, W. J.; Pulido, J. S.; Cameron, J. D. Malignant Melanoma in the 21st Century, Part 1: Epidemiology, Risk Factors, Screening, Prevention, and Diagnosis. *Mayo Clin. Proc.* **2007**, *82* (3), 364–380. https://doi.org/10.4065/82.3.364.

(11) Rigel, D. S. Epidemiology of Melanoma. *Semin. Cutan. Med. Surg.* **2010**, *29* (4), 204–209. https://doi.org/10.1016/j.sder.2010.10.005.

(12) Gomolin, T.; Cline, A.; Handler, M. Z. The Danger of Neglecting Melanoma during the COVID-19 Pandemic. *J. Dermatol. Treat.* **2020**, *31* (5), 444–445. https://doi.org/10.1080/09546634.2020.1762844.

(13) Gualdi, G.; Porreca, A.; Amoruso, G. F.; Atzori, L.; Calzavara-Pinton, P.; De Tursi, M.; Buduo, A. D.; Marino, P. D.; Fabroncini, G.; Fantini, F.; Fargnoli, M. C.; Giannotta, G.; Lacarruba, F.; Lotesoriere, A.; Merli, M.; Micali, G.; Paradisi, A.; Puviani, M.; Quaglino, P.; Rongioletti, F.; Rubatto, M.; Sbano, P.; Scalvenzi, M.; Soglia, S.; Sollima, L.; Villani, A.; Nicola, M. D.; Amerio, P. The Effect of the COVID-19 Lockdown on Melanoma Diagnosis in Italy. *Clin. Dermatol.* **2021**, *39* (5), 911–919. https://doi.org/10.1016/j.clindermatol.2021.05.015.

(14) Hoellwerth, M.; Kaiser, A.; Emberger, M.; Brandlmaier, M.; Laimer, M.; Egger, A.; Bauer, J. W.; Koelblinger, P. COVID-19-Induced Reduction in Primary Melanoma Diagnoses: Experience from a Dermatopathology Referral Center. *J. Clin. Med.* **2021**, *10* (18), 4059. https://doi.org/10.3390/jcm10184059.

(15) Ali, Z.; Yousaf, N.; Larkin, J. Melanoma Epidemiology, Biology and Prognosis. *Eur. J. Cancer Suppl.* **2013**, *11* (2), 81–91. https://doi.org/10.1016/j.ejcsup.2013.07.012.

(16) Batus, M.; Waheed, S.; Ruby, C.; Petersen, L.; Bines, S. D.; Kaufman, H. L. Optimal Management of Metastatic Melanoma: Current Strategies and Future Directions. *Am. J. Clin. Dermatol.* **2013**, *14* (3), 179–194. https://doi.org/10.1007/s40257-013-0025-9.

(17) Widakowich, C.; de Castro, G.; de Azambuja, E.; Dinh, P.; Awada, A. Review: Side Effects of Approved Molecular Targeted Therapies in Solid Cancers. *The Oncologist* **2007**, *12* (12), 1443–1455. https://doi.org/10.1634/theoncologist.12-12-1443.

(18) J, L.; Y, W.; R, L.; X, A.; K, W.; G, S.; Y, T.; J, Z.; J, T. Recent Advances in Targeted Nanoparticles Drug Delivery to Melanoma. *Nanomedicine Nanotechnol. Biol. Med.* **2015**, *11* (3). https://doi.org/10.1016/j.nano.2014.11.006.

(19) Austin, E.; Mamalis, A.; Ho, D.; Jagdeo, J. Laser and Light-Based Therapy for Cutaneous and Soft-Tissue Metastases of Malignant Melanoma: A Systematic Review. *Arch. Dermatol. Res.* **2017**, *309* (4), 229–242. https://doi.org/10.1007/s00403-017-1720-9.

(20) Miller, A. J.; Mihm, M. C. Melanoma. *N. Engl. J. Med.* **2006**, *355* (1), 51–65. https://doi.org/10.1056/NEJMra052166.

(21) Mishra, H.; Mishra, P. K.; Ekielski, A.; Jaggi, M.; Iqbal, Z.; Talegaonkar, S. Melanoma Treatment: From Conventional to Nanotechnology. *J. Cancer Res. Clin. Oncol.* **2018**, *144* (12), 2283–2302. https://doi.org/10.1007/s00432-018-2726-1.

(22) Guan, H.; Nagarkatti, P. S.; Nagarkatti, M. Blockade of Hyaluronan Inhibits IL-2-Induced Vascular Leak Syndrome and Maintains Effectiveness of IL-2 Treatment for Metastatic Melanoma. *J. Immunol.* **2007**, *179* (6), 3715–3723. https://doi.org/10.4049/jimmunol.179.6.3715.

(23) Vuoristo, M.; Jantunen, I.; Pyrhönen, S.; Muhonen, T.; Kellokumpu-Lehtinen, P. A Combination of Subcutaneous Recombinant Interleukin-2 and Recombinant Interferon-α in the Treatment of Advanced Renal Cell Carcinoma or Melanoma. *Eur. J. Cancer* **1994**, *30* (4), 530–532. https://doi.org/10.1016/0959-8049(94)90433-2.

(24) Coit, D. G.; Andtbacka, R.; Bichakjian, C. K.; Dilawari, R. A.; Dimaio, D.; Guild, V.; Halpern, A. C.; Hodi, F. S.; Kashani-Sabet, M.; Lange, J. R.; Lind, A.; Martin, L.; Martini, M. C.; Pruitt, S. K.; Ross, M. I.; Sener, S. F.; Swetter, S. M.; Tanabe, K. K.; Thompson, J. A.; Trisal, V.; Urist, M. M.; Weber, J.; Wong, M. K.; NCCN Melanoma Panel. Melanoma. *J. Natl. Compr. Cancer Netw. JNCCN* **2009**, *7* (3), 250–275. https://doi.org/10.6004/jnccn.2009.0020.

(25) Kuryk, L.; Bertinato, L.; Staniszewska, M.; Pancer, K.; Wieczorek, M.; Salmaso, S.; Caliceti, P.; Garofalo, M. From Conventional Therapies to Immunotherapy: Melanoma Treatment in Review. *Cancers* **2020**, *12* (10), E3057. https://doi.org/10.3390/cancers12103057.

(26) Long, G. V.; Eroglu, Z.; Infante, J.; Patel, S.; Daud, A.; Johnson, D. B.; Gonzalez, R.; Kefford, R.; Hamid, O.; Schuchter, L.; Cebon, J.; Sharfman, W.; McWilliams, R.; Sznol, M.; Redhu, S.; Gasal, E.; Mookerjee, B.; Weber, J.; Flaherty, K. T. Long-Term Outcomes in Patients With BRAF V600-Mutant Metastatic Melanoma Who Received Dabrafenib Combined With Trametinib. *J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol.* **2018**, *36* (7), 667–673. https://doi.org/10.1200/JCO.2017.74.1025.

(27) BHATIA, S.; TYKODI, S. S.; THOMPSON, J. A. Treatment of Metastatic Melanoma: An Overview. *Oncol. Williston Park N* **2009**, *23* (6), 488–496.

(28) Kim, C.; Lee, C. W.; Kovacic, L.; Shah, A.; Klasa, R.; Savage, K. J. Long-Term Survival in Patients with Metastatic Melanoma Treated with DTIC or Temozolomide. *The Oncologist* **2010**, *15* (7), 765–771. https://doi.org/10.1634/theoncologist.2009-0237.

(29) Proietti, I.; Skroza, N.; Bernardini, N.; Tolino, E.; Balduzzi, V.; Marchesiello, A.; Michelini, S.; Volpe, S.; Mambrin, A.; Mangino, G.; Romeo, G.; Maddalena, P.; Rees, C.; Potenza, C. Mechanisms of Acquired BRAF Inhibitor Resistance in Melanoma: A Systematic Review. *Cancers* **2020**, *12* (10), 2801. https://doi.org/10.3390/cancers12102801.

(30) Wong, R. S. Y. Apoptosis in Cancer: From Pathogenesis to Treatment. *J. Exp. Clin. Cancer Res.* **2011**, *30* (1), 1–14. https://doi.org/10.1186/1756-9966-30-87.

(31) Galluzzi, L.; Vitale, I.; Aaronson, S. A.; Abrams, J. M.; Adam, D.; Agostinis, P.; Alnemri, E. S.; Altucci, L.; Amelio, I.; Andrews, D. W.; Annicchiarico-Petruzzelli, M.; Antonov, A. V.; Arama, E.; Baehrecke, E. H.; Barlev, N. A.; Bazan, N. G.; Bernassola, F.; Bertrand, M. J. M.; Bianchi, K.; Blagosklonny, M. V.; Blomgren, K.; Borner, C.; Boya, P.; Brenner, C.; Campanella, M.; Candi, E.; Carmona-Gutierrez, D.; Cecconi, F.; Chan, F. K.-M.; Chandel, N. S.; Cheng, E. H.; Chipuk, J. E.; Cidlowski, J. A.; Ciechanover, A.; Cohen, G. M.; Conrad, M.; Cubillos-Ruiz, J. R.; Czabotar, P. E.; D'Angiolella, V.; Dawson, T. M.; Dawson, V. L.; De Laurenzi, V.; De Maria, R.; Debatin, K.-M.; DeBerardinis, R. J.; Deshmukh, M.; Di Daniele, N.; Di Virgilio, F.; Dixit, V. M.; Dixon, S. J.; Duckett, C. S.; Dynlacht, B. D.; El-Deiry, W. S.; Elrod, J. W.; Fimia, G. M.; Fulda, S.; García-Sáez, A. J.; Garg, A. D.; Garrido, C.; Gavathiotis, E.; Golstein, P.; Gottlieb, E.; Green, D. R.; Greene, L. A.; Gronemeyer, H.; Gross, A.; Hajnoczky, G.; Hardwick, J. M.; Harris, I. S.; Hengartner, M. O.; Hetz, C.; Ichijo, H.; Jäättelä, M.; Joseph, B.; Jost, P. J.; Juin, P. P.; Kaiser, W. J.; Karin, M.; Kaufmann, T.; Kepp, O.; Kimchi, A.; Kitsis, R. N.; Klionsky, D. J.; Knight, R. A.; Kumar, S.; Lee, S. W.; Lemasters, J. J.; Levine, B.; Linkermann, A.; Lipton, S. A.; Lockshin, R. A.; López-Otín, C.; Lowe, S. W.; Luedde, T.; Lugli, E.; MacFarlane, M.; Madeo, F.; Malewicz, M.; Malorni, W.; Manic, G.; Marine, J.-C.; Martin, S. J.; Martinou, J.-C.; Medema, J. P.; Mehlen, P.; Meier, P.; Melino, S.; Miao, E. A.; Molkentin, J. D.; Moll, U. M.; Muñoz-Pinedo, C.; Nagata, S.; Nuñez, G.; Oberst, A.; Oren, M.; Overholtzer, M.; Pagano, M.; Panaretakis, T.; Pasparakis, M.; Penninger, J. M.; Pereira, D. M.; Pervaiz, S.; Peter, M. E.; Piacentini, M.; Pinton, P.; Prehn, J. H. M.; Puthalakath, H.; Rabinovich, G. A.; Rehm, M.; Rizzuto, R.; Rodrigues, C. M. P.; Rubinsztein, D. C.; Rudel, T.; Ryan, K. M.; Sayan, E.; Scorrano, L.; Shao, F.; Shi, Y.; Silke, J.; Simon, H.-U.; Sistigu, A.; Stockwell, B. R.; Strasser, A.;

Szabadkai, G.; Tait, S. W. G.; Tang, D.; Tavernarakis, N.; Thorburn, A.; Tsujimoto, Y.; Turk, B.; Vanden Berghe, T.; Vandenabeele, P.; Vander Heiden, M. G.; Villunger, A.; Virgin, H. W.; Vousden, K. H.; Vucic, D.; Wagner, E. F.; Walczak, H.; Wallach, D.; Wang, Y.; Wells, J. A.; Wood, W.; Yuan, J.; Zakeri, Z.; Zhivotovsky, B.; Zitvogel, L.; Melino, G.; Kroemer, G. Molecular Mechanisms of Cell Death: Recommendations of the Nomenclature Committee on Cell Death 2018. *Cell Death Differ.* **2018**, *25* (3), 486–541. https://doi.org/10.1038/s41418-017-0012-4.

(32) Raineri, A.; Fasoli, S.; Campagnari, R.; Gotte, G.; Menegazzi, M. Onconase Restores Cytotoxicity in Dabrafenib-Resistant A375 Human Melanoma Cells and Affects Cell Migration, Invasion and Colony Formation Capability. *Int. J. Mol. Sci.* **2019**, *20* (23), E5980. https://doi.org/10.3390/ijms20235980.

(33) Saraste, A.; Pulkki, K. Morphologic and Biochemical Hallmarks of Apoptosis. *Cardiovasc. Res.* **2000**, *45* (3), 528–537. https://doi.org/10.1016/S0008-6363(99)00384-3.

(34) Soldani, C.; Scovassi, A. I. Poly (ADP-Ribose) Polymerase-1 Cleavage during Apoptosis : An Update Cell Death Mechanisms : Necrosis and Apoptosis. *Apoptosis* **2002**, *7* (4), 321–328.

(35) Li, X.; Darzynkiewicz, Z. Cleavage of Poly(ADP-Ribose) Polymerase Measured in Situ in Individual Cells: Relationship to DNA Fragmentation and Cell Cycle Position during Apoptosis. *Exp. Cell Res.* **2000**, *255* (1), 125–132. https://doi.org/10.1006/excr.1999.4796.

(36) Madonna, G.; Ullman, C. D.; Gentilcore, G.; Palmieri, G.; Ascierto, P. A. NF-KB as Potential Target in the Treatment of Melanoma. *J. Transl. Med.* **2012**, *10* (1), 1–8. https://doi.org/10.1186/1479-5876-10-53.

(37) Stilmann, M.; Hinz, M.; Arslan, S. Ç.; Zimmer, A.; Schreiber, V.; Scheidereit, C. A Nuclear Poly(ADP-Ribose)-Dependent Signalosome Confers DNA Damage-Induced IκB Kinase Activation. *Mol. Cell* **2009**, *36* (3), 365–378. https://doi.org/10.1016/j.molcel.2009.09.032.

(38) Jordan, P.; Carmo-Fonseca*, M. Molecular Mechanisms Involved in Cisplatin Cytotoxicity. *Cell. Mol. Life Sci. CMLS* **2000**, *57* (8), 1229–1235. https://doi.org/10.1007/PL00000762.

(39) Bhattacharya, S.; Ray, R. M.; Johnson, L. R. STAT3-Mediated Transcription of Bcl-2, Mcl-1 and c-IAP2 Prevents Apoptosis in Polyamine-Depleted Cells. *Biochem. J.* **2005**, *392* (2), 335–344. https://doi.org/10.1042/BJ20050465.

(40) Stilmann, M.; Hinz, M.; Arslan, S. C.; Zimmer, A.; Schreiber, V.; Scheidereit, C. A Nuclear Poly(ADP-Ribose)-Dependent Signalosome Confers DNA Damage-Induced IkappaB Kinase Activation. *Mol. Cell* **2009**, *36* (3), 365–378. https://doi.org/10.1016/j.molcel.2009.09.032.

(41) Siveen, K. S.; Sikka, S.; Surana, R.; Dai, X.; Zhang, J.; Kumar, A. P.; Tan, B. K. H.; Sethi, G.; Bishayee, A. Targeting the STAT3 Signaling Pathway in Cancer: Role of Synthetic and Natural Inhibitors. *Biochim. Biophys. Acta BBA - Rev. Cancer* **2014**, *1845* (2), 136–154. https://doi.org/10.1016/j.bbcan.2013.12.005.

(42) Macias, E.; Rao, D.; Digiovanni, J. Role of Stat3 in Skin Carcinogenesis: Insights Gained from Relevant Mouse Models. *J. Skin Cancer* **2013**, *2013*, 684050. https://doi.org/10.1155/2013/684050.

(43) Guo, Y.-J.; Pan, W.-W.; Liu, S.-B.; Shen, Z.-F.; Xu, Y.; Hu, L.-L. ERK/MAPK Signalling Pathway and Tumorigenesis (Review). *Exp. Ther. Med.* **2020**, *19* (3), 1997–2007. https://doi.org/10.3892/etm.2020.8454.

(44) Abe, M. K.; Saelzler, M. P.; Espinosa, R.; Kahle, K. T.; Hershenson, M. B.; Beau, M. M. L.; Rosner, M. R. ERK8, a New Member of the Mitogen-Activated Protein Kinase Family *. *J. Biol. Chem.* **2002**, *277* (19), 16733–16743. https://doi.org/10.1074/jbc.M112483200.

(45) McCain, J. The MAPK (ERK) Pathway. *Pharm. Ther.* **2013**, *38* (2), 96–108.

(46) Wellbrock, C.; Ogilvie, L.; Hedley, D.; Karasarides, M.; Martin, J.; Niculescu-Duvaz, D.; Springer, C. J.; Marais, R. V599EB-RAF Is an Oncogene in Melanocytes. *Cancer Res.* **2004**, *64* (7), 2338–2342. https://doi.org/10.1158/0008-5472.CAN-03-3433.

(47) Smith, M. P.; Brunton, H.; Rowling, E. J.; Ferguson, J.; Arozarena, I.; Miskolczi, Z.; Lee, J. L.; Girotti, M. R.; Marais, R.; Levesque, M. P.; Dummer, R.; Frederick, D. T.; Flaherty, K. T.; Cooper, Z. A.; Wargo, J. A.; Wellbrock, C. Inhibiting Drivers of Non-Mutational Drug Tolerance Is a Salvage Strategy for Targeted Melanoma Therapy. *Cancer Cell* **2016**, *29* (3), 270–284. https://doi.org/10.1016/j.ccell.2016.02.003.

(48) Courtnay, R.; Ngo, D. C.; Malik, N.; Ververis, K.; Tortorella, S. M.; Karagiannis, T. C. Cancer Metabolism and the Warburg Effect: The Role of HIF-1 and PI3K. *Mol. Biol. Rep.* **2015**, *42* (4), 841–851. https://doi.org/10.1007/s11033-015-3858-x.

(49) Peyssonnaux, C.; Eychène, A. The Raf/MEK/ERK Pathway: New Concepts of Activation. *Biol. Cell* **2001**, *93* (1–2), 53–62. https://doi.org/10.1016/S0248-4900(01)01125-X.

(50) Kunz, M.; Ibrahim, S. M. Molecular Responses to Hypoxia in Tumor Cells. *Mol. Cancer* **2003**, *2* (1), 23. https://doi.org/10.1186/1476-4598-2-23.

(51) Wang, G. L.; Jiang, B. H.; Rue, E. A.; Semenza, G. L. Hypoxia-Inducible Factor 1 Is a Basic-Helix-Loop-Helix-PAS Heterodimer Regulated by Cellular O2 Tension. *Proc. Natl. Acad. Sci.* **1995**, *92* (12), 5510–5514. https://doi.org/10.1073/pnas.92.12.5510.

(52) Tanimoto, K.; Makino, Y.; Pereira, T.; Poellinger, L. Mechanism of Regulation of the Hypoxia-Inducible Factor-1α by the von Hippel-Lindau Tumor Suppressor Protein. *EMBO J.* **2000**, *19* (16), 4298–4309. https://doi.org/10.1093/emboj/19.16.4298.

(53) Haddad, J. J. Antioxidant and Prooxidant Mechanisms in the Regulation of Redox(y)-Sensitive Transcription Factors. *Cell. Signal.* **2002**, *14* (11), 879–897. https://doi.org/10.1016/S0898-6568(02)00053-0.

(54) Kumar, S. M.; Acs, G.; Fang, D.; Herlyn, M.; Elder, D. E.; Xu, X. Functional Erythropoietin Autocrine Loop in Melanoma. *Am. J. Pathol.* **2005**, *166* (3), 823–830. https://doi.org/10.1016/S0002-9440(10)62303-6.

(55) Shweiki, D.; Itin, A.; Soffer, D.; Keshet, E. Vascular Endothelial Growth Factor Induced by Hypoxia May Mediate Hypoxia-Initiated Angiogenesis. *Nature* **1992**, *359* (6398), 843–845. https://doi.org/10.1038/359843a0.

(56) Lim, J.-H.; Lee, E.-S.; You, H.-J.; Lee, J. W.; Park, J.-W.; Chun, Y.-S. Ras-Dependent Induction of HIF-1α785 via the Raf/MEK/ERK Pathway: A Novel Mechanism of Ras-Mediated Tumor Promotion. *Oncogene* **2004**, *23* (58), 9427–9431. https://doi.org/10.1038/sj.onc.1208003.

(57) Sharkia, I.; Hadad Erlich, T.; Landolina, N.; Assayag, M.; Motzik, A.; Rachmin, I.; Kay, G.; Porat, Z.; Tshori, S.; Berkman, N.; Levi-Schaffer, F.; Razin, E. Pyruvate Dehydrogenase Has a Major Role in Mast

Cell Function, and Its Activity Is Regulated by Mitochondrial Microphthalmia Transcription Factor. *J. Allergy Clin. Immunol.* **2017**, *140* (1), 204-214.e8. https://doi.org/10.1016/j.jaci.2016.09.047.

(58) Ratnikov, B. I.; Scott, D. A.; Osterman, A. L.; Smith, J. W.; Ronai, Z. A. Metabolic Rewiring in Melanoma. *Oncogene* **2017**, *36* (2), 147–157. https://doi.org/10.1038/onc.2016.198.

(59) Fischer, G. M.; Vashisht Gopal, Y. N.; McQuade, J. L.; Peng, W.; DeBerardinis, R. J.; Davies, M. A. Metabolic Strategies of Melanoma Cells: Mechanisms, Interactions with the Tumor Microenvironment, and Therapeutic Implications. *Pigment Cell Melanoma Res.* **2018**, *31* (1), 11–30. https://doi.org/10.1111/pcmr.12661.

(60) Borsi, E.; Terragna, C.; Brioli, A.; Tacchetti, P.; Martello, M.; Cavo, M. Therapeutic Targeting of Hypoxia and Hypoxia-Inducible Factor 1 Alpha in Multiple Myeloma. *Transl. Res.* **2015**, *165* (6), 641–650. https://doi.org/10.1016/j.trsl.2014.12.001.

(61) Schafer, K. A. The Cell Cycle: A Review. *Vet. Pathol.* **1998**, *35* (6), 461–478. https://doi.org/10.1177/030098589803500601.

(62) Jiang, Q.; Isquith, J.; Zipeto, M. A.; Diep, R. H.; Pham, J.; Delos Santos, N.; Reynoso, E.; Chau, J.; Leu, H.; Lazzari, E.; Melese, E.; Ma, W.; Fang, R.; Minden, M.; Morris, S.; Ren, B.; Pineda, G.; Holm, F.; Jamieson, C. Hyper-Editing of Cell-Cycle Regulatory and Tumor Suppressor RNA Promotes Malignant Progenitor Propagation. *Cancer Cell* **2019**, *35* (1), 81-94.e7. https://doi.org/10.1016/j.ccell.2018.11.017.

(63) Dang, F.; Nie, L.; Wei, W. Ubiquitin Signaling in Cell Cycle Control and Tumorigenesis. *Cell Death Differ.* **2021**, *28* (2), 427–438. https://doi.org/10.1038/s41418-020-00648-0.

(64) Darzynkiewicz, Z.; Gong, J.; Juan, G.; Ardelt, B.; Traganos, F. Cytometry of Cyclin Proteins. *Cytometry* **1996**, *25* (1), 1–13. https://doi.org/10.1002/(SICI)1097-0320(19960901)25:1<1::AID-CYTO1>3.0.CO;2-N.

(65) Rousseau, F.; Schymkowitz, J. W. H.; Sánchez del Pino, M.; Itzhaki, L. S. Stability and Folding of the Cell Cycle Regulatory Protein, P13suc111Edited by F. E. Cohen. *J. Mol. Biol.* **1998**, *284* (2), 503–519. https://doi.org/10.1006/jmbi.1998.2173.

(66) Malumbres, M.; Harlow, E.; Hunt, T.; Hunter, T.; Lahti, J. M.; Manning, G.; Morgan, D. O.; Tsai, L.-H.; Wolgemuth, D. J. Cyclin-Dependent Kinases: A Family Portrait. *Nat. Cell Biol.* **2009**, *11* (11), 1275–1276. https://doi.org/10.1038/ncb1109-1275.

(67) Lin, Z. P.; Zhu, Y.-L.; Ratner, E. S. Targeting Cyclin-Dependent Kinases for Treatment of Gynecologic Cancers. *Front. Oncol.* **2018**, *8*.

(68) Harbour, J. W.; Luo, R. X.; Santi, A. D.; Postigo, A. A.; Dean, D. C. Cdk Phosphorylation Triggers Sequential Intramolecular Interactions That Progressively Block Rb Functions as Cells Move through G1. *Cell* **1999**, *98* (6), 859–869. https://doi.org/10.1016/S0092-8674(00)81519-6.

(69) Arroyo, M.; Raychaudhuri, P. Retinoblastoma-Repression of E2F-Dependent Transcription Depends on the Ability of the Retinoblastoma Protein to Interact with E2F and Is Abrogated by the Adenovirus E1A Oncoprotein. *Nucleic Acids Res.* **1992**, *20* (22), 5947–5954. https://doi.org/10.1093/nar/20.22.5947.

(70) *Tumor metastasis: Mechanistic insights and therapeutic interventions - Liu - 2021 - MedComm - Wiley Online Library*. https://onlinelibrary.wiley.com/doi/full/10.1002/mco2.100 (accessed 2022-11-11).

(71) Kalluri, R. EMT: When Epithelial Cells Decide to Become Mesenchymal-like Cells. *J. Clin. Invest.* **2009**, *119* (6), 1417–1419. https://doi.org/10.1172/JCI39675.

(72) Lv, Q.; Wang, W.; Xue, J.; Hua, F.; Mu, R.; Lin, H.; Yan, J.; Lv, X.; Chen, X.; Hu, Z.-W. DEDD Interacts with PI3KC3 to Activate Autophagy and Attenuate Epithelial–Mesenchymal Transition in Human Breast Cancer. *Cancer Res.* **2012**, *72* (13), 3238–3250. https://doi.org/10.1158/0008-5472.CAN-11-3832.

(73) Yap, A. S.; Brieher, W. M.; Gumbiner, B. M. Molecular and Functional Analysis of Cadherin-Based Adherens Junctions. *Annu. Rev. Cell Dev. Biol.* **1997**, *13* (1), 119–146. https://doi.org/10.1146/annurev.cellbio.13.1.119.

(74) Thiery, J. P.; Sleeman, J. P. Complex Networks Orchestrate Epithelial–Mesenchymal Transitions. *Nat. Rev. Mol. Cell Biol.* **2006**, *7* (2), 131–142. https://doi.org/10.1038/nrm1835.

(75) Voulgari, A.; Pintzas, A. Epithelial–Mesenchymal Transition in Cancer Metastasis: Mechanisms, Markers and Strategies to Overcome Drug Resistance in the Clinic. *Biochim. Biophys. Acta BBA - Rev. Cancer* **2009**, *1796* (2), 75–90. https://doi.org/10.1016/j.bbcan.2009.03.002.

(76) Haass, N. K.; Smalley, K. S. M.; Li, L.; Herlyn, M. Adhesion, Migration and Communication in Melanocytes and Melanoma. *Pigment Cell Res.* **2005**, *18* (3), 150–159. https://doi.org/10.1111/j.1600-0749.2005.00235.x.

(77) Gonzalez-Mariscal, L.; Namorado, M. C.; Martin, D.; Luna, J.; Alarcon, L.; Islas, S.; Valencia, L.; Muriel, P.; Ponce, L.; Reyes, J. L. Tight Junction Proteins ZO-1, ZO-2, and Occludin along Isolated Renal Tubules11See Editorial by Brown, p. 2652. *Kidney Int.* **2000**, *57* (6), 2386–2402. https://doi.org/10.1046/j.1523-1755.2000.00098.x.

(78) Stevenson, B. R.; Siliciano, J. D.; Mooseker, M. S.; Goodenough, D. A. Identification of ZO-1: A High Molecular Weight Polypeptide Associated with the Tight Junction (Zonula Occludens) in a Variety of Epithelia. *J. Cell Biol.* **1986**, *103* (3), 755–766. https://doi.org/10.1083/jcb.103.3.755.

(79) Fanning, A. S.; Jameson, B. J.; Jesaitis, L. A.; Anderson, J. M. The Tight Junction Protein ZO-1 Establishes a Link between the Transmembrane Protein Occludin and the Actin Cytoskeleton *. *J. Biol. Chem.* **1998**, *273* (45), 29745–29753. https://doi.org/10.1074/jbc.273.45.29745.

(80) Pummi, K.; Malminen, M.; Aho, H.; Karvonen, S.-L.; Peltonen, J.; Peltonen, S. Epidermal Tight Junctions: ZO-1 and Occludin Are Expressed in Mature, Developing, and Affected Skin and In Vitro Differentiating Keratinocytes. *J. Invest. Dermatol.* **2001**, *117* (5), 1050–1058. https://doi.org/10.1046/j.0022-202x.2001.01493.x.

(81) Malminen, M.; Koivukangas, V.; Peltonen, J.; Karvonen, S.; Oikarinen, A.; Peltonen, S. Immunohistological Distribution of the Tight Junction Components ZO-1 and Occludin in Regenerating Human Epidermis. *Br. J. Dermatol.* **2003**, *149* (2), 255–260. https://doi.org/10.1046/j.1365-2133.2003.05438.x.

(82) Langbein, L.; Grund, C.; Kuhn, C.; Praetzel, S.; Kartenbeck, J.; Brandner, J. M.; Moll, I.; Franke, W. W. Tight Junctions and Compositionally Related Junctional Structures in Mammalian Stratified Epithelia and Cell Cultures Derived Therefrom. *Eur. J. Cell Biol.* **2002**, *81* (8), 419–435. https://doi.org/10.1078/0171-9335-00270.

(83) Smalley, K. S. M.; Brafford, P.; Haass, N. K.; Brandner, J. M.; Brown, E.; Herlyn, M. Up-Regulated Expression of Zonula Occludens Protein-1 in Human Melanoma Associates with N-Cadherin and

Contributes to Invasion and Adhesion. *Am. J. Pathol.* **2005**, *166* (5), 1541–1554. https://doi.org/10.1016/S0002-9440(10)62370-X.

(84) Conrad, E.; Polonio-Vallon, T.; Meister, M.; Matt, S.; Bitomsky, N.; Herbel, C.; Liebl, M.; Greiner, V.; Kriznik, B.; Schumacher, S.; Krieghoff-Henning, E.; Hofmann, T. G. HIPK2 Restricts SIRT1 Activity upon Severe DNA Damage by a Phosphorylation-Controlled Mechanism. *Cell Death Differ.* **2016**, *23* (1), 110–122. https://doi.org/10.1038/cdd.2015.75.

(85) Ou, X.; Lee, M. R.; Huang, X.; Messina-Graham, S.; Broxmeyer, H. E. SIRT1 Positively Regulates Autophagy and Mitochondria Function in Embryonic Stem Cells under Oxidative Stress. *Stem Cells Dayt. Ohio* **2014**, *32* (5), 1183–1194. https://doi.org/10.1002/stem.1641.

(86) Fang, Y.; Nicholl, M. B. Sirtuin 1 in Malignant Transformation: Friend or Foe? *Cancer Lett.* **2011**, *306* (1), 10–14. https://doi.org/10.1016/j.canlet.2011.02.019.

(87) Liu, T.; Liu, P. Y.; Marshall, G. M. The Critical Role of the Class III Histone Deacetylase SIRT1 in Cancer. *Cancer Res.* **2009**, *69* (5), 1702–1705. https://doi.org/10.1158/0008-5472.CAN-08-3365.

(88) Byles, V.; Zhu, L.; Lovaas, J. D.; Chmilewski, L. K.; Wang, J.; Faller, D. V.; Dai, Y. SIRT1 Induces EMT by Cooperating with EMT Transcription Factors and Enhances Prostate Cancer Cell Migration and Metastasis. *Oncogene* **2012**, *31* (43), 4619–4629. https://doi.org/10.1038/onc.2011.612.

(89) Sun, T.; Jiao, L.; Wang, Y.; Yu, Y.; Ming, L. SIRT1 Induces Epithelial-Mesenchymal Transition by Promoting Autophagic Degradation of E-Cadherin in Melanoma Cells. *Cell Death Dis.* **2018**, *9* (2), 1–10. https://doi.org/10.1038/s41419-017-0167-4.

(90) Bylund, M.; Andersson, E.; Novitch, B. G.; Muhr, J. Vertebrate Neurogenesis Is Counteracted by Sox1–3 Activity. *Nat. Neurosci.* **2003**, *6* (11), 1162–1168. https://doi.org/10.1038/nn1131.

(91) Kashyap, V.; Rezende, N. C.; Scotland, K. B.; Shaffer, S. M.; Persson, J. L.; Gudas, L. J.; Mongan, N. P. Regulation of Stem Cell Pluripotency and Differentiation Involves a Mutual Regulatory Circuit of the Nanog, OCT4, and SOX2 Pluripotency Transcription Factors With Polycomb Repressive Complexes and Stem Cell MicroRNAs. *Stem Cells Dev.* **2009**, *18* (7), 1093–1108. https://doi.org/10.1089/scd.2009.0113.

(92) Chen, Y.; Shi, L.; Zhang, L.; Li, R.; Liang, J.; Yu, W.; Sun, L.; Yang, X.; Wang, Y.; Zhang, Y.; Shang, Y. The Molecular Mechanism Governing the Oncogenic Potential of SOX2 in Breast Cancer *. *J. Biol. Chem.* **2008**, *283* (26), 17969–17978. https://doi.org/10.1074/jbc.M802917200.

(93) Ben-Porath, I.; Thomson, M. W.; Carey, V. J.; Ge, R.; Bell, G. W.; Regev, A.; Weinberg, R. A. An Embryonic Stem Cell–like Gene Expression Signature in Poorly Differentiated Aggressive Human Tumors. *Nat. Genet.* **2008**, *40* (5), 499–507. https://doi.org/10.1038/ng.127.

(94) Laga, A. C.; Lai, C.-Y.; Zhan, Q.; Huang, S. J.; Velazquez, E. F.; Yang, Q.; Hsu, M.-Y.; Murphy, G. F. Expression of The Embryonic Stem Cell Transcription Factor SOX2 in Human Skin: Relevance to Melanocyte and Merkel Cell Biology. *Am. J. Pathol.* **2010**, *176* (2), 903–913. https://doi.org/10.2353/ajpath.2010.090495.

(95) Kenessey, I.; Keszthelyi, M.; Kramer, Z.; Berta, J.; Adam, A.; Dobos, J.; Mildner, M.; Flachner, B.; Cseh, S.; Barna, G.; Szokol, B.; Orfi, L.; Keri, G.; Dome, B.; Klepetko, W.; Timar, J.; Tovari, J. Inhibition of C-Met with the Specific Small Molecule Tyrosine Kinase Inhibitor SU11274 Decreases Growth and Metastasis Formation of Experimental Human Melanoma. *Curr. Cancer Drug Targets* **2010**, *10* (3), 332–342. https://doi.org/10.2174/156800910791190184.

(96) Czyz, M. HGF/c-MET Signaling in Melanocytes and Melanoma. *Int. J. Mol. Sci.* **2018**, *19* (12), E3844. https://doi.org/10.3390/ijms19123844.

(97) Auyez, A.; Sayan, A. E.; Kriajevska, M.; Tulchinsky, E. AXL Receptor in Cancer Metastasis and Drug Resistance: When Normal Functions Go Askew. *Cancers* **2021**, *13* (19), 4864. https://doi.org/10.3390/cancers13194864.

(98) Jiang, X.; Xie, H.; Dou, Y.; Yuan, J.; Zeng, D.; Xiao, S. Expression and Function of FRA1 Protein in Tumors. *Mol. Biol. Rep.* **2020**, *47* (1), 737–752. https://doi.org/10.1007/s11033-019-05123-9.

(99) Balasubramanian, S.; Efimova, T.; Eckert, R. L. Green Tea Polyphenol Stimulates a Ras, MEKK1, MEK3, and P38 Cascade to Increase Activator Protein 1 Factor-Dependent Involucrin Gene Expression in Normal Human Keratinocytes *. *J. Biol. Chem.* **2002**, *277* (3), 1828–1836. https://doi.org/10.1074/jbc.M110376200.

(100) Efimova, T.; Deucher, A.; Kuroki, T.; Ohba, M.; Eckert, R. L. Novel Protein Kinase C Isoforms Regulate Human Keratinocyte Differentiation by Activating a P38δ Mitogen-Activated Protein Kinase Cascade That Targets CCAAT/Enhancer-Binding Protein α *. *J. Biol. Chem.* **2002**, *277* (35), 31753–31760. https://doi.org/10.1074/jbc.M205098200.

(101) Efimova, T.; LaCelle, P.; Welter, J. F.; Eckert, R. L. Regulation of Human Involucrin Promoter Activity by a Protein Kinase C, Ras, MEKK1, MEK3, P38/RK, AP1 Signal Transduction Pathway *. *J. Biol. Chem.* **1998**, *273* (38), 24387–24395. https://doi.org/10.1074/jbc.273.38.24387.

(102) Eckert, R. L.; Adhikary, G.; Young, C. A.; Jans, R.; Crish, J. F.; Xu, W.; Rorke, E. A. AP1 Transcription Factors in Epidermal Differentiation and Skin Cancer. *J. Skin Cancer* **2013**, *2013*, e537028. https://doi.org/10.1155/2013/537028.

(103) Shaulian, E.; Karin, M. AP-1 in Cell Proliferation and Survival. *Oncogene* **2001**, *20* (19), 2390–2400. https://doi.org/10.1038/sj.onc.1204383.

(104) Angel, P.; Szabowski, A.; Schorpp-Kistner, M. Function and Regulation of AP-1 Subunits in Skin Physiology and Pathology. *Oncogene* **2001**, *20* (19), 2413–2423. https://doi.org/10.1038/sj.onc.1204380.

(105) Mizuno, H.; Cho, Y.-Y.; Zhu, F.; Ma, W.-Y.; Bode, A. M.; Yang, C. S.; Ho, C.-T.; Dong, Z. Theaflavin-3, 3′-Digallate Induces Epidermal Growth Factor Receptor Downregulation. *Mol. Carcinog.* **2006**, *45* (3), 204–212. https://doi.org/10.1002/mc.20174.

(106) Kikuchi, T.; Hagiwara, K.; Honda, Y.; Gomi, K.; Kobayashi, T.; Takahashi, H.; Tokue, Y.; Watanabe, A.; Nukiwa, T. Clarithromycin Suppresses Lipopolysaccharide-Induced Interleukin-8 Production by Human Monocytes through AP-1 and NF-KB Transcription Factors. *J. Antimicrob. Chemother.* **2002**, *49* (5), 745–755. https://doi.org/10.1093/jac/dkf008.

(107) Adhikary, G.; Crish, J.; Lass, J.; Eckert, R. L. Regulation of Involucrin Expression in Normal Human Corneal Epithelial Cells: A Role for Activator Protein One. *Invest. Ophthalmol. Vis. Sci.* **2004**, *45* (4), 1080–1087. https://doi.org/10.1167/iovs.03-1180.

(108) Efimova, T.; Broome, A.-M.; Eckert, R. L. Protein Kinase Cδ Regulates Keratinocyte Death and Survival by Regulating Activity and Subcellular Localization of a P38δ-Extracellular Signal-Regulated Kinase 1/2 Complex. *Mol. Cell. Biol.* **2004**, *24* (18), 8167–8183. https://doi.org/10.1128/MCB.24.18.8167-8183.2004.

(109) Schüle, R.; Rangarajan, P.; Kliewer, S.; Ransone, L. J.; Bolado, J.; Yang, N.; Verma, I. M.; Evans, R. M. Functional Antagonism between Oncoprotein C-Jun and the Glucocorticoid Receptor. *Cell* **1990**, *62* (6), 1217–1226. https://doi.org/10.1016/0092-8674(90)90397-W.

(110) Abate, C.; Patel, L.; Rauscher, F. J.; Curran, T. Redox Regulation of Fos and Jun DNA-Binding Activity in Vitro. *Science* **1990**, *249* (4973), 1157–1161. https://doi.org/10.1126/science.2118682.

(111) Verde, P.; Casalino, L.; Talotta, F.; Yaniv, M.; Weitzman, J. B. Deciphering AP-1 Function in Tumorigenesis: Fra-Ternizing on Target Promoters. *Cell Cycle* **2007**, *6* (21), 2633–2639. https://doi.org/10.4161/cc.6.21.4850.

(112) Diesch, J.; Sanij, E.; Gilan, O.; Love, C.; Tran, H.; Fleming, N. I.; Ellul, J.; Amalia, M.; Haviv, I.; Pearson, R. B.; Tulchinsky, E.; Mariadason, J. M.; Sieber, O. M.; Hannan, R. D.; Dhillon, A. S. Widespread FRA1-Dependent Control of Mesenchymal Transdifferentiation Programs in Colorectal Cancer Cells. *PLOS ONE* **2014**, *9* (3), e88950. https://doi.org/10.1371/journal.pone.0088950.

(113) Milde-Langosch, K. The Fos Family of Transcription Factors and Their Role in Tumourigenesis. *Eur. J. Cancer* **2005**, *41* (16), 2449–2461. https://doi.org/10.1016/j.ejca.2005.08.008.

(114) Ardelt, W.; Shogen, K.; Darzynkiewicz, Z. Onconase and Amphinase, the Antitumor Ribonucleases from Rana Pipiens Oocytes. *Curr. Pharm. Biotechnol.* **2008**, *9* (3), 215–225. https://doi.org/10.2174/138920108784567245.

(115) Mikulski, S. M.; Costanzi, J. J.; Vogelzang, N. J.; McCachren, S.; Taub, R. N.; Chun, H.; Mittelman, A.; Panella, T.; Puccio, C.; Fine, R.; Shogen, K. Phase II Trial of a Single Weekly Intravenous Dose of Ranpirnase in Patients with Unresectable Malignant Mesothelioma. *J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol.* **2002**, *20* (1), 274–281. https://doi.org/10.1200/JCO.2002.20.1.274.

(116) Ardelt, W.; Mikulski, S. M.; Shogen, K. Amino Acid Sequence of an Anti-Tumor Protein from Rana Pipiens Oocytes and Early Embryos. Homology to Pancreatic Ribonucleases. *J. Biol. Chem.* **1991**, *266* (1), 245–251.

(117) Rodríguez, M.; Torrent, G.; Bosch, M.; Rayne, F.; Dubremetz, J. F.; Ribó, M.; Benito, A.; Vilanova, M.; Beaumelle, B. Intracellular Pathway of Onconase That Enables Its Delivery to the Cytosol. *J. Cell Sci.* **2007**, *120* (8), 1405–1411. https://doi.org/10.1242/jcs.03427.

(118) Lee, J. E.; Raines, R. T. Ribonucleases as Novel Chemotherapeutics: The Ranpirnase Example. *BioDrugs* **2008**, *22* (1), 53–58. https://doi.org/10.2165/00063030-200822010-00006.

(119) Iordanov, M. S.; Ryabinina, O. P.; Wong, J.; Dinh, T. H.; Newton, D. L.; Rybak, S. M.; Magun, B. E. Molecular Determinants of Apoptosis Induced by the Cytotoxic Ribonuclease Onconase: Evidence for Cytotoxic Mechanisms Different from Inhibition of Protein Synthesis. *Cancer Res.* **2000**, *60* (7), 1983–1994.

(120) Saxena, S. K.; Sirdeshmukh, R.; Ardelt, W.; Mikulski, S. M.; Shogen, K.; Youle, R. J. Entry into Cells and Selective Degradation of TRNAs by a Cytotoxic Member of the RNase A Family. *J. Biol. Chem.* **2002**, *277* (17), 15142–15146. https://doi.org/10.1074/jbc.M108115200.

(121) Notomista, E.; Catanzano, F.; Graziano, G.; Piaz, F. D.; Barone, G.; D'Alessio, G.; Di Donato, A. Onconase: An Unusually Stable Protein. *Biochemistry* **2000**, *39* (30), 8711–8718. https://doi.org/10.1021/bi000415x.

(122) Kobe, B.; Deisenhofer, J. Crystal Structure of Porcine Ribonuclease Inhibitor, a Protein with Leucine-Rich Repeats. *Nature* **1993**, *366* (6457), 751–756. https://doi.org/10.1038/366751a0.

(123) Furia, A.; Moscato, M.; Calì, G.; Pizzo, E.; Confalone, E.; Amoroso, M. R.; Esposito, F.; Nitsch, L.; D'Alessio, G. The Ribonuclease/Angiogenin Inhibitor Is Also Present in Mitochondria and Nuclei. *FEBS Lett.* **2011**, *585* (4), 613–617. https://doi.org/10.1016/j.febslet.2011.01.034.

(124) Rutkoski, T. J.; Raines, R. T. Evasion of Ribonuclease Inhibitor as a Determinant of Ribonuclease Cytotoxicity. *Curr. Pharm. Biotechnol.* **2008**, *9* (3), 185–189. https://doi.org/10.2174/138920108784567344.

(125) Wu, Y.; Mikulski, S. M.; Ardelt, W.; Rybak, S. M.; Youle, R. J. A Cytotoxic Ribonuclease. Study of the Mechanism of Onconase Cytotoxicity. *J. Biol. Chem.* **1993**, *268* (14), 10686–10693. https://doi.org/10.1016/S0021-9258(18)82252-9.

(126) Kobe, B.; Deisenhofer, J. Mechanism of Ribonuclease Inhibition by Ribonuclease Inhibitor Protein Based on the Crystal Structure of Its Complex with Ribonuclease A. *J. Mol. Biol.* **1996**, *264* (5), 1028–1043. https://doi.org/10.1006/jmbi.1996.0694.

(127) Turcotte, R. F.; Raines, R. T. Interaction of Onconase with the Human Ribonuclease Inhibitor Protein. *Biochem. Biophys. Res. Commun.* **2008**, *377* (2), 512–514. https://doi.org/10.1016/j.bbrc.2008.10.032.

(128) Rybak, S. M.; Pearson, J. W.; Fogler, W. E.; Volker, K.; Spence, S. E.; Newton, D. L.; Mikulski, S. M.; Ardelt, W.; Riggs, C. W.; Kung, H. F.; Longo, D. L. Enhancement of Vincristine Cytotoxicity in Drug-Resistant Cells by Simultaneous Treatment with Onconase, an Antitumor Ribonuclease. *J. Natl. Cancer Inst.* **1996**, *88* (11), 747–753. https://doi.org/10.1093/jnci/88.11.747.

(129) Pavlakis; Vogelzang. Ranpirnase--an Antitumour Ribonuclease: Its Potential Role in Malignant Mesothelioma. *Expert Opin Biol Ther* **2006**, *6(4):391-9*.

(130) Raineri, A.; Prodomini, S.; Fasoli, S.; Gotte, G.; Menegazzi, M. Influence of Onconase in the Therapeutic Potential of PARP Inhibitors in A375 Malignant Melanoma Cells. *Biochem. Pharmacol.* **2019**, *167* (May), 173–181. https://doi.org/10.1016/j.bcp.2019.06.006.

(131) Vasandani, V. M.; Burris, J. A.; Sung, C. Reversible Nephrotoxicity of Onconase and Effect of Lysine PH on Renal Onconase Uptake. *Cancer Chemother. Pharmacol.* **1999**, *44* (2), 164–169. https://doi.org/10.1007/s002800050962.

(132) Libonati, M.; Gotte, G. Oligomerization of Bovine Ribonuclease A: Structural and Functional Features of Its Multimers. *Biochem. J.* **2004**, *380* (Pt 2), 311–327. https://doi.org/10.1042/BJ20031922.

(133) Gotte, G.; Helmy, A. M.; Ercole, C.; Spadaccini, R.; Laurents, D. V.; Donadelli, M.; Picone, D. Double Domain Swapping in Bovine Seminal RNase: Formation of Distinct N- and C-Swapped Tetramers and Multimers with Increasing Biological Activities. *PLOS ONE* **2012**, *7* (10), e46804. https://doi.org/10.1371/journal.pone.0046804.

(134) Bennett, M. J.; Schlunegger, M. P.; Eisenberg, D. 3D Domain Swapping: A Mechanism for Oligomer Assembly. *Protein Sci. Publ. Protein Soc.* **1995**, *4* (12), 2455–2468. https://doi.org/10.1002/pro.5560041202.

(135) Crestfield, A. M.; Stein, W. H.; Moore, S. On the Aggregation of Bovine Pancreatic Ribonuclease. *Arch. Biochem. Biophys.* **1962**, *Suppl 1*, 217–222.

(136) Adinolfi, S.; Piccoli, R.; Sica, F.; Mazzarella, L. BS-RNase Tetramers: An Example of Domain-Swapped Oligomers. *FEBS Lett.* **1996**, *398* (2–3), 326–332. https://doi.org/10.1016/s0014-5793(96)01034-4.

(137) Sorrentino, S.; Libonati, M. Human Pancreatic-Type and Nonpancreatic-Type Ribonucleases: A Direct Side-by-Side Comparison of Their Catalytic Properties. *Arch. Biochem. Biophys.* **1994**, *312* (2), 340–348. https://doi.org/10.1006/abbi.1994.1318.

(138) Wang, X.-M.; Guo, Z.-Y. Recombinant Expression, Different Downstream Processing of the Disulfide-Rich Anti-Tumor Peptide Ranpirnase and Its Effect on the Growth of Human Glioma Cell Line SHG-44. *Biomed. Rep.* **2013**, *1* (5), 747–750. https://doi.org/10.3892/br.2013.138.

(139) Smolewski, P.; Witkowska, M.; Zwolinska, M.; Cebula-Obrzut, B.; Majchrzak, A.; Jeske, A.; Darzynkiewicz, Z.; Ardelt, W.; Ardelt, B.; Robak, T. Cytotoxic Activity of the Amphibian Ribonucleases Onconase and R-Amphinase on Tumor Cells from B Cell Lymphoproliferative Disorders. *Int. J. Oncol.* **2014**, *45* (1), 419–425. https://doi.org/10.3892/ijo.2014.2405.

(140) López-Alonso, J. P.; Gotte, G.; Laurents, D. V. Kinetic Analysis Provides Insight into the Mechanism of Ribonuclease A Oligomer Formation. *Arch. Biochem. Biophys.* **2009**, *489* (1), 41–47. https://doi.org/10.1016/j.abb.2009.07.013.

(141) Gotte, G.; Laurents, D. V.; Merlino, A.; Picone, D.; Spadaccini, R. Structural and Functional Relationships of Natural and Artificial Dimeric Bovine Ribonucleases: New Scaffolds for Potential Antitumor Drugs. *FEBS Lett.* **2013**, *587* (22), 3601–3608. https://doi.org/10.1016/j.febslet.2013.09.038.

(142) Matousek, J.; Gotte, G.; Pouckova, P.; Soucek, J.; Slavik, T.; Vottariello, F.; Libonati, M. Antitumor Activity and Other Biological Actions of Oligomers of Ribonuclease A*. *J. Biol. Chem.* **2003**, *278* (26), 23817–23822. https://doi.org/10.1074/jbc.M302711200.

(143) Fagagnini, A.; Pica, A.; Fasoli, S.; Montioli, R.; Donadelli, M.; Cordani, M.; Butturini, E.; Acquasaliente, L.; Picone, D.; Gotte, G. Onconase Dimerization through 3D Domain Swapping: Structural Investigations and Increase in the Apoptotic Effect in Cancer Cells. *Biochem. J.* **2017**, *474* (22), 3767–3781. https://doi.org/10.1042/BCJ20170541.

(144) Bennett, M. J.; Sawaya, M. R.; Eisenberg, D. Deposition Diseases and 3D Domain Swapping. *Structure* **2006**, *14* (5), 811–824. https://doi.org/10.1016/j.str.2006.03.011.

(145) Notomista, E.; Catanzano, F.; Graziano, G.; Di Gaetano, S.; Barone, G.; Di Donato, A. Contribution of Chain Termini to the Conformational Stability and Biological Activity of Onconase. *Biochemistry* **2001**, *40* (31), 9097–9103. https://doi.org/10.1021/bi010741s.

(146) Bretscher, L. E.; Abel, R. L.; Raines, R. T. A Ribonuclease A Variant with Low Catalytic Activity but High Cytotoxicity. *J. Biol. Chem.* **2000**, *275* (14), 9893–9896. https://doi.org/10.1074/jbc.275.14.9893.

(147) Gotte, G.; Campagnari, R.; Loreto, D.; Bettin, I.; Calzetti, F.; Menegazzi, M.; Merlino, A. The Crystal Structure of the Domain-Swapped Dimer of Onconase Highlights Some Catalytic and Antitumor Activity Features of the Enzyme. *Int. J. Biol. Macromol.* **2021**, *191*, 560–571. https://doi.org/10.1016/j.ijbiomac.2021.09.095.

(148) Wightman, B.; Ha, I.; Ruvkun, G. Posttranscriptional Regulation of the Heterochronic Gene Lin-14 by Lin-4 Mediates Temporal Pattern Formation in C. Elegans. *Cell* **1993**, *75* (5), 855–862. https://doi.org/10.1016/0092-8674(93)90530-4.

(149) Sun, D.; Lee, Y. S.; Malhotra, A.; Kim, H. K.; Matecic, M.; Evans, C.; Jensen, R. V.; Moskaluk, C. A.; Dutta, A. MiR-99 Family of MicroRNAs Suppresses the Expression of Prostate-Specific Antigen and Prostate Cancer Cell Proliferation. *Cancer Res.* **2011**, *71* (4), 1313–1324. https://doi.org/10.1158/0008-5472.CAN-10-1031.

(150) Trabucchi, M.; Briata, P.; Filipowicz, W.; Rosenfeld, M. G.; Ramos, A.; Gherzi, R. How to Control MiRNA Maturation? *RNA Biol.* **2009**, *6* (5), 536–540. https://doi.org/10.4161/rna.6.5.10080.

(151) Ha, M.; Kim, V. N. Regulation of MicroRNA Biogenesis. *Nat. Rev. Mol. Cell Biol.* **2014**, *15* (8), 509–524. https://doi.org/10.1038/nrm3838.

(152) Hoffend, N. C.; Magner, W. J.; Tomasi, T. B. The Modulation of Dicer Regulates Tumor Immunogenicity in Melanoma. *Oncotarget* **2016**, *7* (30), 47663–47673. https://doi.org/10.18632/oncotarget.10273.

(153) Nikfarjam, L.; Farzaneh, P. Prevention and Detection of Mycoplasma Contamination in Cell Culture. *Cell J. Yakhteh* **2012**, *13* (4), 203–212.

(154) Pfaffl, M. W.; Tichopad, A.; Prgomet, C.; Neuvians, T. P. Determination of Stable Housekeeping Genes, Differentially Regulated Target Genes and Sample Integrity: BestKeeper--Excel-Based Tool Using Pair-Wise Correlations. *Biotechnol. Lett.* **2004**, *26* (6), 509–515. https://doi.org/10.1023/b:bile.0000019559.84305.47.

(155) Cafaro, V.; Bracale, A.; Formiggini, F.; Notomista, E.; D'Alessio, G.; Di Donato, A. Protein Engineering of Ribonucleases. *Biochimie* **1998**, *80* (11), 905–909. https://doi.org/10.1016/S0300-9084(00)88887-2.

(156) Levy, D. E.; Darnell, J. E. Stats: Transcriptional Control and Biological Impact. *Nat. Rev. Mol. Cell Biol.* **2002**, *3* (9), 651–662. https://doi.org/10.1038/nrm909.

(157) Borowicz, S.; Van Scoyk, M.; Avasarala, S.; Karuppusamy Rathinam, M. K.; Tauler, J.; Bikkavilli, R. K.; Winn, R. A. The Soft Agar Colony Formation Assay. *J. Vis. Exp. JoVE* **2014**, No. 92, e51998. https://doi.org/10.3791/51998.

(158) Starostina, N. G.; Kipreos, E. T. Multiple Degradation Pathways Regulate Versatile CIP/KIP CDK Inhibitors. *Trends Cell Biol.* **2012**, *22* (1), 33–41. https://doi.org/10.1016/j.tcb.2011.10.004.

(159) Smith, L. K.; Rao, A. D.; McArthur, G. A. Targeting Metabolic Reprogramming as a Potential Therapeutic Strategy in Melanoma. *Pharmacol. Res.* **2016**, *107*, 42–47. https://doi.org/10.1016/j.phrs.2016.02.009.

(160) Fan, H.; Hu, Z.; Wang, S.; Wu, W.; Liu, X.; Geng, H. 5-Aminolevulinic-Acid-Mediated Sonodynamic Therapy Improves the Prognosis of Melanoma by Inhibiting Survivin Expression. *Cancer Biomark.* **2020**, *28* (3), 301–308. https://doi.org/10.3233/cbm-190681.

(161) Girouard, S. D.; Laga, A. C.; Mihm, M. C.; Scolyer, R. A.; Thompson, J. F.; Zhan, Q.; Widlund, H. R.; Lee, C.-W.; Murphy, G. F. SOX2 Contributes to Melanoma Cell Invasion. *Lab. Investig. J. Tech. Methods Pathol.* **2012**, *92* (3), 362–370. https://doi.org/10.1038/labinvest.2011.188.

(162) Laurenzana, A.; Biagioni, A.; Bianchini, F.; Peppicelli, S.; Chillà, A.; Margheri, F.; Luciani, C.; Pimpinelli, N.; Del Rosso, M.; Calorini, L.; Fibbi, G. Inhibition of UPAR-TGFβ Crosstalk Blocks MSC-

Dependent EMT in Melanoma Cells. *J. Mol. Med. Berl. Ger.* **2015**, *93* (7), 783–794. https://doi.org/10.1007/s00109-015-1266-2.

(163) Shi, H.; Liu, L.; Liu, L.-M.; Geng, J.; Chen, L. Inhibition of Tumor Growth by β-Elemene through Downregulation of the Expression of UPA, UPAR, MMP-2, and MMP-9 in a Murine Intraocular Melanoma Model. *Melanoma Res.* **2015**, *25* (1), 15–21. https://doi.org/10.1097/CMR.0000000000000124.

(164) Xie, S.; Price, J. E.; Luca, M.; Jean, D.; Ronai, Z.; Bar-Eli, M. Dominant-Negative CREB Inhibits Tumor Growth and Metastasis of Human Melanoma Cells. *Oncogene* **1997**, *15* (17), 2069–2075. https://doi.org/10.1038/sj.onc.1201358.

(165) Dikshit, A.; Jin, Y. J.; Degan, S.; Hwang, J.; Foster, M. W.; Li, C.-Y.; Zhang, J. Y. UBE2N Promotes Melanoma Growth via MEK/FRA1/SOX10 Signaling. *Cancer Res.* **2018**, *78* (22), 6462–6472. https://doi.org/10.1158/0008-5472.CAN-18-1040.

(166) Uhara, H. Recent Advances in Therapeutic Strategies for Unresectable or Metastatic Melanoma and Real-World Data in Japan. *Int. J. Clin. Oncol.* **2019**, *24* (12), 1508–1514. https://doi.org/10.1007/s10147-018-1246-y.

(167) Wilhelm, S.; Carter, C.; Lynch, M.; Lowinger, T.; Dumas, J.; Smith, R. A.; Schwartz, B.; Simantov, R.; Kelley, S. Discovery and Development of Sorafenib: A Multikinase Inhibitor for Treating Cancer. *Nat. Rev. Drug Discov.* **2006**, *5* (10), 835–844. https://doi.org/10.1038/nrd2130.

(168) Aplin, A. E.; Kaplan, F. M.; Shao, Y. Mechanisms of Resistance to RAF Inhibitors in Melanoma. *J. Invest. Dermatol.* **2011**, *131* (9), 1817–1820. https://doi.org/10.1038/jid.2011.147.

(169) von Euw, E.; Chodon, T.; Attar, N.; Jalil, J.; Koya, R. C.; Comin-Anduix, B.; Ribas, A. CTLA4 Blockade Increases Th17 Cells in Patients with Metastatic Melanoma. *J. Transl. Med.* **2009**, *7*, 35. https://doi.org/10.1186/1479-5876-7-35.

(170) *Anti-CTLA4 Antibody Clinical Trials in Melanoma - PMC*. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2699286/ (accessed 2022-12-01).

(171) Raedler, L. A. Opdivo (Nivolumab): Second PD-1 Inhibitor Receives FDA Approval for Unresectable or Metastatic Melanoma. *Am. Health Drug Benefits* **2015**, *8* (Spec Feature), 180–183.

(172) Specenier, P. Nivolumab in Melanoma. *Expert Rev. Anticancer Ther.* **2016**, *16* (12), 1247–1261. https://doi.org/10.1080/14737140.2016.1249856.

(173) Melero, I.; Grimaldi, A. M.; Perez-Gracia, J. L.; Ascierto, P. A. Clinical Development of Immunostimulatory Monoclonal Antibodies and Opportunities for Combination. *Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res.* **2013**, *19* (5), 997–1008. https://doi.org/10.1158/1078-0432.CCR-12-2214.

(174) Leland, P. A.; Raines, R. T. Cancer Chemotherapy — Ribonucleases to the Rescue. *Chem. Biol.* **2001**, *8* (5), 405–413.

(175) Darzynkiewicz, Z.; Carter, S. P.; Mikulski, S. M.; Ardelt, W. J.; Shogen, K. Cytostatic and Cytotoxic Effects of Pannon (P-30 Protein), a Novel Anticancer Agent. *Cell Tissue Kinet.* **1988**, *21* (3), 169–182. https://doi.org/10.1111/j.1365-2184.1988.tb00855.x.

(176) Westekemper, H.; Freistuehler, M.; Bornfeld, N.; Steuhl, K.-P.; Scheulen, M.; Hilger, R. A. Chemosensitivity of Conjunctival Melanoma Cell Lines to Target-Specific Chemotherapeutic Agents.

Graefes Arch. Clin. Exp. Ophthalmol. Albrecht Von Graefes Arch. Klin. Exp. Ophthalmol. **2013**, *251* (1), 279–284. https://doi.org/10.1007/s00417-012-2083-8.

(177) Mikulski, S. M.; Ardelt, W.; Shogen, K.; Bernstein, E. H.; Menduke, H. Striking Increase of Survival of Mice Bearing M109 Madison Carcinoma Treated with a Novel Protein from Amphibian Embryos. *J. Natl. Cancer Inst.* **1990**, *82* (2), 151–153. https://doi.org/10.1093/jnci/82.2.151-a.

(178) Ehexige, E.; Bao, M.; Bazarjav, P.; Yu, X.; Xiao, H.; Han, S.; Baigude, H. Silencing of STAT3 via Peptidomimetic LNP-Mediated Systemic Delivery of RNAi Downregulates PD-L1 and Inhibits Melanoma Growth. *Biomolecules* **2020**, *10* (2), 285. https://doi.org/10.3390/biom10020285.

(179) Boix, E.; Wu, Y.; Vasandani, V. M.; Saxena, S. K.; Ardelt, W.; Ladner, J.; Youle, R. J. Role of the N Terminus in RNase A Homologues: Differences in Catalytic Activity, Ribonuclease Inhibitor Interaction and Cytotoxicity. *J. Mol. Biol.* **1996**, *257* (5), 992–1007. https://doi.org/10.1006/jmbi.1996.0218.

(180) Iordanov, M. S.; Ryabinina, O. P.; Wong, J.; Dinh, T.-H.; Newton, D. L.; Rybak, S. M.; Magun, B. E. Molecular Determinants of Apoptosis Induced by the Cytotoxic Ribonuclease Onconase: Evidence for Cytotoxic Mechanisms Different from Inhibition of Protein Synthesis1. *Cancer Res.* **2000**, *60* (7), 1983–1994.

(181) Goparaju, C. M.; Blasberg, J. D.; Volinia, S.; Palatini, J.; Ivanov, S.; Donington, J. S.; Croce, C.; Carbone, M.; Yang, H.; Pass, H. I. Onconase Mediated NFKβ Downregulation in Malignant Pleural Mesothelioma. *Oncogene* **2011**, *30* (24), 2767–2777. https://doi.org/10.1038/onc.2010.643.

(182) Qiao, M.; Zu, L.-D.; He, X.-H.; Shen, R.-L.; Wang, Q.-C.; Liu, M.-F. Onconase Downregulates MicroRNA Expression through Targeting MicroRNA Precursors. *Cell Res.* **2012**, *22* (7), 1199–1202. https://doi.org/10.1038/cr.2012.67.

(183) Shao, N.; Wang, D.; Wang, Y.; Li, Y.; Zhang, Z.; Jiang, Q.; Luo, W.; Cao, C. MicroRNA-29a-3p Downregulation Causes Gab1 Upregulation to Promote Glioma Cell Proliferation. *Cell. Physiol. Biochem.* **2018**, *48* (2), 450–460. https://doi.org/10.1159/000491776.

(184) Tarasov, V.; Jung, P.; Verdoodt, B.; Lodygin, D.; Epanchintsev, A.; Menssen, A.; Meister, G.; Hermeking, H. Differential Regulation of MicroRNAs by P53 Revealed by Massively Parallel Sequencing: MiR-34a Is a P53 Target That Induces Apoptosis and G1-Arrest. *Cell Cycle* **2007**, *6* (13), 1586–1593. https://doi.org/10.4161/cc.6.13.4436.

(185) Yan, D.; Zhou, X.; Chen, X.; Hu, D.-N.; Dong, X. D.; Wang, J.; Lu, F.; Tu, L.; Qu, J. MicroRNA-34a Inhibits Uveal Melanoma Cell Proliferation and Migration through Downregulation of c-Met. *Invest. Ophthalmol. Vis. Sci.* **2009**, *50* (4), 1559–1565. https://doi.org/10.1167/iovs.08-2681.

(186) Xu, Y.; Guo, B.; Liu, X.; Tao, K. MiR-34a Inhibits Melanoma Growth by Targeting ZEB1. *Aging* **2021**, *13* (11), 15538–15547. https://doi.org/10.18632/aging.203114.

(187) Kuphal, S.; Winklmeier, A.; Warnecke, C.; Bosserhoff, A.-K. Constitutive HIF-1 Activity in Malignant Melanoma. *Eur. J. Cancer* **2010**, *46* (6), 1159–1169. https://doi.org/10.1016/j.ejca.2010.01.031.

(188) Malekan, M.; Ebrahimzadeh, M. A.; Sheida, F. The Role of Hypoxia-Inducible Factor-1alpha and Its Signaling in Melanoma. *Biomed. Pharmacother.* **2021**, *141*, 111873. https://doi.org/10.1016/j.biopha.2021.111873.

(189) Kim, J.; Tchernyshyov, I.; Semenza, G. L.; Dang, C. V. HIF-1-Mediated Expression of Pyruvate Dehydrogenase Kinase: A Metabolic Switch Required for Cellular Adaptation to Hypoxia. *Cell Metab.* **2006**, *3* (3), 177–185. https://doi.org/10.1016/j.cmet.2006.02.002.

(190) Hatse, S.; Brouwers, B.; Dalmasso, B.; Laenen, A.; Kenis, C.; Schöffski, P.; Wildiers, H. Circulating MicroRNAs as Easy-to-Measure Aging Biomarkers in Older Breast Cancer Patients: Correlation with Chronological Age but Not with Fitness/Frailty Status. *PloS One* **2014**, *9* (10), e110644. https://doi.org/10.1371/journal.pone.0110644.

(191) Ma, G.; Li, G.; Fan, W.; Xu, Y.; Song, S.; Guo, K.; Liu, Z. Circ-0005105 Activates COL11A1 by Targeting MiR-20a-3p to Promote Pancreatic Ductal Adenocarcinoma Progression. *Cell Death Dis.* **2021**, *12* (7), 656. https://doi.org/10.1038/s41419-021-03938-8.

(192) Mao, Z.-J.; Weng, S.-Y.; Lin, M.; Chai, K.-F. Yunpi Heluo Decoction Attenuates Insulin Resistance by Regulating Liver MiR-29a-3p in Zucker Diabetic Fatty Rats. *J. Ethnopharmacol.* **2019**, *243*, 111966. https://doi.org/10.1016/j.jep.2019.111966.

(193) Orgaz, J. L.; Sanz-Moreno, V. Emerging Molecular Targets in Melanoma Invasion and Metastasis. *Pigment Cell Melanoma Res.* **2013**, *26* (1), 39–57. https://doi.org/10.1111/pcmr.12041.

(194) Xiong, Y.; Liu, L.; Qiu, Y.; Liu, L. MicroRNA-29a Inhibits Growth, Migration and Invasion of Melanoma A375 Cells in Vitro by Directly Targeting BMI1. *Cell. Physiol. Biochem. Int. J. Exp. Cell. Physiol. Biochem. Pharmacol.* **2018**, *50* (1), 385–397. https://doi.org/10.1159/000494015.

(195) Tulchinsky, E.; Pringle, J. H.; Caramel, J.; Ansieau, S. Plasticity of Melanoma and EMT-TF Reprogramming. *Oncotarget* **2013**, *5* (1), 1–2. https://doi.org/10.18632/oncotarget.1662.

(196) Rattanasinchai, C.; Llewellyn, B. J.; Conrad, S. E.; Gallo, K. A. MLK3 Regulates FRA-1 and MMPs to Drive Invasion and Transendothelial Migration in Triple-Negative Breast Cancer Cells. *Oncogenesis* **2017**, *6* (6), e345. https://doi.org/10.1038/oncsis.2017.44.

(197) Talotta, F.; Casalino, L.; Verde, P. The Nuclear Oncoprotein Fra-1: A Transcription Factor Knocking on Therapeutic Applications' Door. *Oncogene* **2020**, *39* (23), 4491–4506. https://doi.org/10.1038/s41388-020-1306-4.

(198) Sun, Z.; Hu, W.; Xu, J.; Kaufmann, A. M.; Albers, A. E. MicroRNA-34a Regulates Epithelial-Mesenchymal Transition and Cancer Stem Cell Phenotype of Head and Neck Squamous Cell Carcinoma in Vitro. *Int. J. Oncol.* **2015**, *47* (4), 1339–1350. https://doi.org/10.3892/ijo.2015.3142.

(199) Zhang, Y.; Pan, Y.; Xie, C.; Zhang, Y. MiR-34a Exerts as a Key Regulator in the Dedifferentiation of Osteosarcoma via PAI-1–Sox2 Axis. *Cell Death Dis.* **2018**, *9* (7), 1–13. https://doi.org/10.1038/s41419-018-0778-4.

(200) Hüser, L.; Sachindra, S.; Granados, K.; Federico, A.; Larribère, L.; Novak, D.; Umansky, V.; Altevogt, P.; Utikal, J. SOX2-Mediated Upregulation of CD24 Promotes Adaptive Resistance toward Targeted Therapy in Melanoma. *Int. J. Cancer* **2018**, *143* (12), 3131–3142. https://doi.org/10.1002/ijc.31609.

(201) Adams, B. D.; Wali, V. B.; Cheng, C. J.; Inukai, S.; Booth, C. J.; Agarwal, S.; Rimm, D. L.; Győrffy, B.; Santarpia, L.; Pusztai, L.; Saltzman, W. M.; Slack, F. J. MiR-34a Silences c-SRC to Attenuate Tumor Growth in Triple-Negative Breast Cancer. *Cancer Res.* **2016**, *76* (4), 927–939. https://doi.org/10.1158/0008-5472.CAN-15-2321.

(202) Hu, Z.; Fan, H.; Lv, G.; Zhou, Q.; Yang, B.; Zheng, J.; Cao, W. 5-Aminolevulinic Acid-Mediated Sonodynamic Therapy Induces Anti-Tumor Effects in Malignant Melanoma via P53-MiR-34a-Sirt1 Axis. *J. Dermatol. Sci.* **2015**, *79* (2), 155–162. https://doi.org/10.1016/j.jdermsci.2015.04.010.

(203) Nan, P.; Niu, Y.; Wang, X.; Li, Q. MiR-29a Function as Tumor Suppressor in Cervical Cancer by Targeting SIRT1 and Predict Patient Prognosis. *OncoTargets Ther.* **2019**, *12*, 6917–6925. https://doi.org/10.2147/OTT.S218043.

(204) Chou, K.-Y.; Chang, A.-C.; Tsai, T.-F.; Lin, Y.-C.; Chen, H.-E.; Ho, C.-Y.; Chen, P.-C.; Hwang, T. I.-S. MicroRNA-34a-5p Serves as a Tumor Suppressor by Regulating the Cell Motility of Bladder Cancer Cells through Matrix Metalloproteinase-2 Silencing. *Oncol. Rep.* **2021**, *45* (3), 911–920. https://doi.org/10.3892/or.2020.7910.

(205) Antony, J.; Thiery, J. P.; Huang, R. Y.-J. Epithelial-to-Mesenchymal Transition: Lessons from Development, Insights into Cancer and the Potential of EMT-Subtype Based Therapeutic Intervention. *Phys. Biol.* **2019**, *16* (4), 041004. https://doi.org/10.1088/1478-3975/ab157a.

(206) Zuo, Q.; Liu, J.; Huang, L.; Qin, Y.; Hawley, T.; Seo, C.; Merlino, G.; Yu, Y. AXL/AKT Axis Mediated-Resistance to BRAF Inhibitor Depends on PTEN Status in Melanoma. *Oncogene* **2018**, *37* (24), 3275–3289. https://doi.org/10.1038/s41388-018-0205-4.

(207) Miller, M. A.; Sullivan, R. J.; Lauffenburger, D. A. Molecular Pathways: Receptor Ectodomain Shedding in Treatment, Resistance, and Monitoring of Cancer. *Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res.* **2017**, *23* (3), 623–629. https://doi.org/10.1158/1078-0432.CCR-16-0869.

(208) Wu, J.; Wu, G.; Lv, L.; Ren, Y.-F.; Zhang, X.-J.; Xue, Y.-F.; Li, G.; Lu, X.; Sun, Z.; Tang, K.-F. MicroRNA-34a Inhibits Migration and Invasion of Colon Cancer Cells via Targeting to Fra-1. *Carcinogenesis* **2012**, *33* (3), 519–528. https://doi.org/10.1093/carcin/bgr304.

(209) Yang, S.; Li, Y.; Gao, J.; Zhang, T.; Li, S.; Luo, A.; Chen, H.; Ding, F.; Wang, X.; Liu, Z. MicroRNA-34 Suppresses Breast Cancer Invasion and Metastasis by Directly Targeting Fra-1. *Oncogene* **2013**, *32* (36), 4294–4303. https://doi.org/10.1038/onc.2012.432.

(210) Menegazzi, M.; Gotte, G. Role of the Ribonuclease ONCONASE in MiRNA Biogenesis and TRNA Processing: Focus on Cancer and Viral Infections. *Int. J. Mol. Sci.* **2022**, *23* (12), 6556. https://doi.org/10.3390/ijms23126556.