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BAP1 promotes Stem Cell-like features and three-dimensional spheres formation in Malignant Pleural Mesothelioma

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To you who fall, but don't stay there

BAP1 promotes Stem Cell-like features and three-dimensional spheres formation in Malignant Pleural Mesothelioma

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Introduction

Malignant pleural mesothelioma (MPM) is a rare and highly aggressive tumor of pleural mesothelium tissue associated primarily with extensive commercial use of asbestos in recent decades. From 1940 to 1979, about 27 million US workers were exposed to asbestos with ~3000 deaths nationally per year [1]. Asbestos-correlated MPM affects older people with a male-to-female mortality ratio of ~4:1, as men were historical usual employed in activities involving exposure to asbestos. Despite efforts to discontinue asbestos in commercial businesses, mesothelioma rates have remained stable in the United States since the 90s and in Europe are expected to increase by about 7,5% per year over the next 15 years [1]. Considering the very long latency period, even up to 40 years from exposure to asbestos to the onset of the disease [2], MPM is a more than current problem of the present days. Moreover, the risk shifted from occupational to environmental exposures.

In the past century, many studies reported cases of some individuals that develop malignant mesothelioma (MM) from exposure to even small amounts of asbestos, while others exposed to high doses never developed mesothelioma in their lifetime. Recently, a large mesothelioma study conducted on patients residing in South America reported which were younger, the male-to-female ratio equaled and the percentage of MM patients exposed to asbestos was low and median survival was significantly longer than in Europe and USA [3]. Surprisingly at the time, these data may now be consistent with a high proportion of MM with a genetic predisposition. Later, genetic risk factors related to MPM development have been reported and 3p21.1 site was identified as a region of recurrent chromosomal loss in mesotheliomas [4], [5] . Among identified biomarkers in this smaller region of particular interest is the nuclear deubiquitinase BRCA1 Associated Protein 1 (BAP1) [6]. Germline and somatic BAP1 mutations and loss of BAP1 function are associated with a wide spectrum of cancers and predispose to MM.

BAP1 is historically known as a tumor suppressor whose activity is attributed to its nuclear localization. In the nucleus, it helps to keep genome intact, transcription regulation and DNA repair [7]. Its important role in numerous cellular processes, including metabolism, makes *BAP1* inactivation/mutation or loss a crucial factor in oncogenic transformation [8]. Furthermore, the possible role of *BAP1* in the cytoplasm was unknown until Carbone's team discovered that *BAP1* was located in cytoplasmic areas proximate to the endoplasmic reticulum (ER). Here, BAP1 modulates calcium (Ca^{2+}) release from the ER into the cytosol

and mitochondria, promoting apoptosis, by Inositol 1,4,5-trisphosphate receptor type 3 (IP3R3) [9].

This new role of *BAP1* together with its ability to regulate markers of stemness by post-translational modifications and in particular its deubiquitinating enzyme (DUB) activity has opened new horizons of investigation into the role of *BAP1* in tumorigenesis.

Surprisingly, it is well known that patients with MPM show greater survival when *BAP1* gene is mutated or lost than patients with the wt form of the gene [10]. The reason of an inverse correlation between *BAP1* dysregulation and survival of patients with MM is not yet fully understood. We investigated on the *BAP1* ability to regulate proliferation, migration, spheres formation capacity in vitro, Ca^{2+} flux and stem features in MPM cells to dissect the molecular mechanisms underlying *BAP1*-survival relationship in MPM patients.

The management of MPM remains complex, but *BAP1*/IP3R3 – Ca^{2+} homeostasis and *BAP1*/stem features could be the pathway key to identify a better therapeutic choice of patients with MPM to promote extended survival and a better course of the disease.

1. Chapter 1 - Malignant Pleural Mesothelioma

1.1 Epidemiology

Malignant mesothelioma (MM) is an aggressive tumor of mesothelial origin strongly associated with asbestos exposure. The incidence of MM is linked to uncontrolled asbestos extraction and manipulation, commercial importation and its use in numerous industry sectors. Based on exposures, etiological factors and more, in different countries of the world the incidence and mortality rates vary considerably. Asbestos exposure has an impact in 80% of MM cases and it is the major etiological factor [11]. The first case of MM was reported in the 60s in the context of an epidemic event among American miners that made it possible to establish the association exposure to asbestos-development of the MM disease. Then the incidence increased from the second half of the 20th century and at the time it was estimated that the peak incidence was get around 2020 in Western Europe and North America, while in East Europe, Asia, South America and Africa, the peak is likely to be get in the coming decades [12]. However, approximately 3,000 incident cases of MM are reported in the United States each year [1]. The male-to-female mortality ratio is in favor of the female population, as men were generally exposed to asbestos in workplaces such as mines and industries [13]. Moreover, MM in women has been related to better survival: large data set confirms that survival is better in women than men, independent of confounding factors such as age and stage of disease [14]. This divergence is not yet well understood. Factors such as asbestos exposures and the impact of hormones on host response are still being studied to understand this survival advantage and improve prognosis for patients of both sexes. However, it was also reported that age was not a major determinant of survival [14].

In the USA and in many countries, the MM disease is a current problem. The reason is mainly linked to the large population and the fact that people are living longer. In many countries the population is getting older and generally MM affects mostly older people [13]. In addition, the very long latency period makes MM a global problem. The overall number of new cases and of deaths per year caused by MM continues steadily to increase. Brazil is considered one of the most important producers and exporters of asbestos and in the country the MM mortality rate is quadrupled from 1980 to recent time [15].

The incidence rate increases over the age of 60 , with a mean age of death from MM in the USA of about 73 years [13], [16]. In Latin America, at diagnosis, the median patient age drops to 61 years with 63.2% of patients who are men and only about half had previous exposure to asbestos: Latin American patients were younger than patients from European countries and USA [3].

Mesothelioma incidence and mortality data from some countries are not included in World Health Organization. Eastern countries of the world still used asbestos and dramatic increases in MM incidence and mortality rates are expected to occur in the coming years [17]. The exposed population could be greater than currently considered and the future mortality prospects even higher than expected.

1.2 Histological subtypes

MMs are tumors that affect the thin tissue that covers most of the internal organs, called the mesothelium. The pleura is the most common site of MM origin (~80%), followed by the peritoneum (~20%) [18]. Other areas of the body may be affected to a lesser extent, but the pleura remains the site of onset of the most common mesothelioma, known as malignant pleural mesothelioma (MPM).

The 2021 WHO classifications of MPM, as well as the other MM subtypes, traditionally recognized the three histological subtypes: epithelioid, sarcomatoid and biphasic. Histological classification is important for the prognosis and can also have an impact on the choice of treatment to adopt for MPM patients [19]. Epithelioid histology is the least aggressive and confers the most favorable prognosis. Sarcomatoid MPMs have the worst prognosis and have been associated with a low survival of a few months in patients undergoing surgical treatment [19]. Biphasic MPMs, with mixed epithelioid and sarcomatoid histologies, behave more or less aggressively depending on the percentage of the sarcomatoid component [20]. At least 10% of each component, epithelioid or sarcomatoid is required for definitive diagnosis in biopsies [21]. A higher percentage of epithelial differentiation in biphasic tumors correlates with longer survival and this component is an independent predictor of survival in biphasic MPM [22]. According to the SEER database (The Surveillance, Epidemiology, and End Results - https://seer.cancer.gov/), in the histological types of epithelial, biphasic, and sarcomatoid pleural mesothelioma, the median survival is 14, 10, and 4 months, respectively [19].

1.3 Exposure to environmental carcinogens

1.3.1 Asbestos

Asbestos family includes six different silicate minerals with very small fibers (amphiboles: crocidolite, actinolite, tremolite, anthophyllite, and amosite; serpentine: chrysotile). The high mortality from mesothelioma was recorded around and after the 1960s after the uncontrolled use of asbestos during the world wars. The widespread use of asbestos continued in high resource countries (USA, Europe, and Australia) until the late 1970s and early 1980s, when strict regulations were implemented to limit its use. Italy banned the extraction, use and marketing of asbestos in 1992 (Law 257/92) yet a high number of cases of MPM are expected in the next years. In the last century, the extraction of asbestos was the basis of industrial activity in Northern Italy. The largest European chrysotile quarry was located in Balangero in Piedmont, which remained in operation for 68 years and was definitively closed in 1985 [23]. From the early 1900s, the multinational Eternit began producing asbestos cement and other industrial plants operated for decades in Italian cities. In these areas there are still excesses in the incidence of mesothelioma [24].
Simian virus 40 (SV40) is a DNA tumor virus capable of infecting and transforming human MCs in vitro [25], which has been shown to act as a co-carcinogen with asbestos in transforming cells [26].

1.3.2 Other carcinogenic fibers

Exposure to erionite, an asbestos-like mineral which shares physical characteristics with asbestos, also causes mesothelioma and determined an outbreak in the Cappadocian villages of Turkey [27]. Moreover, laboratory mice exposed to carbon nanotubes exhibited exposure reactions that mimicked asbestos exposure as the initial chronic inflammation. Two fibers that are asbestos-like in nature can pose significant health risks to humans, as reported by exposure to nanoparticles and carbon nanotubes in mice. [28], [29]. Among the 'asbestos-like' fibers there is also fluorine-edenite, a mineral belonging to the subgroup of Ca^{2+} amphiboles [30]. Many studies validate the hypothesis that talc, baby products and cosmetics and other products contaminated with asbestos, cause mesothelioma, as other cancers [31], [32], [33], [34].
Although there are other etiological factors, in humans, asbestos is the main cause of MM and the association with exposure to other mineral fibers, is also well established [35].

1.4 Gene influence in MPM etiopathogenesis

The genetic damage underlying mesothelioma can be caused by exposure to carcinogens. However, MM can be hereditary in nature, can also develop spontaneously or can arise from the combination of several factors.

Only a fraction of asbestos-exposed individuals develop MM, indicating that other factors, possibly of genetic origin, may contribute to the development of this cancer. Additionally, asbestos-related MPM has historically correlated with the male population due to occupational exposure, however, men and women with equivalent exposure to asbestos have a similar incidence of develop MPM [36].

More studies suggest interaction between genetic influence and asbestos exposure in the development of mesothelioma. Patients with pathogenic mutations in genes involved in DNA damage repair developed MPM even though they were exposed to less cumulative amount of asbestos than patients without germline variants [37].

Strong genetic correlation is associated with the risk of develop MM, which creates molecular dysregulations favorable to the development of the oncological process. Genetic risk factors for the development of MPM are mainly related to recurrent mutations in genes with tumor suppressor roles and consequent loss of this function. Studies have shown that in some families the tendency to develop MM is inherited in a Mendelian fashion with an autosomal dominant pattern of inheritance [38].

Several genetic risk factors for the development of MPM have been identified at a specific site on chromosome 3, 3p21.1 [4], [5]. The most studied high-risk factor is a pathogenic germline mutation in the BAP1 gene, which is located precisely in the high-risk chromosomal region for MPM [12]. Furthermore, even somatically, BAP1 is mutated in more than 60% of biological samples from MM cases. In general, mutations affecting BAP1 lead to a loss of the protein of the same name. This is mostly found in epithelioid-type MPMs. In contrast to this, loss of BAP1 is less frequent in sarcomatoid mesotheliomas and, therefore, less useful in distinguishing from benign processes [39], [40].

Genes that have a predisposition to MPM are programmed deathligand 1 (PDL-1), cyclin-dependent kinase inhibitor 2A (CDKN2A), neurofibromatosis type II (NF2), large tumor suppressor kinase 2 (LATS2), TP53, BAP1 and other DNA repair genes [37], [41]–[43]. Approximately 50% of MM tumors carry mutations affecting the NF2 tumor suppressor gene [41], [44]. NF2 is involved in the Hippo pathway, which regulates different cellular process leading to tumor development and progression [45]. Sneddon et al. identified at a

high frequency alterations of BAP1, CDKN2A and NF2 in MM cells, as well as TNF receptor associated factor 7 (TRAF7) and LATS2 alterations (66% and 59% respectively), including novel regions (19p13.3, 8p23.1 and 1p36.32) of interest as MPM markers [42].

1.5 Pathogenic molecular mechanism in MPM

1.5.1 Asbestos - induced Inflammation

Mesothelial cells (MCs) form a monolayer of specialized cells (mesothelium) that line the serous cavities of the body (the pleural, pericardial and peritoneal cavities) and the organs contained within these cavities. MCs are highly susceptible to asbestos cytotoxicity. Many pathogenic events follow the entry of fibers into the human body and can contribute to carcinogenesis also favored by the long latency period between exposure to asbestos and tumor development. Inhalation of asbestos fibers leads as a first response to local inflammation of the mesothelial tissue. This is followed by the immediate production of cytokines and reactive oxygen species (ROS). The lungs and its pleura are the sites of greatest risk for accumulation of harmful fibers. They enter the lungs, persist for a long time in the pleura, and cause repeated cycles of tissue damage and repair, resulting in local inflammation. Over time, inflammation can evolve from acute to chronic, leading to carcinogenesis [46], [47], as the body attempts to fight it. In fact, in the pleural space macrophages try to engulf these fibers without any effect and in doing so release ROS and reactive nitrogen species (RNS). The same defense of the organism can be the cause of the promotion of genotoxic damage with the recruitment of other inflammatory cells and immune. Repeated DNA damage by ROS and RNS can lead to the accumulation of oncogenic mutations in MCs (Figure 1 [48]). Therefore, asbestos induces carcinogenicity through direct and indirect mechanisms by oxidative stress and chronic inflammation, respectively. ROS-induced oxidative stress is considered one of the triggers of asbestos-induced pathogenesis. ROS as DNA-damaging agents increase mutation rates and promote malignant transformation: involve DNA oxidation events, post-translational modifications of histone proteins and DNA methylation. Moreover, inflammatory mediators can promote cell survival by inhibiting apoptotic signals, stimulate MCs proliferation (even in the presence of DNA damage), and promote neo-angiogenesis [49] contributing to tumor development.

The inflammatory process and genetic deficits, with consequent molecular alterations, make MPM a very heterogeneous disease at the molecular level [50].

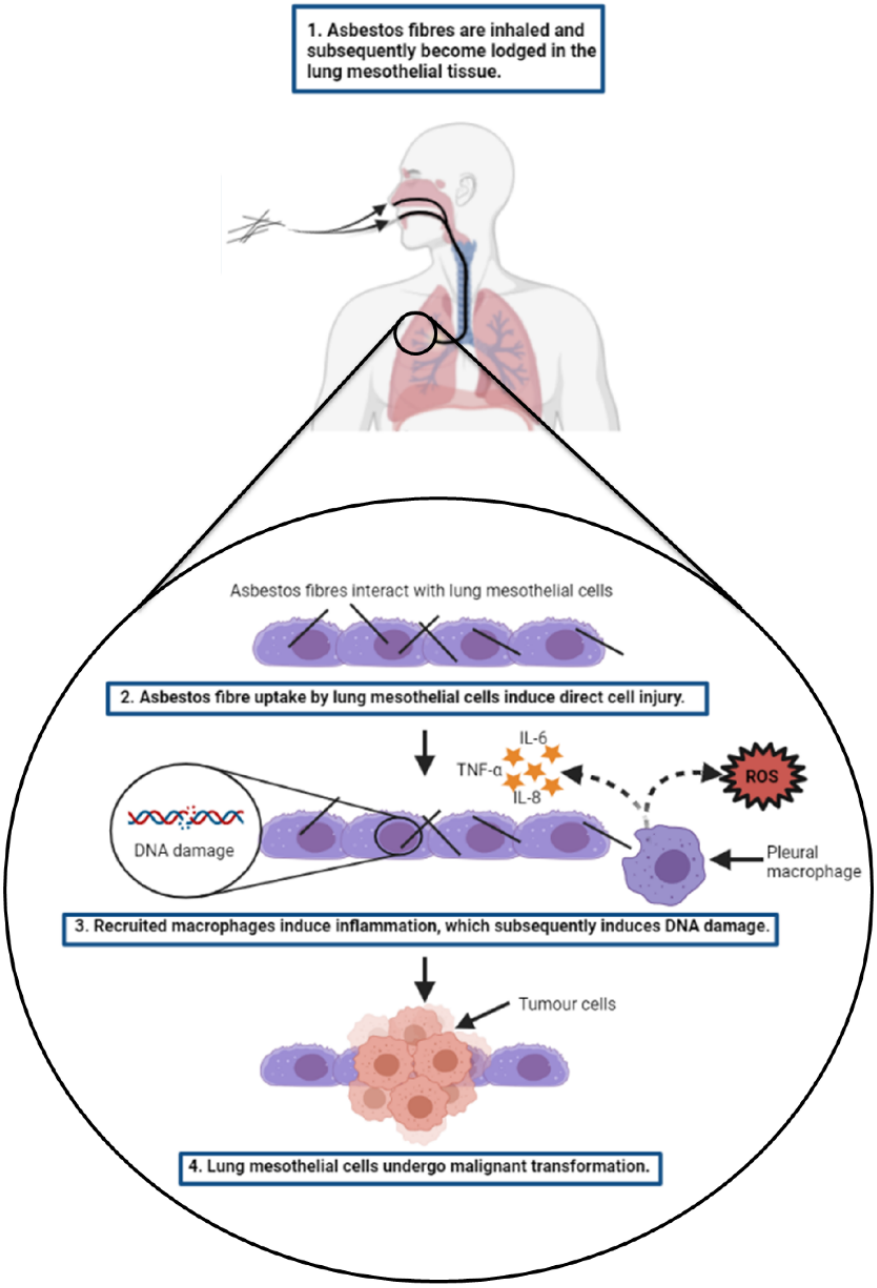

Figure 1 | **Asbestos-induced mesothelial cell injury leading to the development of MPM.** When inhaled, the asbestos fibers, accumulated in the pleura, induce mechanical damage in mesothelial cells with the release of ROS and RNS (asbestos fibers contain iron). This inflammatory process induces cellular DNA damage and impaired cell signaling that promotes malignant transformation of mesothelial cells. Source: B. Johnson et al. 2021 (see bibliography [48]).

1.5.2 Epigenetic modification in asbestos correlated MPM

The etiology of MM is definitely associated in part to genomic mutations but also epigenetic modifications can lead to dysregulation of gene expression.

Parallel to ROS-induced DNA damage, asbestos exposure leads to epigenetic/epigenomic modifications that make a major contribution to malignant onset and evolution. Asbestos exposure induces early changes in the epigenetic modulators miRNA machinery, for this altered miRNA levels can be proposed as biomarkers of early biological effects [51]. MiRNAs are epigenetically regulated in MM and in particular miR-34 family was found downregulated in MM with consequent cell proliferation and invasion of human MCs [52]. MiR-126 is known to inhibit angiogenesis and as a tumor suppressor, by inhibiting the PI3K/AKT pathway, plays a key role in tumor pathogenesis [53]. MiR-126 is epigenetically modulated in MM [54] and methylation-associated silencing of microRNA-126 in MPM s a prognostic factor of poor survival [55].

The formation of ROS, triggered by the permanence of asbestos fibers, can promote hypomethylation events in cells ultimately leading to the expression of methylcytosine dioxygenase enzymes, thus avoiding the interference of DNA methyltransferases (DNMTs). DNA methylation results in the downregulation of gene expression and the addition of methyl groups from cytosine into CpG dinucleotide regions that are concentrated in "CpG islands" at transcription start sites. Inversely, DNA hypomethylation is associated with genomic instability that contribute to malignant transformation with other and multiple genetic alterations [51].

Poly(ADP-ribose) polymerase-1 (PARP1) is involved in asbestos-induced DNA damage with its remodeling chromatin roles and regulating DNA methylation by DNMTs [56]. The exposure to asbestos inhibits PARP1 activity, which results in higher DNA instability, thus causing malignant transformation [51].

1.5.3 Others pathogenic process

Tumor necrosis factor-alpha (TNFA) and nuclear factor-kB (NF-kB) signaling were also involved in MC response to asbestos. Activation of the NF-kB pathway by TNFA allows CDs carrying asbestos-induced DNA damage to evolve into MM [57]. When asbestos and other asbestos-like fibers reach the pleura, or other mesothelial districts, they remain in place for months or even years, triggering an inflammation, which over time becomes chronic, driven by the secretion of the protein of the high mobility group B1 (HMGB1).

Furthermore, inflammasome activation, cytosolic multiprotein oligomers of the innate immune system responsible for the activation of inflammatory responses, induces the activation of NF-κB and the phosphatidylinositol 3-kinase (PI3K) pathways in MCs [58], [59]. This pathway favors the proliferation of MCs which accumulate mutations due to uncontrolled replication or due to the constant presence of asbestos deposits and the related mutagenic effect [60].

Among the factors that lead to the development of the pathogenic process in MPM is the alteration of the Hippo pathway which sees upstream the mutation of the tumor suppressor NF2. NF2 encodes Merlin protein, which is an upstream regulator of the Hippo signal cascade [61]. The Hippo signaling pathway is a normal regulator of organ size, tissue regeneration and stem cell self-renewal. Furthermore, it is involved in tumor formation and progression [45]. This pathway involves, among others, the tumor suppressor LATS, which phosphorylate and inactivate the transcriptional coactivator, Yes-associated protein (YAP), by translocating it from the nucleus to the cytoplasm [62]. MM frequently shows inactivation of Hippo pathway with increased YAP activity in over 70% of MM cell lines [63] which leads to oncogenic transformation.

1.6 Cancer Stem Cells in MPM

Cancer stem cells (CSCs) represent a rare population of cells that have the capacity to initiate and maintain neoplastic tissues creating a heterogeneous cancer cell population. CSCs have capacity to adaptation to adverse environmental conditions, such as hypoxia, through their stem cell-like characteristics as quiescence, ability to repair DNA and metabolic rewiring [64]. CSCs could underlie the origin of malignant tumor cells proliferation and have been isolated from a variety of solid tumors. The presence of CSCs in tumors confers greater resistance to conventional chemotherapy terapy, therefore, compromising long-term survival after therapy [64].

Specific markers characterize CSCs and they include transcription factors, asoctamer-binding transcription factor 4 (OCT4), Krüppel-like Factor 5 (KLF5), SRY (sex determining region Y)-box 2, also known as SOX2, and NANOG, which are essential for maintaining self-renewal of undifferentiated embryonic stem cells. In lung [65] and in prostate [66] cancer, OCT4 has been identified a transcription factor with a role in maintaining the chemo-radio resistant properties.

Cancer cells, compared to non-malignant cells, have the ability to grow under conditions of limited oxygenation. This ability is often related to the increased amount of hypoxia-inducible transcription factor (HIF) family proteins. Commonly, in numerous cancers, such as breast, colon, liver, lung, pancreas, skin, and leukemias, the occurrence of HIF is associated with a poor prognosis (Semenza 2014). Hypoxia acts as a selective insult for genomic alterations that lead to chemotherapeutic resistance observed in CSC populations and HIFs factors are master regulators of oxygen homeostasis, which is disrupted in disorders as cancer [67], [68]. However, little or nothing is known about the impact of hypoxia on the regulation and activity of DUBs and the impact of DUBs on the HIF system. The expression of some CSC markers, as OCT4, in MPM cell lines, including MSTO211H, is significantly higher than those observed in MCs [69]. The drug-resistant properties of CSCs can define MPM as a therapy-resistant neoplasm, therefore CSCs confer chemoresistance properties in MPM cell lines [69]. MPM is highly chemoresistant and subsequently has a poor prognosis, with median survival of ~ 10 months after diagnosi [70], [71]. The resistance is frequently associated to CSCs in tumor [72].

1.7 Metabolic renewal of MPM cells

Metabolic change of tumor cells for a glycolytic profile (called Warburg effect) is a well know hallmark of the malignant phenotype. Indeed, MPM lesions are commonly highly glycolytic. Multiple pathways and numerous factors contribute to the Warburg effect, including PI3K-AKT, HIF, P53, MYC, and AMPK (AMP-activated protein kinase) [73].
In cancer, transcription factor HIF is a master regulator of the glycolytic profile. In a cellular environment with a normal level of O_2, the HIF-1α subunit ubiquitination drive its proteasome degradation. Inversely, O_2 deficiency or hypoxic conditions, generaly found in solid tumors, causes accumulation of HIF-1α and dimerization with the HIF-1β subunit to form the complete transcription factor HIF-1. HIF-1 induces genes transcription, which code for the glucose transporters (GLUTs), pyruvate dehydrogenase kinase 1 (PDK1) and lactate dehydrogenase A (LDHA) involved in the glycolytic phenotype [67]. It follows that tumor cells generate ATP under hypoxic conditions and ROS can enhance the transcription and translation of HIF-1α through the PI3K/AKT/mTOR pathway and can induce, even under normoxic conditions, the stabilization of HIF-1α. The PI3K/AKT pathway is often activated in mesothelioma by ROS-mediated inactivation of phosphatase PTEN [74].

Furthermore, BAP1 gene mutations conditions the metabolites levels involved in glycolysis and tricarboxylic acid cycle (TCA), with decreased mitochondrial respiration and increased glucose consumption and lactate production [9]. BAP1 is known to localize to the endoplasmic reticulum (ER), where it deubiquitynates inositol-(1,4,5)-triphosphate receptor type 3 (IP3R3), resulting in increased Ca^{2+} release from the ER and increased of Ca^{2+} uptake in the mitochondria [9]. Although mitochondrial Ca^{2+} overload is associated with the induction of cell death, moderate increases in intra-mitochondrial Ca^{2+} stimulate the activity of TCA cycle enzymes, thereby promoting mitochondrial respiration (Figure 2 [75]). Thus, low or absent BAP1 activity is associated with reduced electron transport chain (ETC) activity, resulting in a more glycolytic metabolic profile [75] (see chapter 3).

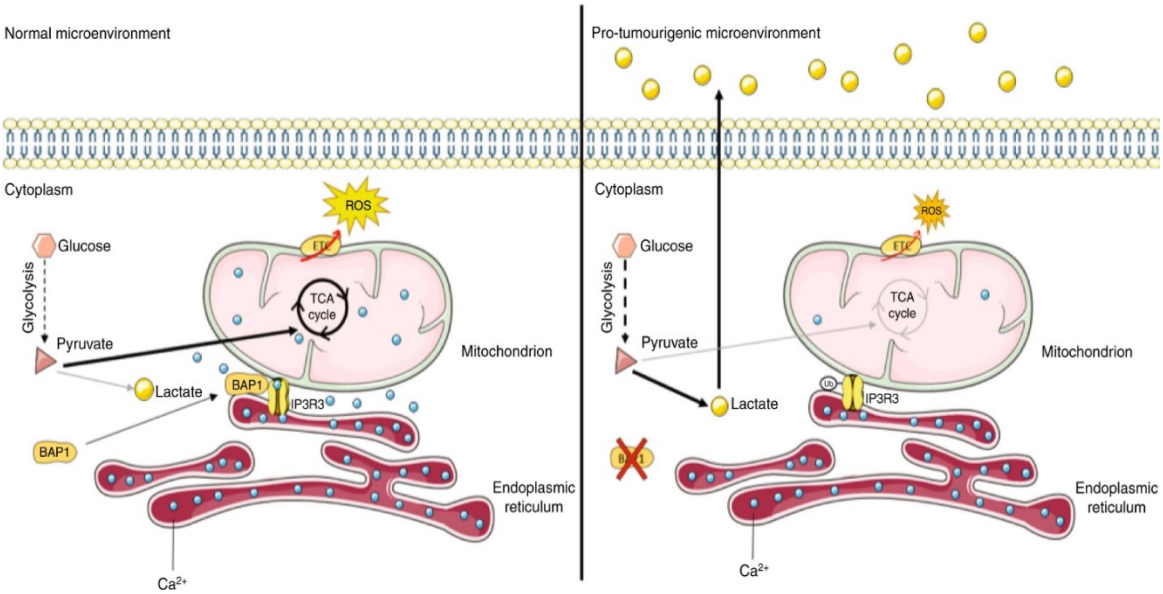

Figure 2 | **Metabolic switch and ROS production controlled by BAP1.** (Left) BAP1 deubiquitylates and activates IP3R3. It follows an increase of Ca^{2+} in the mitochondria; more TCA cycle; improvement of electron transport chain (ETC); production of ROS. (Right) When BAP1 expression is lost or reduced the activity of IP3R3 is impaired due to ubiquitination (Ub) of IP3R3. In this context, the consequence is: the reduction of calcium levels in the mitochondria; reduction of the activity of the TCA cycle and the flow of electrons through the ETC; reduction of ROS production; finally transition to Warburg effect. The lactate excreted in the microenvironment favors the formation of a protumorigenic environment. Source: L. Urso et al. 2020 (see bibliography [75]).

1.8 Diagnosis and treatment approaches

1.8.1 Difficult early diagnosis

Diagnosis of mesothelioma is usually late and depends on the integration of various factors including clinical presentation, imaging techniques, and pathological status. Certainly, early diagnosis would largely improve the chance of curative treatment.

Pleural district is the most affected in MM and pleural biopsies remain the gold standard to confirm the diagnosis. The most common clinical manifestation of MPM is progressive dyspnea and dry cough. Thoracic tomography allows visualization of pleural effusion and detects lymph node involvement [76]. These general clinical signs can easily create confusion in the diagnosis because other pathologies have common symptoms with MPM. The unavailability of an effective screening method to detect the disease at an early stage, the difficulty of discriminating MPM from other diseases with similar symptoms and long latency period hampers MPM early diagnosis. Research is ongoing to identify new biomarkers that might be useful through non-invasive tests to detect mesothelioma at an early stage.

At present it is possible to improve the diagnostic ability, through the integration of cytological and molecular approaches. Cytological and molecular investigations have a high sensitivity and a positive predictive value on the diagnosis. An accurate diagnosis by analysis and research of cyto-histological biomarkers can lead to the identification MPM histological subtype and to improve the patient's prospects of survival [77].

1.8.2 Biomarkers in diagnosis of MPM

Clinically relevant molecular data should be useful to define favorable and unfavorable histologic characteristics [78]. In blood and tissue samples, Mesothelin, fibulin-3, osteopontin, and hyaluronan have been proposed as possible biomarkers for the screening of MPM patients [43] and, in particular, cytokeratin expression can be helpful in the assessment of the amount of sarcomatoid component in biphasic MPM [79].

Using immunohistochemistry (IHC), Cicognetti et al. 2015 evaluated the utility of BAP1 expression in the differential diagnosis between mesothelioma and other mesothelial proliferations as benign mesothelial tumors and reactive mesothelial proliferations. Distinguishing reactive mesothelial proliferation from MM can be difficult, particularly on small biopsies, when it is not always clinically possible to export a large amount of tissue. This alteration should be identified together with other biomarkers to perform a correct

differential diagnosis. BAP1 protein is frequently lost in mesothelioma, especially of epithelioid/biphasic subtype and is commonly associated with homozygous BAP1 deletion [80].

The use of two biomarkers, BAP1 and CDKN2A, improves diagnostic sensitivity and is valuable in establishing the diagnosis of epithelioid mesothelioma. Dacic S. 2021 discussed the importance of identifying the molecular characteristics of mesothelioma tissues for the histological classification of pleural mesothelioma [21]. Morphology is often insufficient for unequivocal diagnosis of mesothelioma in situ and can be often difficult to distinguish malignant and benign mesothelial lesions. BAP1 or CDKN2A homozygous deletion by IHC emerged as specific diagnostic markers of malignancy in mesothelial proliferations and allowed the diagnosis of mesothelioma in fluid specimens and limited tissue samples [21], [81], [82].

Recently, microRNAs (miRNAs) have served as popular biomarkers as their expression is stable in tissue and fluid. MiRNAs are short double-stranded non-coding RNAs that regulate gene expression at the post-transcriptional level and show high sensitivity in detecting exposure to carcinogens. Increased expression of miR-126 and miR-222 was found in subjects currently exposed to asbestos [54] as an adaptive response to exposure. Others miRNA (miR-143, miR-210, and miR-200c) expression in pleural cells effusion has been reported to potentially provide a signature for diagnosing MPM [83].

1.8.3 Surgery and chemotherapy

MPM is resistant also to radiotherapy via unknown mechanisms. The management of MPM remains complex; MPM remains difficult to treat, and has an overall poor prognosis despite current multimodality treatment [71].

Prognostic factors of MPM include age, sex, histologic subtype, health status, symptoms, and explicit laboratory values. Thoracoscopy with multiple pleural biopsies can provide tissue samples suitable for distinguishing histological subtypes of MPM in diagnostic tests. The histological subtype is one of the most important prognostic factor to be taken into consideration for the treatment to be opted for MPM patients: the epithelioid histology has a better prognosis and responds better to treatment than sarcomatoid histology [84].

Treatment of MPM is based on trimodal therapy: surgery, chemotherapy and radiotherapy. Studies have reported that surgical procedures used in the treatment of mesothelioma do not result in a better survival. After the surgical operation there may be a higher rate of

complications but in addition, quality of life at few months was better in the surgically operated patients [85]. Generally, most patients have unresectable tumor mass at diagnosis or are considered inoperable due to age. Systemic therapy is the alternative treatment for inoperable patients. Major improvements have been reported since the introduction of combination therapy with platinum (cisplatin or carboplatin) and antifolates (pemetrexed or raltitrexed). Cisplatin exerts its antitumor activity by triggering apoptosis but given cisplatin's cytotoxicity, DNA, RNA, and protein residues are often compromised. The mechanism of toxicity is a cascade of events starting with induction of oxidative stress, interfering with signal transduction and cellular regulatory mechanisms, such as phosphorylation of p53, upregulation of -p21, phosphorylation of Bcl-2-associated death promoter, resulting in cell cycle arrest [86]. Despite its potent anticancer action, cisplatin chemotherapy has some limitations associated with drug resistance and/or multi-organ toxicity. Combination chemotherapy with cisplatin plus pemetrexed remains the predominant therapeutic regimen. Pemetrexed is a cytostatic antifolate drug, a cornerstone in the treatment of lung cancer, that inhibits several enzymes in the de novo pathways of pyrimidine and purine biosynthesis. The efficacy of pemetrexed in combination with cisplatin for MPM has been reported in studies which showed median survival increased by approximately 4 months in patients who received cisplatin plus permetrexed [87].
Considering the heterogeneity of the MPM, identifying the molecular profile of the tumor could influence prognosis and patient-tailored treatment options. From the first clinical studies, immunotherapies and therapies directed against antigens associated with cancer and oncogenic alterations are emerging as promising treatments to focus on for the near future [71].

1.8.4 Immunotherapy

The biology of mesothelioma is conditioned by the intrinsic tumor heterogeneity and also by the tumor microenvironment. The inflammatory component often found to be associated with mesothelioma may influence survival [88] and Immunotherapy has expanded treatment options for MPM tumors. The presence of significantly elevated numbers of monocytes and macrophages has been demonstrated in non-epithelioid tumors [89] to emphasize the association between higher monocyte counts and shorter survival.
Furthermore, tumors with elevated macrophage levels, positive CD163 levels, and low T-lymphocyte infiltration, had the worst prognosis than MPM patients with CD163-positive

tumor-associated macrophages and CD20-positive B-lymphocyte infiltration showed a better prognosis [88], [90], [91].

2. Chapter 2 - *BAP1* and Tumorigenesis

2.1 *BAP1* is a deubiquitinase enzyme

2.1.1 Post-translational Ubiquitin signaling

Ubiquitin (Ub) signaling is a conserved and dynamic process in which protein substrates are rapidly modified by a series of ubiquitin molecules in the form of a chain and ligases act in a coordinated process [92].

Ubiquitination is a complex process regulated by the E1, E2, and E3 enzymes that in successive step activate and conjugate Ub to substrates [93]. Ub is activated by the consumption of a molecule of ATP, through the creation of a high-energy thioester bond between the carboxyl terminal glycine of Ub and a cysteine residue present on the E1 enzyme. Subsequently, Ub is transferred to catalytic cysteine present in the active site of an E2 enzyme in a trans-thio esterification reaction. The last step requires the intervention of a ub-protein ligase enzyme, or E3, capable of interacting with E2 and, specifically, with the substrate to be labeled with Ub and therefore to be degraded. RING E3 domain mediate a direct transfer of Ub to the substrate from Ub-charged E2. In mammalian cells, there is a larger number of E2 and E3 enzymes, therefore the Ub network is more complex. Their variability guarantees extreme substrate specificity to the entire process.

Post-translational Ub protein modifier process impacts on protein activity, localization, or stability and influences numerous pathways such as proteasomal degradation, DNA damage repair and protein translation and trafficking [93]–[95]. To regulate this reversible process, DUBs remove Ub from protein substrates to regulate the functions, frequently in response to environmental changes and stress [96].

2.1.2 BAP1 molecular structure

BAP1 (BRCA1-Associated Protein 1) enzyme is a DUB, therefore it is involved in the removal of Ub from proteins regulating stability and function. *BAP1* is a Ub COOH-terminal hydrolase that was identified as a protein that bound to the RING finger domain of BRCA1, a tumor suppressor, hence the name of the protein [97].

The *BAP1* gene is located on chromosome 3p21.3 and with its 17 exons is expressed in all human tissues [97]. *BAP1* encoded protein has a molecular weight of 90 kDA and consists

of 729 amino acids and is the largest member of the Ub carboxyl hydrolase (UCH) subfamily of DUBs. In addition to *BAP1*, UCH subclass consists of others three members (UCHL1, UCHL3, UCHL5) that have close homology in catalytic domain [98]. *BAP1* protein contains an N-terminal catalytically active Ub carboxyl hydrolase domain (UCH 1-240), an unstructured nonorganized region (NORS 241-598), a C-terminal domain (CTD 599-699), and a nuclear localization signal (NLS). *BAP1* is unique among UCH because it has a long C-terminal tail, which contains two NLS (NLS1 at 656–661 and NLS2 at 717–722) [97], [99]. Furthermore, in the middle portion of *BAP1* there is a host cell factor 1 (HCF1) binding domain (HBM) and its CTD contains a coiled-coil motif for interaction with sex combs-like proteins ½ (ASXL1/2), and also various binding regions to numerous proteins [100]. *BAP1* UCH domain is highly conserved throughout evolution, as its C-terminal hydrolase, while *BAP1* has a large insertion in the middle of the protein of vertebrate [101]. The central portion of the enzyme contains binding motifs for several chromatin-associated proteins [102].

2.2 "*BAP1* cancer syndrome"

"*BAP1* cancer syndrome" is inherited in an autosomal dominant pattern [103] caused by heterozygous germline mutation in the *BAP1* gene on chromosome 3p21 [6]. Similar to inactivation of other tumor suppressors, affected individuals inherit a non-functional *BAP1* allele, while the remaining functional allele can be inactivated in later years. All carriers of inherited heterozygous germline *BAP1*-inactivating mutations (*BAP1* +/−) developed one and often several *BAP1* −/− malignancies in their lifetime [104]. *BAP1* inactivating mutations were initially identified in a particular lung cancer cells know as tumor non-small cell lung carcinoma (NSCLC) and individuals carrying heterozygous BAP1 mutations are at high risk for developing a wide variety of cancers [97]. Germline mutations of *BAP1* confer increased susceptibility for the development of several tumors: malignant mesothelioma (27% MM), uveal melanoma (24% UVM), cutaneous melanoma (17% CM), clear renal cell carcinoma (10% ccRCC), basal cell carcinoma and squamous cell carcinoma (7% BCC and SCC) breast cancer (6%), lung adenocarcinoma (3%), Meningioma (2%) and other cancer types [8], [105]–[108] (Figure 3 [108]).

The tumor types frequently encountered in "*BAP1* cancer syndrome" have the characteristic of being rare and of high molecular complexity. Therefore, family histories are very powerful, allowing a clinical diagnosis of *BAP1* cancer syndrome that can be verified by

genetic testing. In terms of prognosis, *BAP1* germline mutations are associated with poor prognosis in UM, CM and RCC, while in mesothelioma they are associated with less aggressive disease [105].

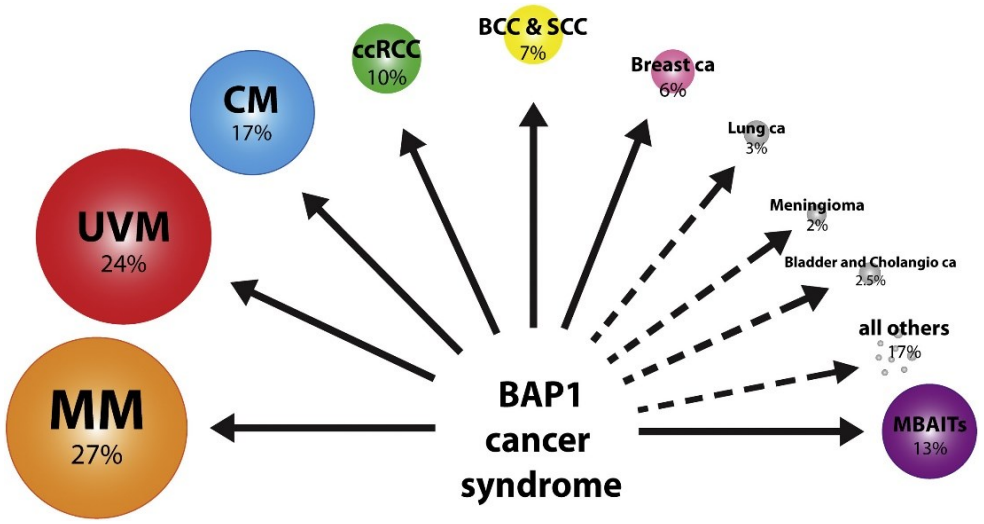

Figure 3 | **Cancer incidence in germline BAP1 mutation carriers**. MM, malignant mesothelioma; UVM, uveal melanoma; CM, cutaneous melanoma; ccRCC, clear cell renal cell carcinoma: BCC, basal cell carcinoma; SCC, squamous cell carcinoma; ca, cancer; MBAIT, atypical intradermal tumors with melanocytic BAP1 mutation. Source: M. Carbone et al. 2022 (see bibliography [106]).

2.2.1 BAP1 germline mutations

NLS is located at the carboxyl terminus of the *BAP1* protein, therefore, all truncating mutations are pathogenic for the loss of the nuclear portion [1]. The truncated *BAP1* protein remains in the cytoplasm where it is degraded to amyloid [109]. To translocate from the cytoplasm to the nucleus, *BAP1* must deubiquitylate itself [110]. A truncation mutant found in lung cancer cells results in *BAP1* that fails to localize to the nucleus, because of the loss of one of the two predicted nuclear targeting motifs that is required for its nuclear localization [99]. Mutations in the UCH domain (the *BAP1* deubiquitylating domain) can also be pathogenic when they cause loss of deubiquitylating activity. Most families with *BAP1* cancer syndrome carry truncating mutations in the NLS domain [108]. Loss of *BAP1*

nuclear staining is evidence of malignancy while mutations in other portions of the protein are less frequently pathogenic as insertions, deletions, frameshift, nonsense and missense mutations [8], [99]. Typically missense mutations and truncating mutations affect deubiquitinating activity and nuclear localization, respectively alternating the tumor suppressor effects of *BAP1* [99].

Missense mutations in the UCH domain of *BAP1* induce structural instability and aggregation of β-amyloid in vitro. The aggregates accumulate in the cytoplasm and upregulate the heat shock protein response, especially of Hsp90, which is known to be overexpressed in tumors [100]. Furthermore, Mashtalir et al. show that the ubiquitin-conjugating enzyme UBE2O multi-monoubiquitinates the NLS of *BAP1*, thereby inducing its cytoplasmic sequestration [110].

2.2.2 BAP1 somatic mutations

The pathogenic role of *BAP1* loss in these malignancies is very complex. Germline mutations in *BAP1* have been associated with "*BAP1* cancer syndrome", including MPM, but affected individuals develop cancer sporadically without a family hereditary [104]. Somatic *BAP1* mutations are much more common in MPMs tumors than in other *BAP1* dependent malignancies, although the frequency of *BAP1* mutations varies widely among different tumor types [111]. Bott et al. 2011 identifies somatic inactivating mutations in *BAP1* in twenty percent of MPM tumor samples considered in his study. Somatic *BAP1* mutations are found in sporadic MMs, therefore in MMs that occur in individuals who do not carry germline *BAP1* mutations. Loss of *BAP1* immunohistochemical expression is highly concordant with BAP1 somatic mutation [40]. More than 60% of sporadic mesotheliomas exhibit biallelic inactivation of BAP1 in their tumor cells [40], [111]. Also in this case, like the germline mutations of BAP1, to be compromised in a mutation affects the translocation / loss of *BAP1* from the nucleus. These are truncating mutations that cause deletion of the NLS located at the carboxy terminus of the *BAP1* protein or these mutations impair the deubiquitylation activity of *BAP1* [110].

Nevertheless, the significant improved survival of patients with mesothelioma in carriers of germline *BAP1* mutations is not observed in patients with mesothelioma carrying somatic biallelic *BAP1* mutations: this suggests that heterozygous germline *BAP1* mutations influence the microenvironment, including possibly the immune response, rendering the host more resistant to mesothelioma growth [108].

2.3 *BAP1* nuclear antitumor activity

BAP1 is the most frequently mutated deubiquitinase in human cancers and is a major tumor suppressor reported in cancers. The relationship between *BAP1* and cancer was suggested immediately in the first studies. *BAP1* was initially identified as a nuclear protein that interacts with BRCA1 and enhances the growth suppressive effect of the tumor suppressor BRCA1 [97]. BRCA (acronym for «BReast Cancer») is a tumor suppressor gene that produces a protein called «breast cancer susceptibility protein 1», whose role is the control of the cell cycle. *BAP1* bounds to the RING finger domain of BRCA1 and contributes to the E3 activity of BRCA1/BARD1, therefore *BAP1* inhibits the E3 ligase activity of BRCA1/BARD1, in breast and ovarian tumors [112]. *BAP1* is a key player in the BRCA1 growth suppression pathway, and it is itself a tumor suppressor gene.

In lung cancer cell lines, it has been demonstrated that *BAP1* overexpression drastically reduces tumorigenicity and it was found to be mutated or deleted in some cases of lung cancer [99]. Moreover, *BAP1* functions as a tumor suppressor in pancreatic cancer by promoting the activity of the Hippo tumor suppressor pathway [113], [114].

BAP1 loss in UVM patient samples is associated with upregulated gene expression of multiple cell adhesion molecules (CAM), including E-cadherin (CDH1), cell adhesion molecule 1 (CADM1), and syndecan-2 (SDC2) which may regulate metastatic traits [115]. Therefore, loss-of-function *BAP1* mutations are associated with UVM metastasis and poor prognosis. *BAP1* mutant in UVM tumors have an elevated glycolytic gene signature compared to *BAP1* wild type (wt) UVM tumors [116].

Recent studies show that *BAP1* loss in cancer cells causes pivotal changes in cellular metabolism [116], [117]. For example, mesothelioma cells that carry germline *BAP1* mutations have upregulated aerobic glycolysis, process also known as the 'Warburg effect' [9], as reported in *BAP1* mutant ccRCC [118].

BAP1 is a tumor suppressor that is believed to mediate its effects through chromatin modulation, change cellular metabolism, transcriptional regulation, DNA damage response pathway, cell cycle control, regulated cell death and possibly via the ubiquitin-proteasome system.

The numerous mechanisms that characterize the antitumor activity of *BAP1*, all requiring the nuclear localization of the *BAP1* protein, are summarized in figure 4 [119].

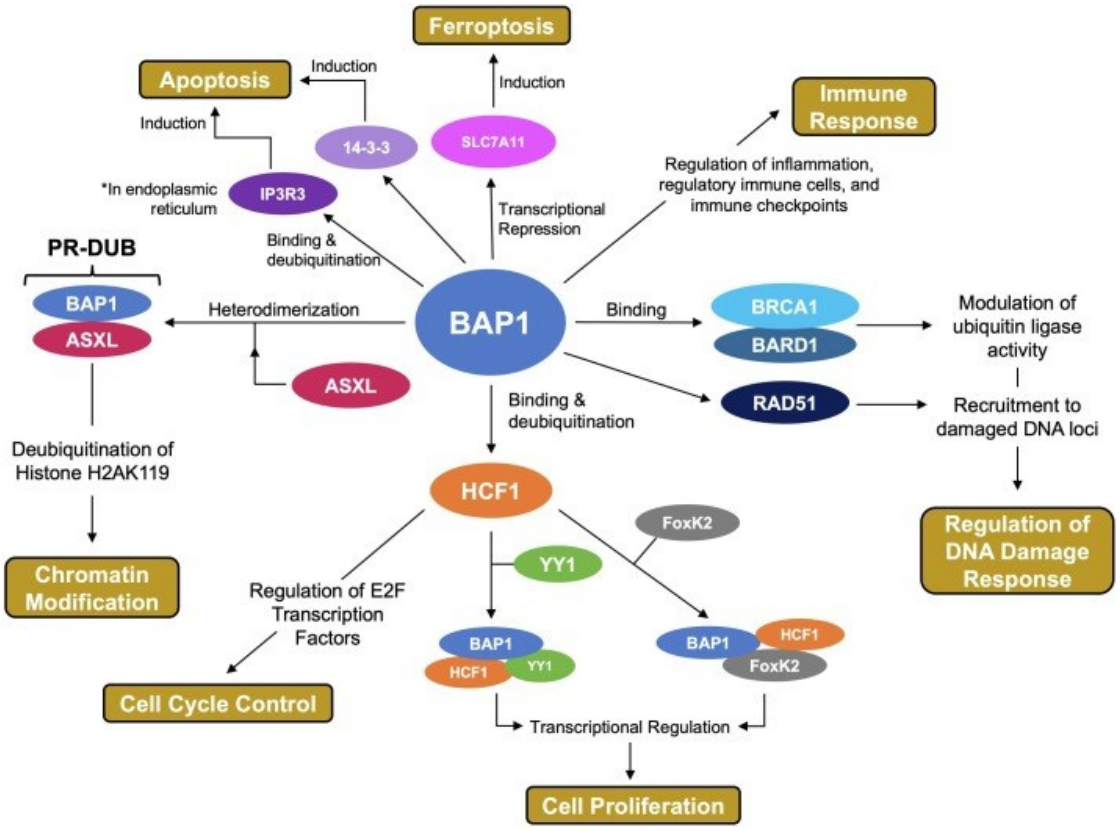

Figure 4 | **Functional roles of BAP1.** BAP1 regulates DNA damage repair through BRCA1, BARD1 and RAD51 and regulates cell cycle and cell proliferation by interacting with HCF1. BAP1 binds to ASXL to form the PR-DUB complex, which is responsible for regulating chromatin through the deubiquitination of histone H2A. BAP1 is associated with a number of cell death pathways (antitumor role of BAP1 in cytoplasm) and is implicated in immune regulation. Sourse: B.H. Louie et al. 2020 (see bibliography [117]).

2.3.1 BAP1 is transcriptional regulator in chromatin-associated complex

Studies establish a direct link between *BAP1* and the transcriptional control of genes. BAP1 activates transcription in a manner dependent on its enzymatic activity and through it regulates the expression of a variety of genes involved in numerous cellular processes. *BAP1* regulates cell growth and proliferation [120] and it is well known its involvement in epigenetic modification of chromatin [102]. Moreover, *BAP1* regulates DNA repair and replication through chromatin structure regulation and, in protein complex, binds to both promoters and enhancers at the chromatin level [121], confirmed its involvement in the regulation of gene transcription.

*BAP1*transcriptional activity is likely to be more complex due to its association with chromatin-associated proteins that participate to recruitment of *BAP1* to specific chromatin loci [122], [123]. *BAP1* assembles multiprotein complexes containing numerous transcription factors and cofactors, including HCF-1 [124], [125] and the transcription factor Yin Yang 1 (YY1) [126] and the multifaceted transcription factor forkhead box proteins K1/2 (FOXK1/2) [127]. HCF-1 is a transcriptional cofactor found in a number of important regulatory complexes and it is known as a component of the "death from cancer" signature that strongly predicts the prognosis of a variety of human cancers. Moreover, HCF-1 is a chromatin-associated protein thought to both activate and repress transcription by linking appropriate histone-modifying enzymes to a subset of transcription factors [128]. In nucleus, *BAP1* deubiquitinates HCF-1 and form multi-protein complexes that control transcriptional regulation [126].

The additional ASXL1–3 proteins were found as essential core subunits within *BAP1* complex [129]. The primary function for ASXLs is to stabilize and link *BAP1* complex to the nucleosome [130]. ASXLs proteins form mutually exclusive complexes with *BAP1*, due to a sequence similarity present at the N-terminal domain of ASXLs, which directly interacts with *BAP1*'s CTD [130].

2.3.2 BAP1 is transcriptional co-activator and co-repressor

BAP1 acts as a transcriptional co-activator through its deubiquitination activity on histone H2A mono-ubiquitinated (H2Aub) [131]. *BAP1* has conserved functional origins and was initially identified its DUB activity on histone H2Aub in Drosophila [101]. Histone H2Aub is a histone modification mediated by the Polycomb Repressive Complex 1 (PRC1) [101], an E3- ligase that facilitates the monoubiquitination of histone H2A in lysine 119, leading to altering chromatin architecture and gene silencing [131]. The mammalian Polycomb Repressive DeUBiquitinase (PR-DUB) complexes catalyze removal of mono-Ub on lysine 119 of histone H2A (H2AK119ub1) through a multiprotein core comprised of *BAP1*, HCFC1, FOXK1/2, and OGT in combination with either of ASXL1, 2, or 3. PR-DUB and PRC1 belong to Polycomb group (PcG) families involved in gene regulation at the chromatin level. Therefore, PR-DUB plays an antagonistic role to PRC1 and *BAP1* plays a widespread role in maintaining a delicate balance of H2A ubiquitination, regulating the chromatin architecture, and affecting expression of genes, many of which may be implicated in cancer pathways [131]. Mutations in PR-DUB components are frequent in

cancer and *BAP1*, in PR-DUB complex, is dependent on the ASXLs proteins and FOXK1/2 in facilitating gene activation across the genome and maintains expression of genes important for general functions such as cell metabolism and homeostasis [123]. Moreover, *BAP1* and ASXL1/2/3 are required for normal cell proliferation and for the expression of a common set of genes [129].

BAP1 is primarily a transcriptional co-activator, but it is known that it can also act as a transcriptional co-repressor. How BAP1 ensures these two opposing roles and BAP1's direct target genes still remains an open question. *BAP1* depletion or inactivation induced up/down-regulation of numerous genes associated with cell cycle, DNA repair, metabolism and apoptosis [123], [132]. *BAP1*, in repressor complex, deubiquitinates and stabilities the transcriptional co-repressor NCoR1, increasing chromatin recruitment, with transcriptional repression of γ-globulin gene. *BAP1* maintains NCoR1 at sites in the β-globin locus and promotes fetal-adult developmental switch at the human β-globin gene locus [120].

2.3.3 BAP1 role in cell cycle

BAP1 is involved in binding and regulation of transcriptional factors that in turn are involved in a number of processes that control the cell cycle and proliferation.

BAP1 has multiple roles in the coordination cell proliferation and it is well know that Ub mediated is critical in this event [133]. *BAP1* possesses growth inhibitory activity: *BAP1* exerts its tumor suppressor functions by affecting the cell cycle, speeding the progression through the G1-S checkpoint, and inducing cell death via a process that has characteristics of both apoptosis and necrosis [99].

Interaction with HCF-1, a cell-cycle regulator composed of HCF-1N and HCF-1C, is critical for the *BAP1*-mediated growth regulation [124]. HCF1 is known to be involved in regulating transcription and promoting cell-cycle progression through the G1/S phase by recruiting H3K4 histone methyltransferases to the E2F1 transcription factor so that genes required for S-phase can be transcribed [128]. Depletion of *BAP1* results in accumulation of HCF-1(C), therefore *BAP1* helps to control cell proliferation by regulating HCF-1 protein levels and by associating with genes involved in the G1-S transition [125]. Studies have demonstrated that *BAP1* function is necessary for the transition of cells from the G1 to S phase of the cell cycle, with knockdown of *BAP1* causing cells to be arrested in the G1 phase and is accompanied by a decrease in the expression of S phase genes in UVM [134]. Additionally, *BAP1* was shown to bind to gene promoters targeted by E2F1 transcription

factors and this localization is dependent on HCF1 [134]. Moreover, *BAP1* knockdown leads to increased H2AK119ub levels, by deubiquitination, on E2F responsive promoters thus promoting transcriptional activation and proper progression through the G1/S phase of the cell cycle [134]. Given the known role of *BAP1* in regulatory ubiquitination of histones, the findings suggested transcriptional deregulation as a pathogenic mechanism [6]. Given the robust associations between *BAP1*/HCF-1 and HCF-1/E2Fs, it is reasonable to speculate that *BAP1* influences cell proliferation at G1/S by co-regulating transcription from HCF-1/E2F-governed promoters [135]. Therefore, *BAP1* plays a major part in regulating HCF1-mediated control of the cell cycle. *BAP1* forms a ternary complex with HCF1 and YY1, which control the expression of genes involved in cell proliferation [126]. *BAP1* binds to the zinc fingers of YY1 and HCF1 through its HBM domain and, this complex, is recruited to target promoters, thus altering expression of genes such as COX7C, which is a component of the mitochondrial respiratory chain. More recently, another study demonstrated a different ternary complex between *BAP1*, HCF1, and FoxK2, a transcription factor involved in proper control of cell proliferation and cell cycle control [127].

Ventii et al. hypothesizes that BAP1 might promote cell cycle transition from G1 to S with cumulative DNA damage, ultimately leading to growth arrest and cell death [99]. Transcription factors that associate with BAP1 to form protein complexes, such as FOXK1/2 and YY1, can also direct BAP1 recruitment to specific chromatin loci [123], [127].

In summary, it appears that *BAP1* is involved in a number of multiprotein complexes that involve HCF1 and several other factors that are important for regulating cell-cycle control and proliferation [136].

2.3.4 BAP1 in DNA damage response

BAP1 controls distinct cellular activities by direct modulating DNA repair. Inhibition of *BAP1* expression by short hairpin RNA resulted and in retardation of S-phase progression [112]. Therefore, depletion of *BAP1* led to an S-phase arrest and increased susceptibility to DNA damage [112]. *BAP1* might also indirectly contribute to DNA repair through the coordination of gene expression. Well-known tumor suppressor responsible for coordinating the DNA damage response via homologous recombination (HR) [137].

Nishikawa et al. discover that the interaction between *BAP1* and the BRCA1/BARD1 complex may in fact be necessary to mediate repair of DNA damage*BAP1* was shown to bind the BRCA1/BARD1 complex, which is an E3 ubiquitin ligase that regulates the HR pathway of DNA damage repair [112]. *BAP1* modulates the function of the BRCA1/BARD1 complex by binding to the RING finger domain on BARD1 and inhibiting its E3 ligase function. It also plays an antagonistic role by deubiquitinating sites of BRCA1/BARD1 ubiquitination [112]. Furthmore, *BAP1* near chromatin DNA double stranded breaks recruits HR factors BRCA1 and RAD51 to facilitate homologous recombination DNA repair [7], [132]. *BAP1* promotes the assembly of RAD51 foci to facilitate DNA repair and replication fork progression: *BAP1* is able to interact with chromatin remodeling complex, INO80, facilitating the anchorage of the complex on chromatin through H2Aub and promoting the progression of the DNA replication fork [138].

2.4 *BAP1* modulates cellular metabolism

The Warburg effect, observed in most cancer cells, produces energy predominantly through aerobic glycolysis which consists of a high level of glucose uptake and glycolysis followed by lactic acid fermentation which occurs in the cytosol. In normal cells the "normal" citric acid cycle and oxidative phosphorylation in the mitochondria is balanced with other energy-producing processes but cancer cells prefer the less efficient process of aerobic glycolysis [139].

The reduction in the levels of the *BAP1* protein show a typical Warburg effect [140]. Metabolomics and in vitro analyzes of primary cultured fibroblasts of *BAP1* +/− family members revealed that a reduction in *BAP1* protein levels shifts cellular metabolism from oxidative phosphorylation to aerobic glycolysis. Furthermore, in primary fibroblasts from individuals with heterozygous *BAP1* +/− mutations, aerobic glycolysis/lactate secretion was increased and mitochondrial respiration/ATP synthesis was decreased compared with control (*BAP1* +/+) family members. Bononi et al. suggests a potential new tumor-promoting role for the Warburg effect that precedes malignancy. Furthermore, *BAP1* deletion also impairs several metabolic pathways. Using a genetically engineered inducible *BAP1* knockout mouse model, cic demonstrates that cholesterol biosynthesis was increased, while gluconeogenesis and lipid homeostasis proteins were decreased in the liver [141]. Furthermore, through the O-linked β-N-acetylglucosamine (O-GlcNAc) transferase

(OGT)/HCF1 complex, *BAP1* regulates gluconeogenesis by modulating the stability of the transcriptional coactivator PGC1α, a master regulator of gluconeogenesis [142]. HCF-1 recruits OGT to O-GlcNAcylate PGC-1α and O-GlcNAcylation facilitates the binding of the deubiquitinase *BAP1*, thus protecting PGC-1α from degradation and promoting gluconeogenesis. Therefore, *BAP1* contributes to the maintenance of metabolic homeostasis.

2.5 *BAP1* role in development and cell differentiation

Numerous and diverse scientific evidence suggests that BAP1 is a critical regulator of cell self-renewal. The cell fate of multiple tissues and in multiple stages of development is precisely regulated by *BAP1*. *BAP1* has been shown to be required for the development and homeostasis of several mammalian tissues. Being present in various tissues, their response to a possible inactivation of *BAP1* could have a different impact on the oncogenic transformation. Thus, *BAP1* promotes the expression of key developmental genes that regulate the transition from pluripotency to differentiation. This role is also associated with the interaction with chromatin and in particular with histone H3K27: *BAP1* determines histone deacetylation in the regulatory regions of the gene involved in cell differentiation. [143]. *BAP1* deficiency determined histone deacetylation by histone deacetylase (HDAC) activity with disturb of normal expression of genes regulating embryonic lineages [143].
Depletion of *BAP1* in UVM cells resulted in a loss of differentiation and increase of stem-like properties, including expression of stem cell markers, increased capacity for self-replication, and enhanced ability to grow in stem cell conditions [144]. In melanocytic, in vivo studies, *BAP1* is involved in the maintenance of a normal cell phenotype, while the depletion of *BAP1* protein levels result in dedifferentiation of cells and the acquisition of a more primitive, stem cell like phenotype [144].
BAP1 is known to be central to the control of early placentation in mice. Downregulation of *BAP1* protein is associated with a gain in invasiveness and embryonic lethality, demonstrated by the epithelial-mesenchymal transition (EMT) trigger during trophoblast differentiation [130], [145]. Moreover, the function of *BAP1* in suppressing EMT progression is dependent on the binding of *BAP1* to ASXL1/2 proteins, to form the PR-DUB complex, and the molecular function of *BAP1* in regulating trophoblast differentiation is conserved in mice and humans [130]. Deletion of *BAP1* in the hematopoietic system

causes myelodysplastic syndrome (MDS) in mouse, but similar features are found in the human MDS [145].

BAP1 modulate hematopoietic stem cell (HSC) self-renewal and hematopoiesis in a post-translational modification way that, like any other protein, may influence the activity of *BAP1* in this context. The deglutamylate *BAP1* modification stabilize the protein and promotes HSC self-renewal and hematopoiesis *BAP1* depended [146]. Moreover, *BAP1* inactivation impairs normal HSC differentiation and this is accompanied by an increased proliferation of *BAP1*-deficient myeloid progenitors [145]. Other studies underline *BAP1* involved in different mechanisms of regulation in myeloid and lymphoid lineages through thymus development and proliferative responses of T lymphocytes [147].

2.6 *BAP1* can promote malignant progression

The role of *BAP1* in tumor progression may be more complex than its presumed tumor suppressor function. Cell cycle regulation is reported in numerous studies as *BAP1* role more controversial. One study reported that *BAP1* depletion slows S-phase, while another showed that *BAP1* expression accelerates S-phase entry [99], [112]. In other words, *BAP1* is involved in the transition from G1 to S phase and this G1/S transition is a function of HCF-1N. Interestingly, *BAP1* is not essential for cell proliferation. A number of cancer cell lines are viable without *BAP1* [97] and the lack of *BAP1* is even beneficial to those cells for tumor proliferation and formation [99] which likely have further genetic alterations or epigenetic in other genes. *BAP1* may have a dual role in growth control; it is involved in the regulation of the normal cell cycle, while preventing uncontrolled cell growth, thus ensuring proper cell proliferation. The enzymatic activity of *BAP1* is also required for the malignant progression, as reported in myeloma and breast cancer [148], where *BAP1* depletion results in growth retardation [125] and G1 to S transition delay [102], [124].

This can be possibly explained by cell-context, cell-type or *BAP1* specific functions and its interacting partners. In addition, *BAP1* complex could mediate the chromatin recruitment of other epigenetic complexes, such as methyltrasferase MLL3 and COMPASS complex to activate transcription [121] and oncogenic function of BRD4/ASXL3/*BAP1* epigenetic axis at active chromatin enhancers in small cell lung cancer (SCLC) -A subtype [149].

2.6.1 BAP1 stabilizes KLF5 transcription factor in cancer

Krüppel-like Factor 5 (KLF5) belongs to a transcription factor family involved in in disease development. KLF5 plays roles cell stemness, proliferation, apoptosis, autophagy, and migration [150] and it is mainly involved in embryonic development and different tissue [151], [152]. The complex and numerous functions of KLF5 involve multiple signal pathways, including Receptor Tyrosine Kinase (RTK), hormone, Transforming Growth Factor β 1 (TGF-β), receptor NOTCH, (Nuclear factor kappaB) NF-κB signaling pathways [150].

Past studies report that KLF5 promotes cell proliferation and migration in breast cancer and promote glioblastoma angiogenesis [153] by regulating several key target genes including growth factors and proteins involved in cell cycle as cyclin D1 [154] and cyclin E1 [155], [156]. In pancreatic cancer, KLF5 activates the transcription of E2F transcription factor 1 (E2F1) and RAD51 recombinase and it is highly expressed in tissue samples from three short-surviving patients with pancreatic cancer [157].

KLF5 promotes tumorigenesis in most cancers and the expression of KLF5 is abnormal in a variety of solid tumors, such as breast cancer [148], melanoma [158], prostate cancer [159], pancreatic cancer [157], NSCLC [156], and glioblastoma [153]. KLF5 is regulated by multiple post-transcriptional modifications, including ubiquitination. HDAC promoted the acetylation of KLF5, which decreased the association between BAP1 and KLF5. Thus, KLF5 acetylation promotes ubiquitination and proteasomal degradation in breast cancer [160]. *BAP1* deubiquitinates and stabilizes KLF5 transcription factor in BC and form a transcription complex to regulate the expression of target genes which promote cell proliferation and metastasis by inhibiting p27 gene expression [148]. *BAP1* did not stabilize acetylated KLF5 [160]. KLF5 forms a complex with *BAP1*/HCF-1/OGT1 to regulate the transcription of Fibroblast growth factor-binding protein 1 (FGF-BP1) and p27 and cell cycle progression [148]. In melanoma, the high expression of *BAP1* also indicates a poor prognosis for patients, which promotes tumor progression by hindering KLF5 ubiquitination *in vivo* [158]. In lung cancer, KLF5 was subjected to deubiquitination/ubiquitination modification by *BAP1*/WW Domain Containing E3 Ubiquitin Protein Ligase 1 (WWP1) and activated PI3K/AKT/mTOR signaling pathway to inhibit melanoma cell autophagy [161]. *BAP1* antagonizes WWP1-mediated transcription factor KLF5 ubiquitination and inhibits autophagy to promote melanoma progression [158]. Recently, Wang et al. report that cell proliferation and migration were significantly enhanced in esophageal carcinoma cells overexpressing *BAP1*. In addition, the expression

of KLF5 transcription factor, CyclinD1, and FGF-BP1 was increased by *BAP1* overexpression and decreased by *BAP1* knockdown with proliferation and migration reduction [162].

KLF5 is associated with CSC-like properties. KLF5 knockdown suppressed sphere-formation activity in colorectal cancer (CRC) cell lines [163], [164] and it plays vital roles in disease development and regulates the expression of a wide range of target genes, such as stem marker NANOG [152], [165].

A summary of *BAP1*-KLF5 action in promoting tumorigenesis is shown in Figure 5.

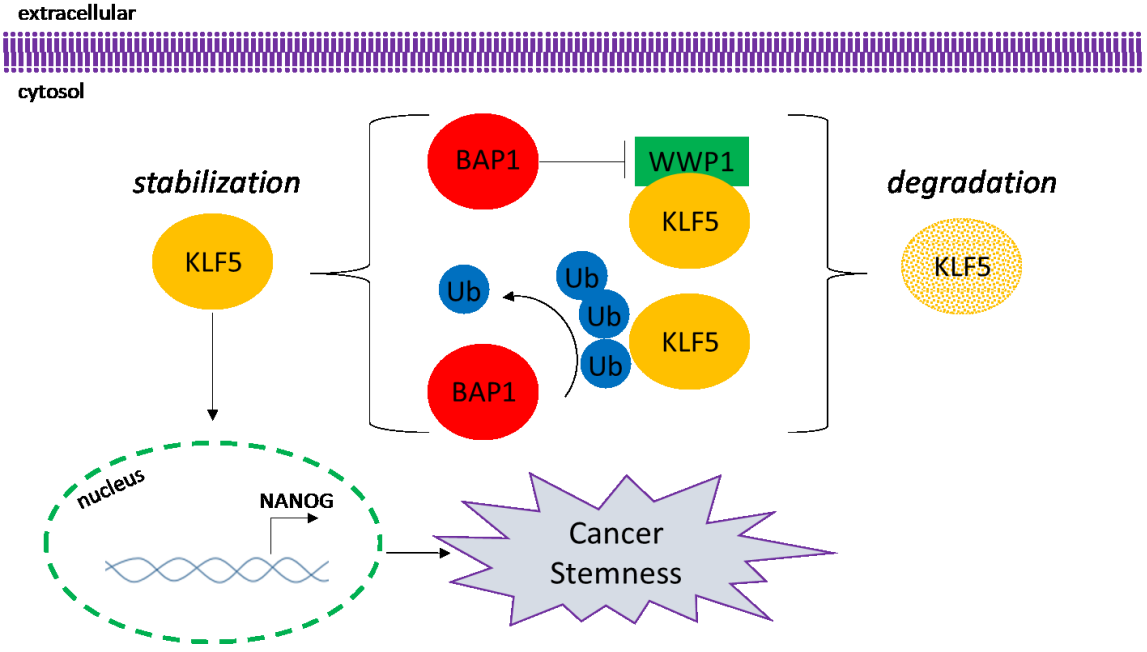

Figure 5 | **BAP1 stabilizes KLF5 by deubiquitination.** BAP1 stabilizes KLF5 by removing the ubiquitin chain and inhibition WW1 factor. KLF5 as transcription factor regulates the expression of stem marker NANOG with promotion of characteristics of stemness in cells.

3. Chapter 3 - *BAP1* in MPM evolution

3.1 *BAP1* alteration in genetic MPM

In a 2001 study, Carbone's team reported mesothelioma clustering in some US and Turkish families in which up to 50% of the members developed mesothelioma [38]. Testa et al. prospectively studied USA families, in Wisconsin and in Louisiana, with high incidence of mesothelioma. The family members had neither been exposed to erionite nor had occupational exposure to asbestos, so Carbone and colleagues set out to identify putative mesothelioma susceptibility genes. Approximately 50% of family members had inherited *BAP1* mutations [1]. In *BAP1* mutation carriers, in patients with minimal or no exposure to asbestos, pleural and peritoneal mesotheliomas presented with a male:female ratio of 1:1 and a pleural:peritoneal ratio of 1:1 [17], [37], [166].

3.1.1 BAP1 mutations increase susceptibility to asbestos: role in cellular transformation

Germline-inactivating mutations of BAP1 predispose to MM and certain other cancers and represent a model for gene–environment interactions in oncogenesis [8]. Individuals with BAP1 mutations exposed to asbestos have a predominance of developing MM. Alternatively, a single mutation to the BAP1 gene is sufficient to cause mesothelioma [1]. The reduced levels of *BAP1* determine a tumor phenotype predominant in genotoxic/stress conditions underlining the involvement of *BAP1* in the modulation of gene-environment interactions in carcinogenesis [9]. *BAP1* mutation carriers are predisposed to the tumorigenic effects of asbestos [167].

In mice, MCs with *BAP1* +/− have a significant downregulation in the mRNA and protein levels of a E2F target gene known as the Retinoblastoma tumor suppressor (RB) [167]. MM cells show biallelic inactivation of *BAP1*, consistent with its proposed role as a recessive cancer susceptibility gene. Moreover, MM cells derived from *BAP1* +/− treated with asbestos acquire a biallelic inactivation of *BAP1* with a more incidence on RB downregulation [167]. In parallel, in human, knockdown of *BAP1* in mesothelioma cell lines expressing *BAP1 wt* resulted in proliferation defects with an accumulation of cells in the S phase and also downregulated E2F regulated genes [168].

Exposure to carcinogenic fibers may significantly increase the risk of MM in genetically predisposed individuals carrying germline *BAP1* mutations, possibly via alterations of the inflammatory response [169]. *BAP1* mutations cooperate with asbestos in chronic inflammatory process promoted by the extracellular release of HMGB1 [170]. *BAP1* forms a trimeric protein complex with HMGB1 and with HDAC1 that modulates HMGB1 acetylation and its release. Reduced *BAP1* levels caused increased ubiquitylation and degradation of HDAC1, leading to increased acetylation of HMGB1 and its active secretion that in turn promoted MC transformation [170].

3.2 *BAP1* regulates apoptosis in cellular cytoplasm

After DNA damage caused by asbestos (or other carcinogens as ultraviolet light, radiation, or chemotherapy) *BAP1* regulates both DNA repair and apoptosis [9]. The balance between DNA damage and cell death is the crossroads that determines the final effect: the more DNA-damaged cells that survive exposure, the higher the risk that one of them could develop into a malignant tumor. It is possible that the malignancies most frequently associated with the *BAP1* cancer syndrome, such as mesothelioma, arise from tissues in which Ca^{2+}-induced apoptosis plays a critical role in cellular transformation.

3.2.1 ER Ca^{2+} flux to mitochondria

Ca^{2+} is a regulator and cofactor of several fundamental cellular processes. Gene transcription, secretion, apoptosis, cell proliferation and differentiation, and metabolism are just some of the major Ca^{2+}-dependent processes [171]. The intracellular concentration of Ca^{2+} ($[Ca^{2+}]$) is tightly controlled by complex interactions between different channels, pumps and transporters. Changes in $[Ca^{2+}]$ modulate a variety of intracellular functions and dysregulation leads to various pathological conditions.

The ER is the major store of intracellular Ca^{2+} which releases Ca^{2+} to the cytoplasm and other organelles, through ryanodine receptors (RyRs) [172] and inositol 1,4,5-triphosphate (IP3) receptors (IP3Rs) [173]. In particular, IP3Rs are the most ubiquitous intracellular Ca^{2+} channels that control Ca^{2+} release from the ER to the mitochondria and cytoplasm [173].

In response to extracellular stimuli such as hormones, growth factors, and neurotransmitters that bind to G-protein-coupled receptors (as well as to receptor tyrosine kinases), inositol

1,4,5-triphosphate (IP3) is produced by hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) in the cell membrane through phospholipase C (PLC) and bound IP3R to trigger the opening of the channel [174]. Ca^{2+} is released in specific areas of the ER (called MAMs - mitochondria associated membranes) that are in close contact with the outer mitochondrial membrane. at the level of MAMs Ca2+ flows into the intermembrane space of the mitochondrion through the voltage-gated anion channel (VDAC) and then is actively transported into the mitochondria by the mitochondrial uniporter channel (MCU). Transient Ca^{2+} release promotes mitochondrial Krebs cycle and ATP production while excessive accumulation triggers mitochondria-related apoptotic process [175].

3.2.2 BAP1 deubiquitinates IP3R3 Ca^{2+} channel

BAP1 was originally identified as a nuclear protein and all known activities of *BAP1* have been associated to its nuclear localization and chromatin-associated complex [8]. Moreover, recently function of *BAP1* was also identified in the cytoplasm, and in particular in mesothelioma, subcellular fractionation, immunofluorescence and microscopy assays revealed that extra nuclear *BAP1* localization in the ER [9]. BAP1 located in the ER regulates Ca^{2+} signaling-dependent cellular activities such as cell death [9], [17]. It is logical to deduce and underline that the mechanisms regulating the localization of BAP1, in the nucleus or in the cytoplasm, could play a critical role in the nuclear translocation of BAP1 and the regulation of its function. This regulation predicts a balance between ubiquitination of the NLS of BAP1, which leads to its sequestration in the cytoplasm, and BAP1 auto-deubiquitylation. [110].

Bononi et al. found that the cytoplasmic *BAP1* localizes at the ER in primary fibroblasts from members of the two USA families skin biopsies, in which originally discovered that *BAP1* mutations causing '*BAP1* cancer syndrome'. Pathogenic *BAP1* mutations resulted in loss of *BAP1* nuclear localization, impairing the activity of BAP1 in the nucleus, where *BAP1* regulates DNA repair, chromatin assembling, and transcription [111]. *BAP1* in cytosol binds, deubiquitylates, and stabilizes IP3R3, thus regulating ER Ca^{2+} release and into the mitochondria [9].

Thus, cells with reduced or absent *BAP1* activity accumulate more DNA damage, as they cannot properly repair the DNA [9], [132] and, at the same time, they cannot execute apoptosis, which normally eliminates cells that contain genetic mutations, to prevent cancer (Figure 6). As a consequence of the altered mitochondrial metabolism caused by reduced

Ca^{2+} levels, cells with *BAP1* mutations derive energy largely through aerobic glycolysis, the so-called Warburg effect, a metabolic shift that favors malignant growth [140]. Subsequently, Zhang et al reported that cells with reduced *BAP1* activity also have impaired ferroptosis [176], providing an additional mechanism by which *BAP1*-mutated cells escape cell death [177].

The double activities of *BAP1,* in the nucleus and cytoplasm, define the strong tumor suppressor activity of this deubiquitylase. Decreased BAP1 levels lead to increased DNA damage and impaired apoptosis, due to decreased nuclear and cytoplasmic activity of BAP1, respectively. Furthermore, low levels of BAP1 are related to increased cellular transformation, and in this case, the cause is related to decrease both nuclear and cytoplasmic activities.

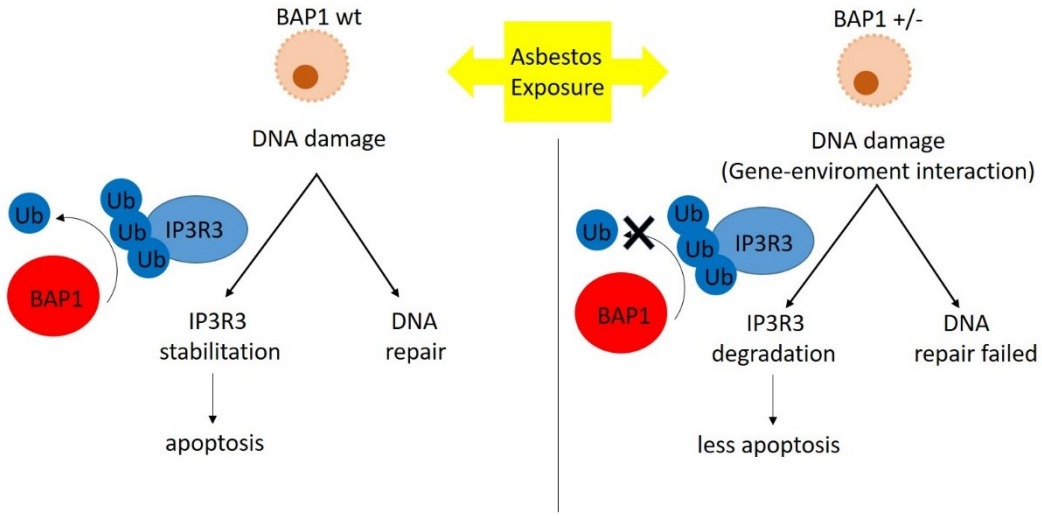

Figure 6 | **BAP1 interference in cellular response to asbestos exposure.** (left) BAP1 helps cells resist environmental insults such as the presence of asbestos fibers. The intact and normally expressed BAP1 gene (BAP1 wt) promotes the repair of asbestos-induced DNA damage and stabilizes the IP3R3 channel with activation of the mitochondrion-dependent apoptotic process. (right) The lack of expression of the BAP1 gene makes the cell less able to respond to the asbestos-induced DNA damage with the accumulation of further DNA damages which add up to the first ones. The IP3R3 calcium channel is degraded and mitochondrion-calcium dependent apoptosis is reduced.

3.3 *BAP1* loss expression predicts longer survival in mesothelioma patients

BAP1 is a diagnostic marker of mesothelioma [1] and germline mutations favor the development of mesothelioma and of other cancers, but, conversely, for reasons that currently are unclear, these same mutations appear to mitigate aggressive tumor growth as these patients live much longer. Some studies observed a significantly improved survival among MPM in carriers of germline mutations [178], [179]. Improved survival has been found for mesothelioma, not for other malignancies developing in carriers of germline *BAP1* mutations.

Bauman et al. 2014 tested the hypothesis that MM associated with germline *BAP1* mutations has a better prognosis compared with sporadic MM and in 2015 a work entitled "Loss of expression of *BAP1* predicts longer survival in mesothelioma" was published [10]. Meta-analysis study found that MM patients with germline *BAP1* mutations have an overall 7-fold increased long-term survival, independently of sex and age [104].

3.4 Targeting *BAP1* mutations for chemotherapy

There is still no effective therapy for MPM. Moreover, the prognosis is poor and conventional chemotherapy entails remarkable toxic side effects [76], [180]. Mesothelioma cells have a characteristic highly unstable karyotype [181] however the use of genetic markers in patients with MPM would allow prediction of response to chemotherapy.

In MPM, *BAP1* alterations is a negative predictor of response to chemotherapy and could possibly be used as a useful biomarker for the therapeutic decision to be taken. Tanaka et al. reports that *BAP1* mutations may interfere with anti-proliferative effects of statins, an antitumor in MM [182]. Moreover, alterations of *BAP1* may influence individual sensitivity to cisplatin chemotherapy, possibly through modulation of apoptosis and transcriptional regulation of the *BAP1*-HCF1/E2F1 axis [183]. Therefore, it has been proposed that *BAP1* status is a useful biomarker to stratify patients for based chemotherapy [183].

BAP1 is an attractive therapeutic target and prognostic biomarker because it is the most frequently mutated gene in mesothelioma. Many of the pathways controlled by BAP1 are already being targeted by drugs already in development or work is underway to create new drugs.

HDAC inhibitors (HDACi) have emerged as a promising new class of multifunctional anticancer agents with the ability to suppress cancer cell migration, invasion, metastasis, and angiogenesis [176]. Histones are among the *BAP1* targets and *BAP1* downregulation

or knockdown in mesothelioma cell lines increases the sensitivity for HDAC inhibitors, leading to cell death [184].

BAP1 loss induce methylation at the amino terminal of core histone H3 [185]. This activity is influenced by *BAP1* binding to ASXL1 [102] and in *BAP1*-mutant cell lines, ASXL1 inhibition abrogates tumor growth [185].

BAP1 modulates double-strand DNA damage repair [132] therefore, cells with *BAP1* mutations are frequent inactivation of DNA repair genes that contributes to genomic instability. more sensitive to both radiation and treatment with a PARP inhibitor [56], [168], [186]. *BAP1* offers potential for the use of synthetic lethal approaches targeting DNA repair factors and in mesotheliomas patients with germline mutations in *BAP1* are more sensitive to PARP inhibitors has been recently reported [187], [188].

Two actionable targets, ribonucleotide reductase regulatory subunit M1 (RRM1) and M2 (RRM2), were validated, and their inhibition, mediated by gemcitabine or hydroxyurea, was more cytotoxic to *BAP1*mutant/deleted cell lines. These data indicate that *BAP1* regulates RRM2 levels during replication stress and that patients could be stratified for gemcitabine treatment, depending on *BAP1* status [189]. A parallel study demonstrates that mesothelioma cells with functional *BAP1* were more sensitive to gemcitabine treatment compared with cells bearing mutated and nonfunctional *BAP1* [190]. Together, these independent studies indicate that it may be possible to identify those patients with mesothelioma who are more likely to respond to gemcitabine based on *BAP1* status. Preclinical evidence suggests that *BAP1* wt status increases sensitivity to gemcitabine [190]. The *BAP1* mutant cells were significantly less sensitive than *BAP1* wt cell lines to the clinically relevant drug gemcitabine [190].

Recent study assessed the efficacy of the anti–PD-L1 antibody durvalumab with platinum-based chemotherapy in patients with unresectable pleural mesothelioma and with germline alterations in cancer predisposing genes, especially those involved in DNA repair, as *BAP1* [191].

4. Chapter 4 - Materials and Methods

4.1 Cell cultures

Mesothelioma MSTO-211H and REN cells were purchased from the American Type Culture Collection (ATCC). MSTO-211H mesothelioma cell line were cultured in RPMI1640 (SigmaAldrich) whereas REN cells in DMEM (SigmaAldrich). All cell lines were cultured with standard supplements and conditions: 10% FBS, 1% Penicillin/Streptomycin and 1% L-Glutammine at 37°C in at 5% CO2.

The human MSTO-211H MPM cell line derives from a tumor of biphasic histotype whereas REN from a tumor of epithelioid histotype. Both MPM cell lines have *BAP1*1 wt (MSTO $^{BAP1\ wt}$ and REN $^{BAP1\ wt}$).

4.2 Lentiviral Vectors

To stably transduce MPM cell lines we used Lentiviral Vectors (LVs) technology. The easily transfectable human embryonic kidney cell line (HEK293), grown in DMEM, was used for the production of replication-incompetent lentiviral particles of 3rd generation. The pLKO.1 vector was used to clone short hairpin (sh)RNA sequences targeting *BAP1*. HEK293 cells were transfected (through Lipofectamine LTX - ThermoScientific) with the pLKO.1 plasmid along with the packaging plasmid psPAX2 (Addgene #12260) and the envelope plasmid pMD2.G (Addgene #12259) all containing the different components necessary for viral assembly. 48 hours after trasfection, we collected the cell medium with the lentiviral particles in suspension at 24, 48 and 72 hours post transfection. These aliquots were collected and used for MPM cell infection (1 ml for 100 dish). Once introduced, the puromycin resistance marker encoded in pLKO.1 allows for the selection of stably transduced clones. To generate MSTO-211H and REN stably silenced for *BAP1* expression, we have cloned into the pLKO.1 the sh*BAP1* (shRNA TRCN0000007374 – Broad Institute) sequence. To select stably silenced clones we used puromycin (SigmaAldrich) 3 µg/µl and 4 µg/µl for MSTO 211-H and REN cells, respectively. The control was generated with the plasmid construct pLKO.1 shRNA scr, a negative control vector containing non-targeting scrambled shRNA. In the text we indicated the MSTO 211-H and REN cells *BAP1* silenced with the LV *BAP1* acronym, while the control is identified with the LV-scr (scramble) abbreviation.

4.3 Adenovirus particles

Recombinant adenoviral vectors were used to introduce Aequorin (AEQ) protein in MPM cells to detect Ca^{2+} flux (see after). Adenoviruses were made available by Paolo Pinton Lab. Signal Transduction - University of Study to Ferrara. The virus is added to target cells, the DNA cargo is delivered into cells where it enters the nucleus and remains as episomal DNA without integration into the host genome. HEK293 cells have once again proved to be an excellent system for the replication of adenoviruses. 48 hours after HEK infection, cells were harvested and pelleted in Tris HCl 0,1 M pH 8,5. Rapid thermal shock allowed viral particles extraction.

We used adenovirus with AEQ gene at a ratio of 1:1000 in MPM cell culture discs, as recommended. We used the cells in the experiments 24 hours after adenovirus infection.

4.4 Cell transfection

We make use of siRNAs to obtain a transient MSTO 211-H cell line silenced for the *BAP1* gene. siRNA o shRNA (short interfering RNA) interferes with the expression of specific genes with complementary nucleotide sequences by degrading mRNA after transcription, preventing translation. We used siRNA mix (Qiagen; 1027416) 25 nM and HiPerFect Transfection Reagent (Qiagen) for *BAP1* transient silencing in MPM cells, following the siRNA protocol by Qiagen. After 48 hours transfection, MSTO cells were checked for silencing of the *BAP1* gene and used for the experiments used siRNA negative (Qiagen) cell transfected as control for RNAi experiments.

Sh RNAs were used to obtain a cell line stably silencing the Ca^{2+} channel IP3R3 in the REN mesothelioma cell line. REN cells at 80% confluence were transfected with 4 μg of pSilencer5.1-shRNA IP3R3 through Lipofectamine™2000 (ThermoScientific) following the manufacturer's instructions. After 48h, the cells were selected with puromycin at a previously optimized concentration of 4 μg/μl. After selection, we obtain IP3R3 stably silenced REN clones, which we labelled as clone 1 and 2 or as REN IP3R3-sil. Clone 2 shows better gene silencing and will therefore be chosen for experiments in which a commercial untargeted shRNA (sh control or sh ctr) is used as a negative control (Life Technologies). Successful silencing following wither siRNA and shRNA transfection assay was analyzed by western blotting and qRT-PCR.

4.5 Protein extraction

From each cell culture plate, adherent cells were washed with cold 1X PBS and harvested by scraping.. To extract total proteins cells were resuspended in cell-lysis buffer (50 mM HEPES pH7.5, 1% Triton X-100, 150 mM NaCl, 5 mM EGTA) containing 1X of 25X Protease Inhibitors Cocktail (P8340, Sigma Aldrich, Saint Louis, MO, USA) and of 100X Phosphatase Inhibitor Cocktail 2 (P5726, Sigma Aldrich). Following 30 min on ice in lysis buffer, lysates were centrifuged at 13,700 rpm for 20 minutes at 4°C and the clear supernatant was collected for subsequent use or stored at −80°C.

4.6 Western blotting and antibody

Bio-Rad protein assay was used to determine protein concentration in each sample and equal protein aliquots (50 μg) were resolved by SDS-PAGE, transferred to nitrocellulose membranes, immuno-blotted with adequate primary antibodies, detected with conjugated secondary antibodies and revealed by enzymatic chemiluminescence reagent (Immobilon ECL Ultra Western HRP Substrate – Millipore WBULS0500) and detected by ImageQuant™ 500. In particular, the following antibodies were used : Anti-*BAP1* (C-4) sc28383 SantaCruz; Purified Mouse Anti-IP3R-3 - DB Biosciences 610312; Anti-KLF5; Anti-GAPDH CellSignaling 2118.

4.7 Growth curve

For growth curve analysis, 1×10^4 cells were plated in 60 mm tissue culture dishes. The cells were counted at 24 hours, corresponding to time zero (t0). Cell count was then performed every 48 hours over a 6-day time span using a Burker chamber. Cell counts were reported as a growth curve over time.

4.8 MTS assay

To assess cell viability, the suitable cell density was identified as 1×10^3 cell/well for MSTO-211H and 1.2×10^3 cell/well for REN in 96-well plates. After 24 hours, 20 μl/well of the MTS solution was added to the 100 μl of RPMI or DMEM cell media, for MSTO 211-H and REN respectively, and cells were incubated for 2 hours. Then the absorbance was detected at 490 nm with a 96-well plate reader (Biorad).

4.9 Clonogenic assay

500 cells/well were seeded in 6-well plates for both MPM cell line. Ten days after plating the colonies were clearly visible. Colonies were fixed with methanol and stained with crystal violet for 20 minutes, washed with PBS (Phosphate-buffered saline) 1X and air-dried. Cell colonies were counted and then photographed.

4.10 Scratch-wound

The scratch wound assay was performed to study cell migration. Cells were grown to maximum confluence in 60 mm plates. 24 hours later, a scratch was made in the cell monolayer using a sterile pipette tip; a gentle wash with sterile 1X PBS was performed to remove non-adherent cells. The width of the scratch, corresponding to the wound margins, was measured (in pixel values) at baseline (t0) and after 24 hours. Wound width was measured as the mean distance between wound edges in 3 random areas. The migration rate was calculated using the formula: migration rate = (D0 −D1) /D0, with D0 and D1 representing the width of the wound respectively at 0 and 24 h.

4.11 Sphere assay

We evaluated the ability of cells to form 3D spheres in RPMI1640 or DMEM supplemented with 20 ng/mL epidermal growth factor (EGF, Invitrogen) and fibroblast growth factor (FGF, BD) respectively, and 20 μg/ml insulin (Sigma) to mimic stem cell medium (Endoh et al. 2019). Cells were seeded at a concentration of 1×10^5 cell/well in 96-wells ultralow attachment plates (Corning Life Sciences, Acton, MA, USA). After a centrifugation at 1300 rpm for 5', cells were cultured in a humidified incubator at 21% O2, 5% CO2 at 37 °C. We evaluated sphere-forming ability by counting the number of ≥50 μm spheres in each well. The cells were cultured for 7-10 days and the images of spheres were captured by microscope (100 X magnification) in at least three random fields.
The diameter (D=(Dmax+Dmin)/2) and volume of the spheres (V=(4/3)*π(D/2)3) were measured with ImageJ64 software.

4.12 Real-time quantitative reverse transcription (qRT)-PCR

qRT-PCR was used for the *BAP1* and stemness markers (OCT4, NANOG, SOX2 and KLF5) mRNA expression analysis. 500 ng of total RNA were retro transcribed using the

iScript cDNA Synthesis kit (Bio-Rad) while cDNA was amplified in the 7900HT Fast Real-Time PCR using the Power Sybr Green Mix (Applied Biosystems). Gene expression was calculated using the $2-\Delta\Delta ct$ method relatively to controls (β-Actin). RT-PCR data are shown as histograms reporting the fold of change of genes mean expression ± relative standard deviation (s.d.), relatively to the control.
Primer sequences are listed in Table 1.

Gene	RTF	RTR
OCT4	5′- CTGGGGGTTCTATTTGGGA - 3′	5′- TGTTGTCAGCTTCCTCCACC - 3′
SOX2	5′- CGCAGCAAACTTCGGG - 3′	5′- GGACCACACCATGAAGGC – 3′
NANOG	5′- GTCTCTCCTCTTCCTTCCTCC - 3′	5′- CAGAAGTGGGTTGTTTGCC - 3′
KLF5	5′- AACTCACAAAACATCCAACCTG - 3′	5′- CAACCAGGGTAATCGCAGTA - 3′
β-Actin	5′- CAGGGCGTGATGGTGGGC - 3′	5′- CTCGGTCAGCAGCACGG - 3′
BAP1	**5′- CAAGGAGGAGGTAGAGAAGAG - 3′**	**5′- CATCGTAGTTGTGGGTCCTT - 3′**

Table 1 | **Primers sequences.** Sequences of qRT-PCR primers used to analyze the mRNA expression of various protein-coding genes. RTF = Real time forward; RTR= Real time reverse.

4.13 Ca^{2+} flux measure – AEQ

Ca^{2+} concentration ($[Ca^{2+}]$) measurements with luminescent proteins AEQ. AEQ are Ca^{2+}-sensitive photoprotein, mutants of Green Fluorescent Protein (GFP) that detect Ca^{2+} in different cellular organelles. AEQ reacts with Ca^{2+} ions through the oxidation of the prosthetic group, called coelenterazine, with emission of light. A special luminescence

reader (aequorinometer or luminescence plate readers) detect the light. Cells grown on 13-mm round glass coverslips (in 24-wells plate) were transduced with AEQ encoded by an adenoviral construct (mitochondria-targeted AEQ, mtAEQ). Adenoviruses were made available by Paolo Pinton Lab. Signal Transduction - University of Study to Ferrara. The coverslips with the cells were incubated with coelenterazine 5μM for 1.5–2 hours, and then transferred to the perfusion chamber. All AEQ measurements were performed in Krebs-Ringer buffer (135mM NaCl, 5mM KCl, 1mM $MgSO_4$, 0.4mM KH_2PO_4, 5.5mM glucose, 20mM HEPES, pH 7.4) supplemented with 1mM $CaCl_2$. The central event is the perturbation of the Ca^{2+} resting conditions, with the generation of changes in mitochondrial [Ca^{2+}]. The perturbing agent is chosen by considering the source of Ca^{2+} (intracellular deposits such as ER o the extracellular medium) and the cell type, because the receptors capable of activating Ca^{2+} waves may differ in type and expression, depending on the cell model. For AEQ assay, we will use the Ca^{2+} -perturbing agents, bradykinin (BK) for REN cells and histamine (HIST) for MSTO-211-H cells. BK and HIST IP3R3 agonists were added to the same medium at a concentration of 1 μM and 100 μM, respectively (as recommended by Paolo Pinton'team). The experiments were terminated by lysing the cells with the Triton X-100 detergent in a hypotonic Ca^{2+}-rich solution (10mM $CaCl_2$ in H_2O), thus discharging the remaining AEQ pool. Light emission induced upon cell lysis is directly proportional to the whole amount of AEQ expression. The light signal was calibrated into [Ca^{2+}] by an algorithm based on the Ca^{2+} response curve of AEQ.

4.14 Statistics

Statistical analyses were performed using the GraphPad Software 5.01. The results were expressed as the mean ± standard deviation (s.d.) from three independent experiments, as indicated. To compare the means of two matched groups, we used paired two-sided Student's t test. P value < 0.05 was considered statistically significant. The other statistical analyses are specifically detailed in each figures legend.

5. Chapter 5 – Results

5.1 *BAP1* silencing reduces mesothelioma cell proliferation

To assess *BAP1* role on mesothelioma tumorigenic features, we silenced its expression on two mesothelioma cell lines of different histotype: the MSTO 211H deriving from a mesothelioma of biphasic histotype and REN deriving from a mesothelioma of epithelial histotype. To stably silence *BAP1* expression, we infected cells with lentiviruses expressing either shRNAs against *BAP1* or a non-targeting scramble sequence as control. Following puromycin selection cells were analyzed for *BAP1* expression levels both at the mRNA and protein levels through real time qRT-PCR and Western blot respectively. *BAP1* expression was indeed effectively silenced (Figure 7a, b). Then we analyzed the effect of *BAP1* silencing on MSTO 211H and REN cell proliferation and viability. We performed a growth curve counting cell number every other day until day 6 after plating. LV *BAP1* silenced MSTO 211-H and REN cells showed reduced proliferation compared with LV scr. Similar results were observed through the analysis of cell viability by MTS assay performed at 24, 48 and 72h after plating (figure 7c, d).

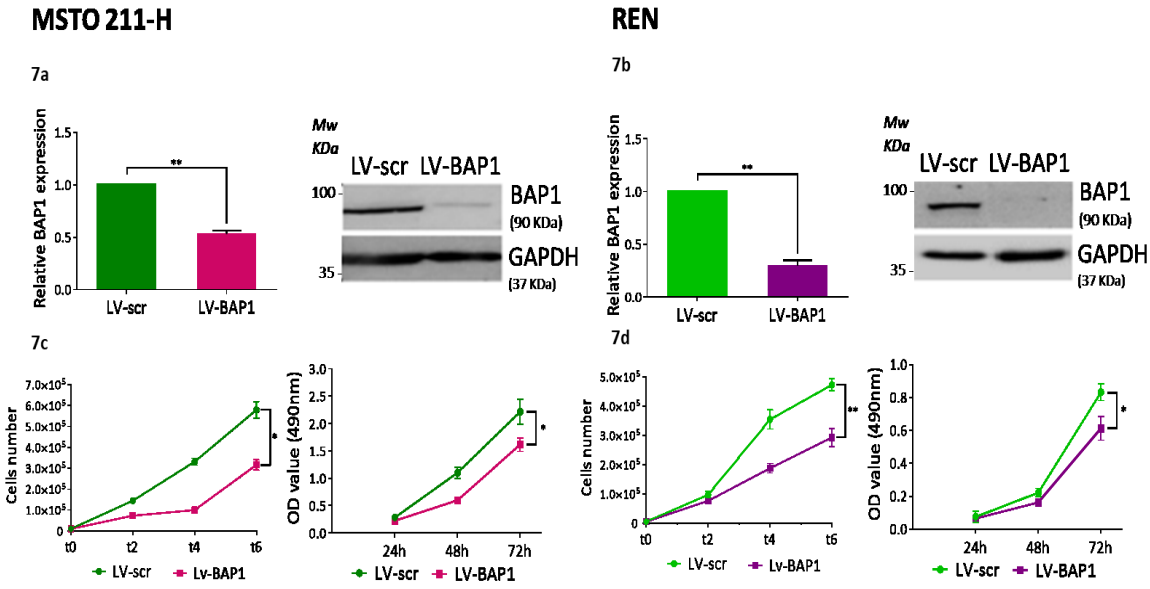

Figure 7 | ***BAP1* silencing in MPM cells affects proliferation and viability.** *BAP1*, silencing in MPM cell lines MSTO211H and REN (a, b), reduces proliferation, as assessed by cell growth analysis and cell viability by MTS assay (c, d). Data are shown as mean ± s.d. P value is calculated using two-tailed unpaired Student's t-tests. *P<0.05; **P<0.01

5.2 *BAP1* silencing reduces mesothelioma cell ability to form colonies and cell migration

Clonogenic assay or colony formation assay is an *in vitro* cell survival assay based on the ability of a single cell to grow into a colony. Clonogenic assay is the method of choice to determine cell long-term proliferation ability. The colony formation capacity of MSTO 211H and REN cells, silenced for *BAP1* expression, is visually reduced than ctr cells, as shown in Figure 8a, b. The colony numbers show a lower percentage of colonies formed in LV *BAP1* cells than in the control cells. Data are reported for both cell lines, MSTO and REN. It is evident, and particularly significant in REN cells, that when *BAP1* expression is decreased, the ability to form colonies from single cells in vitro is almost lost.

The ability of mesothelioma cells to migrate was tested by the scratch test. Similarly to proliferation and viability, also migration is reduced when the *BAP1* gene is silenced in cells. The wound in the assay remains wider and at 24 hours, the migration is slowed down in both silenced MSTO 211-H and REN cells than control cells (Figure 8c, d). MPM cells with lower *BAP1* gene expression migrate less.

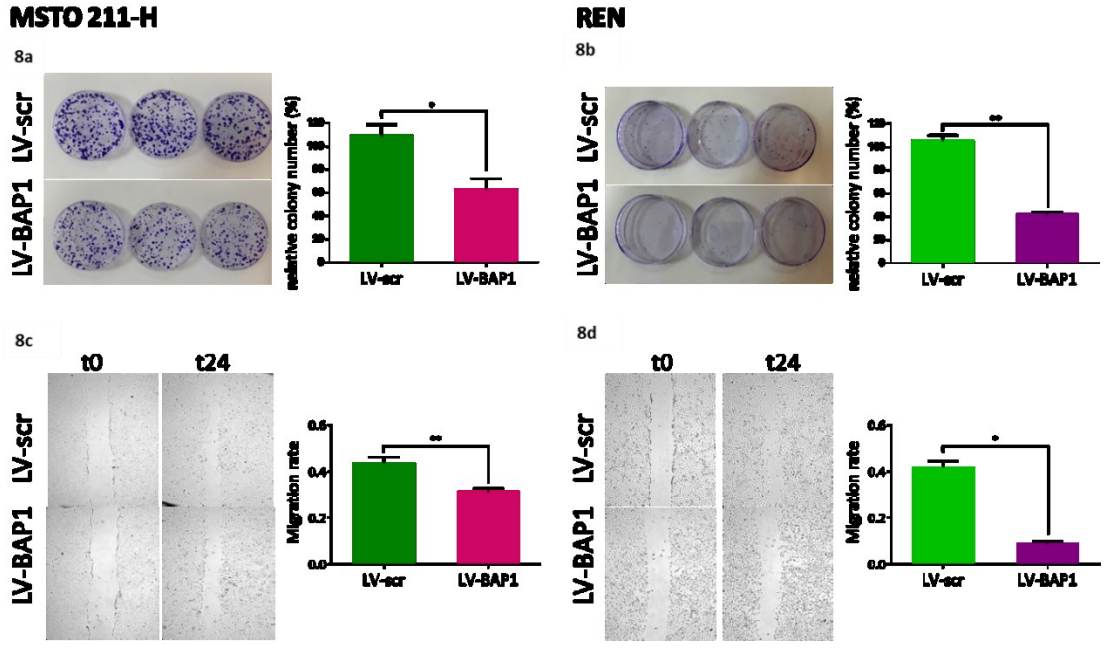

Figure 8 | **Clonogenic and migration assay in MPM cells.** MSTO 211-H and REN LV *BAP1 in vitro* clonogenic assay and relative colony number (a, b). The width of the scratch, between two margins, was measured in pixel values, at t0 and after 24 hours (t24). MPM cells with *BAP1* protein levels reduced show minor migration than LV-scr (c, d). Data are shown as mean±s.d. P value is calculated using two-tailed unpaired Student's t-tests. *P<0.05; **P<0.01.

5.3 *BAP1* silencing affects spheres formation ability in MPM cells

In vitro sphere formation assay is a method commonly used to observe the three-dimensional (3D) cell growth ability and is commonly used to identify CSCs, a sub-population of cells, identified in most tumors, responsible for the initiation, recurrence, metastatic potential, and resistance to therapy of different malignancies. Compared to 2D culturing, 3D sphere formation more closely mimics some aspects of the *in vivo* tumor growth conditions. By this technique, we have observed a lower ability of *BAP1* silenced MPM cells to form spheres. The number of spheres, expressed as a percentage, is reduced to half in both LV *BAP1* REN and MSTO 211-H cells when compared with their respective control cells (Figure 9a, b). Simultaneously with a reduction in the number of spheres, we observed a reduction in the diameter and volume of the spheres in LV *BAP1* REN and MSTO 211-H cells compared with controls. The reduction is very evident and significant for the REN cells.

5.4 Sphere formation is impaired in MPM cells silenced for the type 3 inositol-1,4,5-trisphosphate receptor (IP3R3)

In MCs *BAP1* has been shown to localize at the ER where it binds and deubiquitylates IP3R3 achieving its stabilization and subsequently its modulation of Ca^{2+} release from the endoplasmic reticulum into the cytosol and mitochondria, promoting apoptosis [9]. Therefore, we focused on the most studied Ca^{2+} ER channel isoform, IP3R3, and wondered if a change in IP3R3 levels could affect the ability of mesothelioma cells to form 3D spheres. We stably silenced IP3R3 in REN cells and selected clone 2 in which we achieved the best silencing (Figure 10a). We then assessed the capacity to form 3D spheres of REN IP3R3-sil cl2 compared to the control cells. As shown in Figure 10b, when IP3R3 channel is silenced in REN cells the formation of sphere is completely inhibited, suggesting that when the IP3R3-mediated Ca^{2+} flux from the ER to the mitochondria is impaired in MPM cells, they are unable to grow as 3D spheres when grown on low attachment plates.

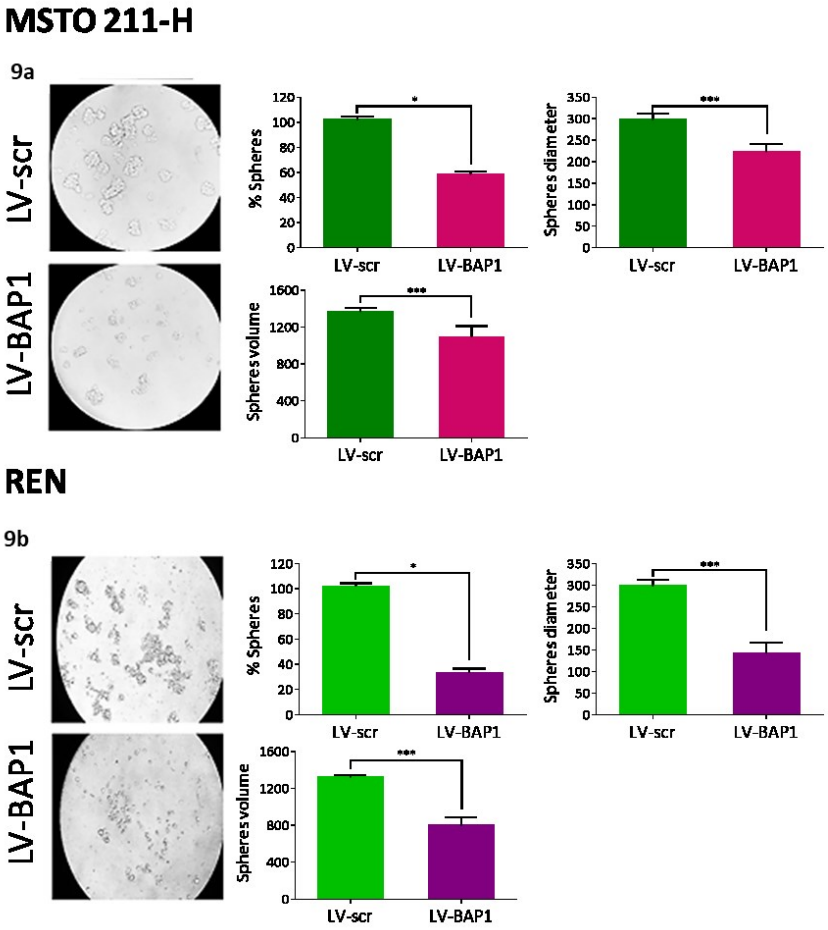

Figure 9 | **Spheres formation by *BAP1* status modulation.** On 48 hours after the start of 3D culture, small tumor sphere formation was observed. Both *BAP1*-silenced MPM lines, in low attachment plates, tend to form smaller and fewer spheres than LV scr. 100x microscope images (a, b). The diameter, volume and percentage number of spheres formed were measured ad show by histograms. Data shown as mean±s.d. P value is calculated using two-tailed unpaired Student's t-tests. *P<0.05; ***P<0.001.

5.5 Ca^{2+} flux in MPM: [Ca^{2+}] mitochondrial level in REN IP3R3-sil clone 2

In the course of the experiments, we found that it was difficult to measure the Ca^{2+} fluxes in the cells treated with LVs. This was probably due to the fact that infections and/or selection may affect the mitochondrial and ER networks thereby affecting the Ca^{2+}-transmission between these cellular organelles. Consistently, by analyzing the morphology of the mitochondria by fluorescence microscopy with selective probes for the mitochondrial

compartment, we observed that they appeared clustered and not in the typical rod shape of a healthy mitochondrial population (data not shown). However, we were able to measure Ca^{2+} flux in clone 2 REN IP3R3-sil, generated following transfection and selection rather than through lentiviral shRNA transduction. Ca^{2+} measurements were made using AEQ, which is a bioluminescent Ca^{2+} probe, by a luminometer. The [Ca^{2+}] in Figures 10c is a function of the mitochondrial Ca^{2+} uptake following the opening of the BK agonist-induced IP3R3 channel and consequent release of Ca^{2+} from the ER. Therefore, for how the experiment takes place, it is a process strictly dependent on the IP3R3 channel.

We recorded mitochondrial [Ca^{2+}] values of 6 and 4.1 μM, for REN ctr and clone 2 respectively (Figure 10c). The low ER-mitochondrial Ca^{2+} flux, and the subsequent lower accumulation in the mitochondrion are due to deficient IP3R3 levels in clone 2, consistently with its previously described role in MCs.

Therefore, low mitochondrial Ca^{2+} uptake following IP3R3 silencing further supports the idea that Ca^{2+}signalling is implicated in sphere formation.

5.6 ER to mitochondria Ca^{2+} flux is reduced in *BAP1 -silenced* MSTO 211-H

The difficulties encountered in measuring the Ca^{2+}levels in LVs-silenced MPM cells made us opt for another silencing technique, albeit in transient. We use *BAP1* siRNAs to obtain MSTO 211-H cells transiently silenced for the *BAP1* gene (Figure 10d). After 48 hours siRNA transfection, mitochondrial Ca^{2+} levels were measured by luminometer (or aequorinometer) and the use of the AEQ probe. In Figure 10e, the [Ca^{2+}] is the consequence of the mitochondrial Ca^{2+} uptake following the opening of the HIST agonist-induced IP3R3 channel and release of Ca^{2+} from the ER. Again, we can argue that the accumulation of Ca^{2+} in the mitochondria is a process strictly dependent on the IP3R3 channel. The mitochondrial [Ca^{2+}] values of 4.9 and 3.7 μM, for MSTO siRNA ctr and *BAP1* respectively (Figure 10e), demonstrate the low ER-mitochondrial IP3R3-dependent Ca^{2+} flux and the consequent lower Ca^{2+} accumulation in the mitochondrion in cells deficient in *BAP1*.

Thus, in 211-H MSTOs, the low mitochondrial Ca^{2+} uptake after *BAP1* silencing supports the hypothesis that Ca^{2+} homeostasis might be implicated in 3D sphere formation.

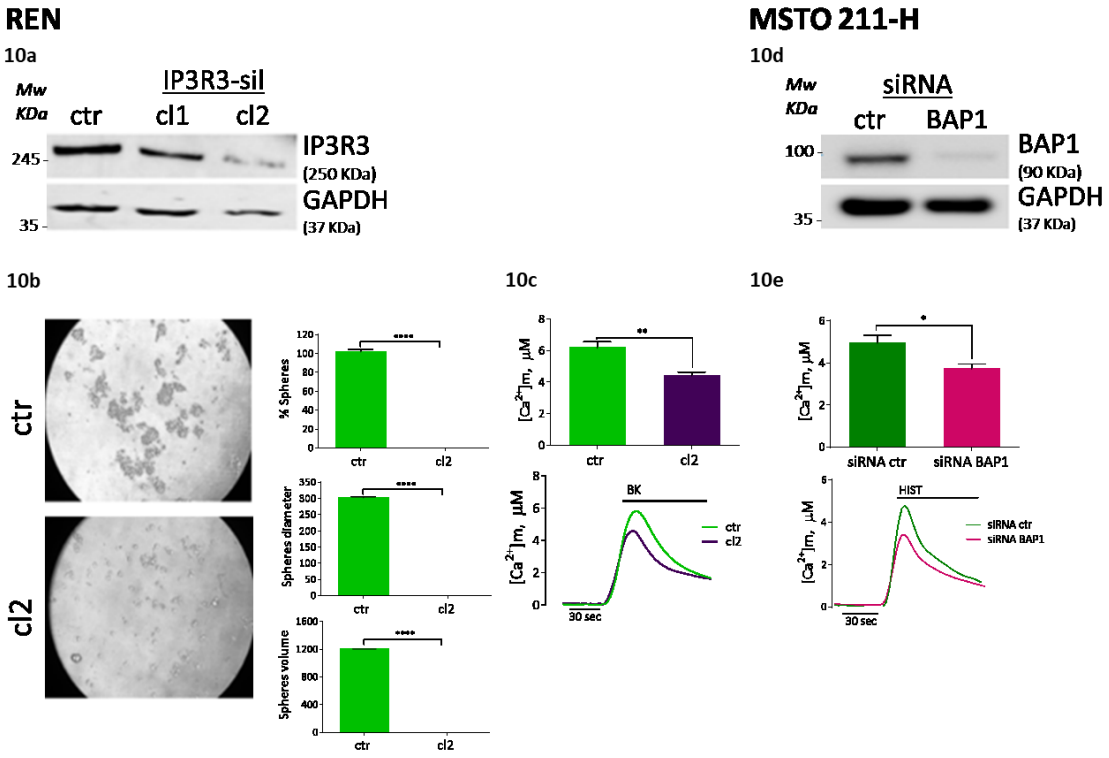

Figure 10 | **Sphere formation in REN IP3R3-sil and mitochondrial [Ca^{2+}] in REN IP3R3sil and MSTO siRNA *BAP1*.** Silencing of the IP3R3 calcium channel in REN cells, as in western blot (a), shows a total inability of the cells to form spheres in 100X image microscopy (b). The lack of spheres is associated with reduced mitochondrial [Ca^{2+}] in REN IP3R3-sil cells (c). REN IP3R3-sil cells stimulated with 1 µM bradykinin (BK) show reduced ER mitochondrial Ca^{2+} concentrations ([Ca^{2+}]m). MSTO 211-H *BAP1* cells silenced by siRNA (d) show reduced [Ca^{2+}]m after 100 µM histamine (HIST) stimulation (e). Data shown as mean±s.d. P value is calculated using two-tailed unpaired Student's t-tests. *P<0.05; **P<0.01; ****p<0.0001.

5.7 Stem cell markers level reduced in MPM

So far, we observed that *BAP1* silencing reduces mesothelioma cell tumorigenic features and their ability to form 3D spheres. Therefore tested the *BAP1* capacity to regulate the mRNA levels of various CSC markers. We set out to assess whether *BAP1* silencing could affect the levels of known CSC markers in MSTO 211-H and REN cells: KLF5, SOX2, OCT4 and NANOG. Upon BAP1 silencing, the mRNA levels drop for all four stem

markers for MSTO 211-H cell line (Figure 11b). In particular, we underline the reduction of KLF5 protein level (Figure 11a, b) in both MPM cell lines. *BAP1* regulates the expression levels of major stem markers in MPM cells in vitro and its reduction, in MSTO 211-H and REN cells, results in being associated with lower stem characteristics than in the control cell.

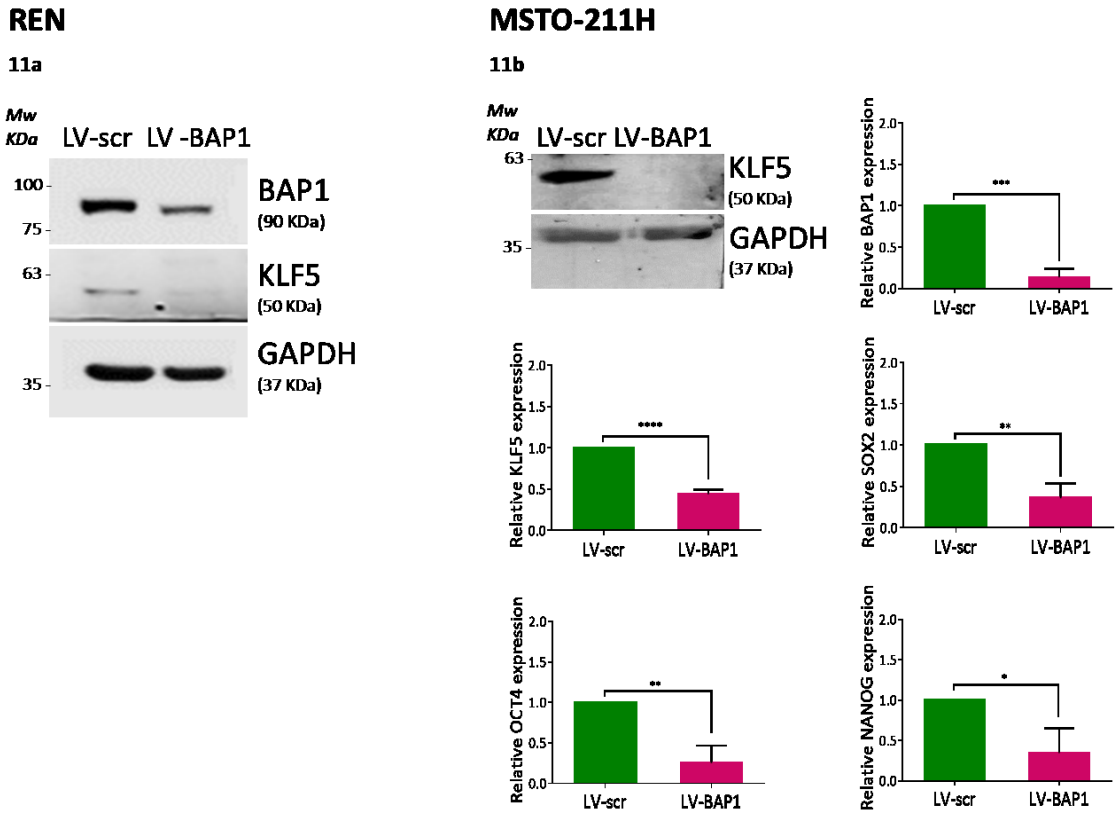

Figure 11| **KLF5 marker modulation by *BAP1*.** Western blot of KLF5 levels in MPM cells (a, b). REN and MSTO 211-H LV-*BAP1* cells reduce KLF5 protein level than LV-scr. Decreased mRNA levels of main stem cell markers in MSTO 211- cells, detected by RT qPCR are showed in b on the right. Data shown as mean±s.d. P value is calculated using two-tailed unpaired Student's t-tests. *P<0.05; **P<0.01;***p<0.001; ****p<0.0001.

Discussion

Here, we tried to identify the molecular mechanisms that could explain why *BAP1* loss is related to a longer survival in MPM patients. Germline and somatic *BAP1* mutation increases survival life months of genetic MPM patients and *BAP1* role to predict prolonged survival in MM patients is widely discussed [1], [10]. The improved survival of MPM patients can be attributed to several factors: the epithelioid histotype, the main subtype of mesothelioma, is less aggressive; through investigations of patients with family histories of mesothelioma and the identification of the mutated *BAP1* gene, the main cause of genetic MM, could lead to an early diagnosis of the disease; MM patients often do not die of mesothelioma but develop further tumors, which can be aggressive, therefore, survival data may not account for this observation. However, while the correlation between *BAP1* loss and longer survival in mesothelioma patients is reported in the literature, the molecular causes interconnecting the two events are still unknown.

To elucidate the inverse relationship between mutated *BAP1* and patients' survival, first we generated a cellular model system with modulated *BAP1* status. To stably transduce MPM cell lines we used LVs technology and obtained MSTO 211-H and REN LV *BAP1* and respective control, LV scr. In this MPM cell lines, the ability of *BAP1* to regulate viability *in vitro* has been tested. *BAP1* establishes contacts with various proteins and itself is part of numerous protein complexes capable of regulating the status of chromatin and orchestrates the transcription of genes involved in fundamental cellular processes, such as proliferation and migration. In numerous studies, *BAP1* role in the regulation of cell cycle and proliferation is reported as more controversial [99], [112]. The role of *BAP1* in tumor progression may be more complex than its presumed tumor suppressor function. Misaghi et al. report that *BAP1* also plays positive roles in cell proliferation and *BAP1* depletion inhibits cell proliferation as does overexpression of a dominant negative mutant of *BAP1* [125]. We report that MPM cells with lower expression of *BAP1* protein proliferate less than negative control. *In vitro*, this result is confirmed by lower capacity of migration and to form colonies by single cell, in both cellular MPM model with *BAP1* downregulated. The scratch test shows a lower ability of *BAP1*-silenced cells to close the wound and when single cells are plated, they show a lower ability to create colonies than control cells.

So far, our viability test experiments (growth curves, migration tests and colony assays) have been performed on a two-dimensional (2D) cell model in which flat monolayer cells

are cultured. 2D culture is still the most commonly used for the research of cell-based assays but it not accurately describes the environment in which cancer cells reside *in vivo*. Three-dimensional (3D) culture systems have ability to mimic tissue-like structures more effectively than monolayer cultures and allow to study the natural characteristics and architectures of cancerous [192]. We test the ability to regulate cell proliferation and growth in the 3D system by generating cell spheres *in vitro*. MSTO 211-H and REN cells are plated on special low attachment plates and the formation of spheres, in LV-scr and LV-*BAP1*, is observed. In the mesothelioma lines studied, the spheres are smaller in number, volume and diameter when the *BAP1* gene is silenced. Therefore, this test of viability also validates that in *BAP1*-silenced MPM lines show slowed growth *in vitro*. These first observations, lead us to conclude that the reduced growth, tested in 2D and 3D systems, could be associated with a lower capacity of the tumor mass to grow and acquire more aggressive characteristics, as spread from an initial or primary site to a different or secondary site. *BAP1* may regulate metastatic traits, as reported in breast cancer and hepatocellular carcinoma cells where *BAP1* promotes proliferation and metastasis [115], [148].

BAP1 role in the cytosolic district recently opened up new areas of research. *BAP1*, generally mutated or lost in genetic mesothelioma, is correlated with low levels of IP3R3 and DUB activity play a principal role in this mechanism [9]. Bononi et al. proposes that the high incidence of tumors carrying mutations in *BAP1* result from the combined nuclear and cytoplasmic activity of *BAP1*. Cytoplasmic *BAP1* can deubiquitylate and stabilize IP3R3, which modulates Ca^{2+} release from ER and enhances apoptosis in fibroblasts from mesothelioma patients tissues [9].

Based on the study mentioned above, our interest focused on the possible *BAP1*-IP3R3 relationship, still poorly documented in mesothelioma. The survey was made possible by the collaboration with Paolo Pinton Lab. Signal Transduction - University of Study to Ferrara. The Ca^{2+} detection assay used consists in introducing into the cells AEQ, a bioluminescent protein capable of binding Ca^{2+} and emitting light following a structural modification (see materials and methods for details). A luminometer captures the light and converts it into Ca^{2+} concentrate ($[Ca^{2+}]$). Here, we report mitochondrial $[Ca^{2+}]$ thanks to the use of a particular AEQ mitochondrial localized (mtAEQ). Mitochondrial $[Ca^{2+}]$ corresponds to mitochondrial Ca^{2+} uptake measured only after inducing Ca^{2+} release from the ER by BK, an agonist that acts upstream of the IP3R3 Ca^{2+} channel receptor. In this context, the observed Ca^{2+} variations detected are strictly connected to the Ca^{2+} release from the ER induced by the IP3R3 receptor activity.

From the first Ca^{2+} measurements experiments we encountered some difficulties. Both cytosolic and mitochondrial [Ca^{2+}] levels detected upon agonist stimulation were particularly low, variable and not quantifiable, thereby avoiding to have reliable comparisons based on *BAP1* status in the MPM lines considered. We associate these difficulties at the lentiviral system used to achieve *BAP1* silencing in MPM cell lines. This observation was found in particular for the MSTO 211-H LV infected. It is possible that lentiviral particles may have damaged the cells' mitochondria, as seen by the poor Ca^{2+} flux from ER to mitochondria and the high levels of mitochondrial markers damage. Furthermore, the morphology of the mitochondria appeared clustered and spherical in shape as opposed to the typical long-linked rod shape of mitochondria in healthy cells. Therefore, LV technique negatively affected our study of Ca^{2+} flux in MPM lines and in the future, we recommend using other silencing systems on which to base Ca^{2+} homeostasis studies. We already plan to use silencing techniques that exploit siRNAs, as reported below for a part of the experiments on Ca^{2+}.

Taking account of these problems and to clarify the relationship between Ca^{2+} and IP3R3 in MPM cells, we created an REN cell line IP3R3-silenced identified as clone 2 or IP3R3-sil REN. This clone was selected from REN cells transfected with an IP3R3 silencing plasmid and subsequently amplified and controlled.

It is well known that IP3Rs are ER channels that control Ca^{2+} release from the ER to the cytoplasm and the mitochondria [173] and in particular, Bononi et al. confirmed the specificity of the *BAP1* interaction with IP3R3. Hence the choice of silencing just subtype-3 of the IP3R Ca^{2+} ER channels. Previously, we discussed how in REN *BAP1* silenced cells, sphere formation is reduced when compared to control REN cells. Similarly, IP3R3 low levels expression in REN cells makes it appear the cells isolated and do not organized to form spheres. The capacity of REN cells is totally inhibited if the IP3R3 gene is silenced. We obtain a similar result, about ability to form spheres, in REN cells both if silenced for *BAP1* and for IP3R3.

Moreover, we associate the inability to form spheres of the REN IP3R3-sil cells at [Ca^{2+}] in mitochondria. We report the [Ca^{2+}] mitochondrial measures when IP3R3 channel is silenced. As expected, REN IP3R3-sil cells show reduced ER-mitochondria Ca^{2+} flux than control cells, evidenced by lower [Ca^{2+}] in mitochondria. In the light of the results obtained, we hypothesize a correlation between *BAP1* and IP3R3 in MPM, as well as in other cancer cellular systems where the nature of the *BAP1*-IP3R3 link is to be found in the DUB activity of *BAP1* [9]. *BAP1* could deubiquitinate and stabilize IP3R3 also in MPM cells. The

reduction of *BAP1* gene expression would lead to a lower stabilization of IP3R3 on the ER with consequent lower Ca^{2+} flux from the ER to the mitochondria, lower Ca^{2+}-related mitochondrial activity, including apoptosis, and overall lower cell viability. In general, the decreased viability of *BAP1*-silenced MPM cells, reported as decreased proliferation and migration, could be attributed to the interaction of *BAP1* with IP3R3.

A possible relationship between *BAP1* and IP3R3 was indirectly demonstrated in REN mesothelioma cell line, still leaving doubts for the other line examined.

As already discussed, the use of LVs invalidated the Ca^{2+} measurement experiments. to overcome this problem and establish a link between *BAP1* and IP3R3 also for MSTO cell lines, we silenced the *BAP1* gene using siRNA constructs. Therefore, we use a small RNA that interferes with the complementary nucleotide sequences of *BAP1* and degrades its mRNA after transcription, such that translation does not occur, ultimately inhibiting *BAP1* gene expression.

MSTO 211-H siRNA *BAP1* cells move less Ca^{2+} from the ER to the mitochondrion than siRNA ctr. This is reported as less mitochondrial Ca^{2+} accumulation when the *BAP1* gene is not expressed in MSTO by siRNA. As for the REN IP3R3sil, also here we can immediately link the lower Ca^{2+} flux ER to mitochondria to the lower presence of spheres in vitro when the *BAP1* gene is silenced in MSTO 211-H lines. The formation of 3D spheres goes hand in hand with mitochondrial Ca^{2+} uptake. We hypothesize a IP3R3 dependence of on *BAP1* in the studied mesothelioma lines for the chain of these events: first, the lower mitochondrial [Ca^{2+}] is dependent on the lower Ca^{2+} flux from the ER; second, the release of Ca^{2+} from the ER mainly depends on the IP3R3 channel in the experiments conducted, since, thirdly, the opening of the IP3R3 channel is mediated by an agonist (BK for REN and HIST for MSTO 211-H).

Other investigations will continue in the future to confirm this correlation and pathway correlated.

Our study continues pointing to the different ability of MPM cells to generate spheres *in vitro*. 3D cellular model is considered as a suitable system to monitor the CSC features [192]. Recent studies have demonstrated that the spherical 3D culture system is a highly efficient method to separate and distinguish CSCs from other tumor cells [193]. One study reported an MPM pattern as a culture model to identify stemness characteristics and differences in response to hypoxia that are more evident in 3D culture-derived cells [192]. Moreover, data indicate that in a MPM cells grown in 3D culturing model exhibit enhanced expression of genes involved in CSC properties.

We proved that *BAP1* silenced MPM cells create fewer spheres than control cells therefore we hypothesize the possible impairment of *BAP1* in regulating stem features in MPM cell lines.

Moreover, we hypothesize that *BAP1*, as a DUB, can act on the post-transcriptional modification of factors involved in the stemness process. In breast cancer, it is already known that *BAP1* regulates KLF5, the major stem cell markers, by deubitiquitination [148]. Therefore, we investigate whether KLF5-*BAP1* regulation could also be involved in MPM and whether *BAP1* affects stemness.

We report KLF5 levels decreased in MSTO-211H and REN cells, with *BAP1* less level *in vitro*. Moreover, we had the possibility to verify the decrease in KLF5 level also with RT qPCR, an analysis then extended to the other major stem markers, reported in the literature as altered in mesothelioma [150]. All the stem markers considered (OCT4, SOX2, NANOG and KLF5) are reduced in MSTO 211-H LV-*BAP1* compared to LV-scr control. We concluded that KLF5, as well as other markers of stemness, may be a direct target of the deubiquitinase activity of *BAP1*, as demonstrated in other tumors. *BAP1*-KLF5 correlation could be the cause of the lower viability of *BAP1*-silenced cells, in terms of proliferation, migration and sphere formation *in vitro*. Therefore, the spheres have less stemness features when *BAP1* is less expressed in MPM cells. MPM cells in reduced expression of the *BAP1* gene condition form fewer spheres, a sign of less stem characteristic. Therefore, *BAP1*-silenced MPM cells could have lower stem characteristics, as established by lower levels of stem markers and lower replicative capacity than MPM cells with normal *BAP1* levels.

We have often said in this work that, according to recent findings, BAP1 regulates IP3R3 levels throughout its deubiquitinase enzymatic activity. However, these results were only achieved in MCs and fibroblasts with BAP1 mutations. The novelty of this work is to analyze the contribution of this molecular axis in the context of mesothelioma by linking the BAP1-IP3R3 axis to stemness capacity by analyzing the contribution of different cancer stem cell markers. Interestingly, it was shown here that by silencing BAP1 it is possible to reduce the mRNA of several cancer stem cell markers. Considering these results, it could be possible that in mesothelioma BAP1 modulates the expression of IP3R3 levels only at the transcriptional level since the relationship between protein levels remains uncertain in mesothelioma cells.

Conclusion and future prospective

To date, the molecular basis of the inverse relationship between mutation/loss of the *BAP1* gene and increased survival in patients with *BAP1*-related genetic MM is unknown. Our work lays the foundations for understanding this twisted and inverse relationship. We show that *BAP1* promotes the formation of 3D spheres *in vitro* and with them the increased expression of stem markers. Moreover, the relationship between *BAP1* and IP3R3, and the related Ca^{2+} homeostasis, could also be valid for MPM cells, as demonstrated in literature for other cell model. The reduction proliferation, migration, clones formation, 3D spheres, stem cell traits, (such as reduced expression levels of the stem cell marker KLF5), in MPM *BAP1* silencing could explain a lower mitochondrial and therefore cellular viability, which confers characteristics of less aggressiveness to tumor cells and could ensure greater survival in MPM patients with reduced expression levels of the *BAP1* gene.

This work could be the first step in understanding the inverse relationship between *BAP1* and survival in MPM patients we will continue with other experiments to establish with certainty how *BAP1* governs cell viability, which could be the cause of better survival in genetic MPM patients with *BAP1* loss or mutations.

In the future, we would like to establish what kind of relationship exists between BAP1 and IP3R3, thus understand whether the deubiquitinase activity of BAP1 acts on IP3R3 also in malignant mesothelioma cells. We design deubiquitinating assays and experiments with cycloheximide. We know that the Ca^{2+} isoform 3 family of IP3Rs channels is the most known to be involved in *BAP1*-related Ca^{2+} flux disturbances in cancer. However, we also intend to evaluate the impairment of the other two isoforms of the ER Ca^{2+} channel family in mesothelioma. We also know that *BAP1* regulates both DNA repair and apoptosis. The balance between DNA damage and cell death is the crossroads that determines the final effect: the more DNA-damaged cells survive, the greater the risk that one of them could turn into a malignant tumor. It is possible that mesothelioma, most frequently associated with BAP1 mutations, derives from tissues in which Ca^{2+}-induced apoptosis plays a critical role in cellular transformation. To this end, we will study the effects of BAP1 deficiency on cell death by apoptosis and autophagy. Given the contribution of *BAP1* to the maintenance of metabolic homeostasis, we will test the possible effect of *BAP1* on MC metabolism by studying glycolysis and oxidative phosphorylation balance and ATP production systems by monitoring basal mitochondrial ATP content and production of mitochondrial ATP as a result of oxidative phosphorylation. Finally, we plan to evaluate

the role of *BAP1* on mitochondrial activities, including ATP production, as mitochondrial structural and functional alterations, resulting from the possible altered mitochondrial Ca^{2+} homeostasis. We will perform some in vivo experiments and experiments in primary samples of human mesothelioma.

Abbreviation list

$[Ca^{2+}]$ - Ca^{2+} concentration

AEQ - Aequorin

AMPK -AMP-activated protein kinase

ASXL1/2 - sex combs-like proteins ½

BAP1 - BRCA1 Associated Protein 1

BCC - basal cell carcinoma

BK - bradykinin

BRCA - BReast cancer

Ca^{2+} - calcium

CADM1cell adhesion molecule 1

CAM - cell adhesion molecules

ccRCC - clear renal cell carcinoma

CDH1 E-cadherin

CDKN2A - cyclin-dependent kinase inhibitor 2A

CM - cutaneous melanoma

CRC - colorectal cancer

CSCs - Cancer stem cells

CTD - C-terminal domain

DNMTs - DNA methyltransferases

DUB - deubiquitinating enzyme

EMT - epithelial-mesenchymal transition

ER - endoplasmic reticulum

ETC - electron transport chain

FGF-BP1 - Fibroblast growth factor-binding protein 1

FOXK1/2 - forkhead box proteins K1/2

GFP - Green Fluorescent Protein

GLUTs - glucose transporters

H2AK119ub1 - monoubiquitination on lysine 119 of histone H2A

H2Aub - H2A monoubiquitination

HBM - host cell factor 1 binding domain

HCF1 - host cell factor 1

HDACi - HDAC inhibitors

HIF - hypoxia-inducible transcription factor

HIST - histamine

HMGB1 - high mobility group protein B1

HR - homologous recombination

HSC - hematopoietic stem cell

IHC - immunohistochemistry

IP3 - inositol 1,4,5-triphosphate

IP3R3 - Inositol 1,4,5-trisphosphate receptor type 3

KLF5 - Krüppel-like Factor 5

LATS - large tumor suppressor

LDHA - lactate dehydrogenase A

LV - Lentiviral Vector

MAMs - mitochondria associated membranes

MBAIT - atypical intradermal tumors with melanocytic BAP1 mutation

MC - Mesothelial cell

MCU - mitochondrial uniporter channel

MDS- myelodysplastic syndrome

miRNA - microRNA

MM - Malignant Mesothelioma

MPM - Malignant pleural Mesothelioma

mtAEQ - mitochondria-targeted AEQ

NF2 - Neurofibromatosis type II

NF-kB nuclear factor-kB

NLS - nuclear localization signal

OCT4 - octamer-binding transcription factor 4

O-GlcNAc - O-linked β-N-acetylglucosamine

OGT - O-linked β-N-acetylglucosamine transferase

PARP1 - Poly(ADP-ribose) polymerase-1

PDK1 - pyruvate dehydrogenase kinase 1

PDL-1 - programmed deathligand 1

PI3K - phosphatidylinositol 3-kinase

PIP2 - phosphatidylinositol 4,5-bisphosphate

PIP2- of phosphatidylinositol 4,5-bisphosphate

PLC - phospholipase C

PRC1 - Polycomb Repressive Complex 1

PR-DUB - Polycomb Repressive DeUBiquitinase

RB -Retinoblastoma tumor suppressor

RNS - nitrogen species

ROS - reactive oxygen species

RRM1 and RRM2 - ribonucleotide reductase regulatory subunit M1 and M2

RTK - Receptor Tyrosine Kinase

SCC - squamous cell carcinoma

SCLC - small cell lung cancer

Scr - scramble

SDC2 - syndecan-2

Sh - short hairpin

SOX2 or SRY – sex determining region Y-box 2

SV40 - Simian virus 40

TCA - tricarboxylic acid cycle

TGF-β - Transforming Growth Factor β 1

TNFA Tumor necrosis factor-alpha

TRAF7 - TNF receptor associated factor 7

Ub – ubiquitin

UCH - carboxyl hydrolase

UVM - uveal melanoma

VDAC - voltage-gated anion channel

wt – wild type

WWP1 - WW Domain Containing E3 Ubiquitin Protein Ligase 1

YAP - Yes-associated protein

YY1 - Yin Yang 1

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