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RESEARCH

# BAP1 Protein is a Progression Factor in Malignant Pleural Mesothelioma

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**Abstract** Human malignant pleural mesothelioma (MPM) is an aggressive cancer due to former asbestos exposure with little knowledge about prognostic factors of outcome and resistance to conventional therapy. BRCA1-associated protein 1 (BAP1) is a tumor suppressor gene that is frequently lost in MPM. Germline mutations of *BAP1* predispose to several different tumors including malignant mesothelioma. Our study aimed to clarify if asbestos exposure has an influence on BAP1 expression and if BAP1 expression could be used as a prognostic factor of outcome. An immunohistochemical staining for BAP1 was performed on 123 MPM tissue samples and the expression levels have been correlated with asbestos exposure and overall survival time. BAP1 expression was not associated with asbestos exposure but we detected a significant effect of BAP1 expression on overall survival time - the higher the BAP1 expression (non-mutated BAP1), the shorter the overall survival. *BAP1* mutation has been linked to non-asbestos induced familial mesotheliomas, which usually belong to the long survivor group and BAP1 is most probably functioning differently than in sporadic cases. Further investigations need to be performed to characterize the BAP1 mutations and to identify the BAP1 downstream targets in MPM.

**Keywords** BRCA1-associated protein1 (BAP1) · Malignant pleural mesothelioma · Asbestos exposure · Overall survival

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## Introduction

Malignant mesothelioma is a primary tumor of the pleura, peritoneum and other serous membranes. The most frequent localization is the pleura followed by peritoneum [1]. The knowledge of prognostic factors for outcome is poor and malignant pleural mesothelioma (MPM) becomes resistant to conventional therapy early on [2].

The incidence is increasing worldwide over the past 30 years with an expected peak in Europe in 2020 – the United States reached its peak incidence in 2004 [3–5]. Mesotheliomas are supposed to represent less than 1 % of all cancers [6] and they are usually diagnosed in middle aged men exposed to asbestos in the workplace [7]. Asbestos exposure is the main risk, but Simian virus 40 infection and inheritance of susceptibility genes seems to play a role. Asbestos causes genetic modifications, and as a consequence, the upregulation of cell survival and growth signaling pathways, as well as the expression of other proteins that favor the resistance of MPM to apoptosis and chemotherapy [8–10]. The long latency period in MPM pathogenesis as well as the absence of early symptoms are responsible for the late diagnosis of the disease [1]. Prognosis for patients is dismal, almost 90 % of patients die of disease within the first 2 years after diagnosis despite therapy [11, 12].

In recent years, the incidence of mesothelioma cases without apparent association to asbestos exposure is rising [13]. Rare cases of familial mesothelioma do occur associated with germ line mutation of *BAP1* (BRCA1 associated protein-1) [14]. BAP1 is a 729 residue, nuclear-localized deubiquitinating enzyme with an ubiquitin carboxy-terminal hydrolase function [15]. It has been suggested to be a tumor suppressor gene with a role in cell proliferation and growth inhibition [16, 17]. *BAP1* is located on chromosome 3p21, a region, which is deleted in several cancers like mesothelioma, cutaneous and uveal melanoma and cancers of the lung and breast [18]. *BAP1* mutations have been identified in several tumors in the last years: It was reported that germline *BAP1* mutations predispose to the development of several distinctive epithelioid melanocytic tumors as well as to cutaneous melanomas [19]. Abdel-Rahman et al. suggested that germline *BAP1* mutations predispose to lung adenocarcinoma, meningioma and other cancers [20]. The results of another study indicated that germline *BAP1* mutations cause a BAP1 cancer syndrome, predominantly characterized by malignant mesothelioma, uveal and cutaneous melanoma as well as atypical melanocytic tumors, which they call "melanocytic *BAP1*-mutated atypical intradermal tumors" (MBAITs) [21].

Testa et al. stated that *BAP1* germline mutations are associated with uveal melanoma and malignant mesothelioma. They suggest that individuals with uveal melanoma who carry germline *BAP1* mutations have a high risk of developing mesothelioma and they hypothesize that when individuals with *BAP1* mutations are exposed to asbestos, mesothelioma predominates [14]. Wiesner et al. reported that peritoneal mesothelioma was diagnosed in a patient who harbored a *BAP1* germline mutation (c.1305delG) and had no known history of asbestos or erionite exposure and that mesotheliomas were inherited in a family over three generations and none of them being exposed to asbestos [22].

Based on these previously published results we aimed to clarify if asbestos exposure has an influence on BAP1 expression and furthermore if BAP1 expression could be used as a prognostic factor of outcome.

## Materials and Methods

### Subjects

A total of 123 human malignant mesothelioma samples were used for the present study. Fifty-seven of these samples were kindly provided by Dr. Thomas Mairinger, Berlin. The patients gave informed consent and the study was approved by the local ethics Committee of the Medical University (No. 24–135). Survival data were available for all 123 patients. In 52 patients exposure data for asbestos fibers were available – 21 of them had a history of asbestos exposure. For major clinicopathological characteristics (gender, histological subtype, treatment) see Table 1.

### Histopathology

All samples were routinely fixed in 4 % neutral-buffered formalin, paraffin-embedded and afterwards dehydrated according to standard protocols. Four μm thick sections of FFPE tissue were deparaffinized with xylol and dehydrated with graded ethanols before Hematoxylin-Eosin (H&E) staining was performed. The stained reference sections were histologically verified and tumor areas with more than 85 % tumor cells were indicated with a pen on the slide and used to locate the tumor on the tissue block for further tissue microarray construction.

### Tissue Microarray (TMA) Construction and Immunohistochemistry

Two tissue microarrays have been constructed: one with the samples from the Medical University of Graz (named "Graz") and another with the samples provided by Dr. Thomas Mairinger (named "Berlin"). For each case, three to five tissue cylinders (depending on the availability of material) with a diameter of 0.6 mm were punched from the marked tumor areas and assembled into a new paraffin block by using a manual instrument (Beecher Instruments Sun Prairie, Wisconsin, USA). Normal adjacent pleura was used for comparison. Four μm thick sections were cut from the TMA block and used for immunohistochemical staining with the BAP1 (C-4)

**Table 1** Patient characteristics; $n = 123$ human malignant mesothelioma samples

| Characteristic | *n* (%) |
|---|---|
| Gender | |
| Male | 89 (72 %) |
| Female | 34 (28 %) |
| Histology | |
| Epithelioid | 108 (88 %) |
| Sarcomatoid | 1 (1 %) |
| Biphasic | 14 (11 %) |
| Treatment | |
| Operation | 123 (100 %) |
| Chemotherapy (cisplatin+pemetrexed) | 39 (32 %) |
| Chemotherapy (carboplatin+pemetrexed) | 20 (16 %) |
| Chemotherapy (platin+pemetrexed) | 5 (4 %) |
| Chemotherapy (pemetrexed) | 4 (3 %) |
| No chemotherapy | 11 (9 %) |
| No data concerning chemotherapy | 44 (36 %) |

antibody (Santa Cruz Biotechnology, Santa Cruz, USA, #sc-28383, 1:100) according to standard methods.

### Analysis of Stained Slides

The stained slides were scanned using a slide scanner (ScanScope AT, Aperio, Vista, USA). Immunohistochemical analysis was carried out by an experienced pathologist (H.H.P.). Protein expression levels were recorded semi-quantitatively and the staining scores were calculated by multiplying the staining intensity (from 0 to 3+, no staining to strong staining) by percentage (0–100 %) of positive cells. To verify the conventional analysis done by a pathologist, the scanned slides were also evaluated and analyzed using the software Tissue Studio® (Definiens, Munich, Germany). Tumor cells were defined by nuclear size, chromatin pattern (granularity) as well as cytoplasmic features. Tumor cells were identified on these features. Staining intensity and percentage of stained tumor cells were evaluated as optical densities. Three groups of tumor cells were created using cut offs by staining intensity scores.

### Statistical Analyses

Statistical tests were performed two-sided. P-values below 0.05 were considered significant. The Wilcoxon-Mann–Whitney test was used for group comparisons. Survival rates were estimated by the Kaplan-Meier method. Cox proportional hazards regression with score criterion was used to test for continuous and categorical risk factors. This procedure is the log-rank test if two groups are compared. In multivariate Cox proportional hazards regression marginal (type-II) likelihood ratio tests were calculated with R-package aov version 2.0-16. The association between Tissue Studio® assessments and IHC score was measured by the Spearman correlation coefficient and the corresponding statistical test. R 2.15.1 (http://www.r-project.org ) was used for calculations.

## Results

### Immunohistochemical Analysis

Sixty percent of the MPM cases used in the present study were not positively stained by the anti-BAP1 antibody. No nuclear staining (see Fig. 1a) indicates epigenetic downregulation of BAP1 or BAP1 mutation-positive tumors whereas specific nuclear staining (see Fig. 1b) is achieved for BAP1 mutation-negative tumor samples. If negative staining is equivalent to real *BAP1* mutations needs to be clarified in a subsequent study. Antibody staining was also seen in all normal mesothelial cells adjacent to the tumor (internal positive control). The immunohistochemical analysis was only carried out by one pathologist and was therefore repeated with the software Tissue Studio® to verify the results.

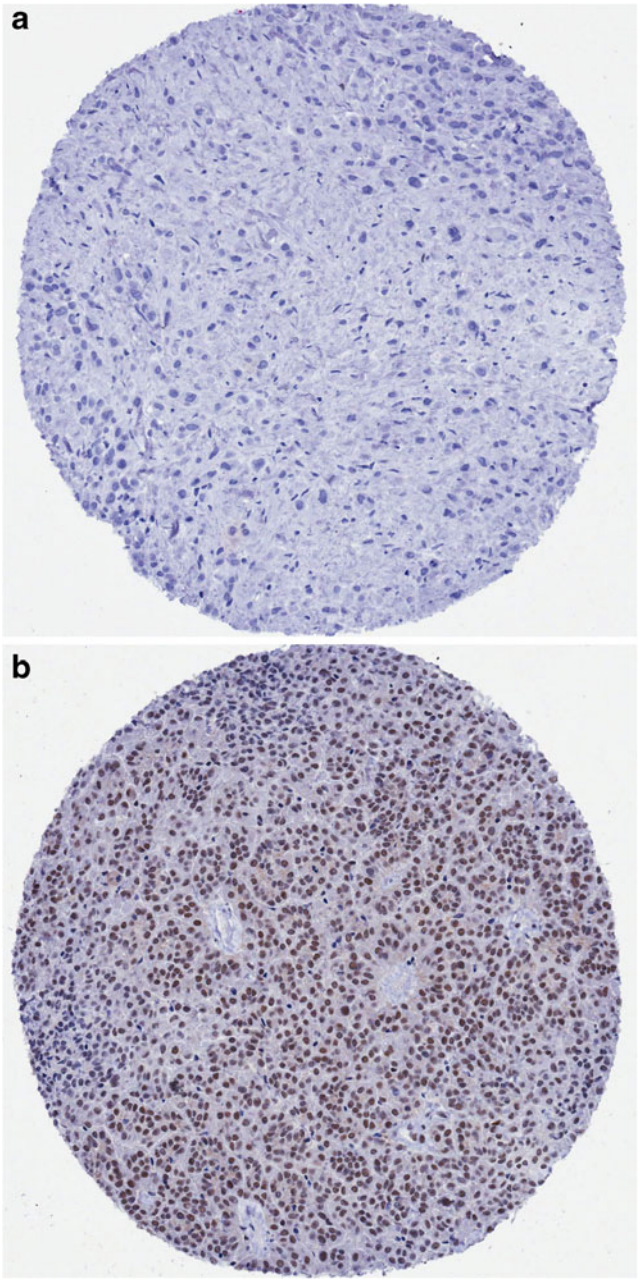


**Fig. 1** Immunohistochemical staining of tissue microarray sections with anti-BAP1 antibody (40× magnification). No nuclear staining was observed among tumor samples with epigenetic downregulation of BAP1 or BAP1 mutation positive samples (**a**) whereas specific, nuclear staining was observed among BAP1 mutation-negative tumor samples (**b**)

Comparison of staining results between manual evaluation and computer-based evaluation using Tissue Studio® showed an excellent correlation (see Fig. 2). Therefore we used the results of the conventional manual analysis further on. However, there are problems to be solved with computer based evaluation. Nuclear features such as size, granularity and shape can show overlaps between tumor cells and reactive

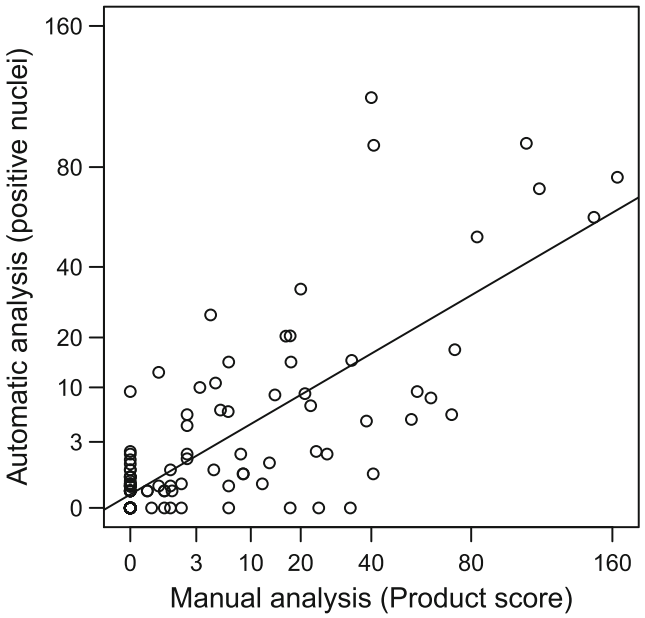


**Fig. 2** Comparison between manual analysis and computer-based analysis of immunohistochemical staining. Staining scores (calculated by multiplying the staining intensity by percentage of positive cells) are shown as results of the manual evaluation and are correlated with the total number of positively stained nuclei detected by the software Tissue Studio®

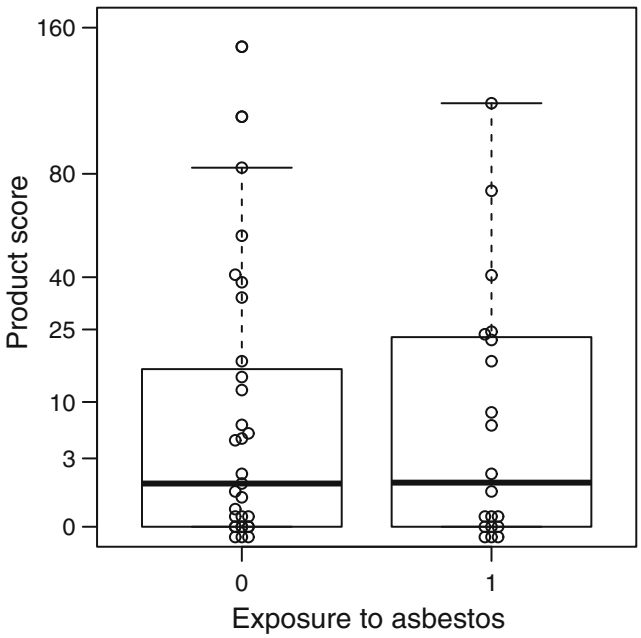


**Fig. 3** Dot plot with overlayed box and whiskers plot for the influence of asbestos exposure on BAP1 expression (product scores of immunohistochemistry). If the patient has been exposed to asbestos or not, has no significant influence on the BAP1 expression ($p = 0.93$)

stroma. This means that the software sometimes includes stroma cells into tumor cell numbers or also excludes tumor cells because of smaller nuclear size. Therefore the learning ability of the software was used to give satisfying results, which will be even more important in experiments using different antibodies.

## Asbestos Exposure and BAP1 Expression

One of the aims of the present study was to clarify if asbestos exposure has an influence on BAP1 expression. Fifty-two samples were used for this analysis and we found that it has no statistically significant effect on the BAP1 expression whether the patient has been exposed to asbestos or not ($p = 0.93$) (see Fig. 3).

## Overall Survival and BAP1 Expression

Due to the number of MPM cases available for the present study, we had to construct two tissue microarrays. They have been analyzed separately with respect to overall survival time (see Fig. 4). No statistically significant difference in survival time was found ($p = 0.57$) between the two tissue microarrays and therefore all samples have been combined for further analyses.

There was a significant effect of BAP1 expression score on overall survival time ($p = 0.0014$, score test of Cox regression). None of the additional clinical risk factors age, sex and subtype was significant. In multivariate Cox proportional hazards regression comprising the risk factors mentioned before only BAP1 expression score was significant ($p = 0.013$, marginal likelihood ratio test). In order to visualize the effect, patients were split into two groups at BAP1 score 50 (see

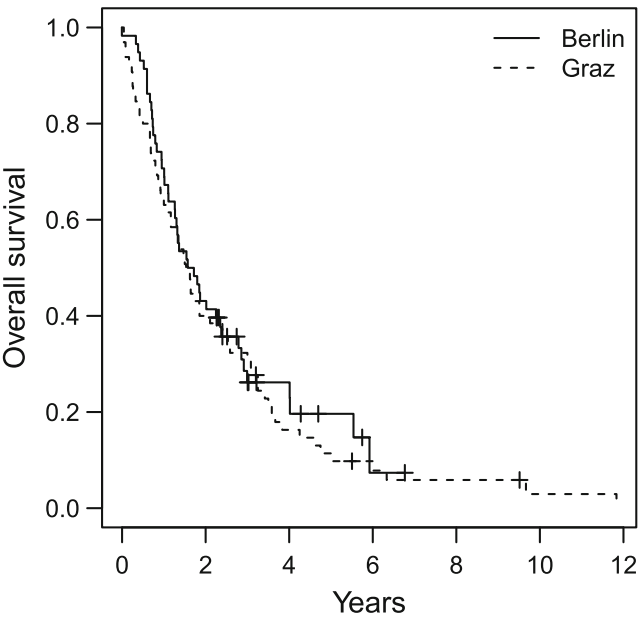


**Fig. 4** Comparison of overall survival time between the two tissue microarrays (Berlin = 57 samples from Berlin, Graz = 66 samples from the Medical University of Graz). There is no statistically significant difference ($p = 0.57$) between the two microarrays concerning the overall survival time. Therefore all samples have been combined for further analyses

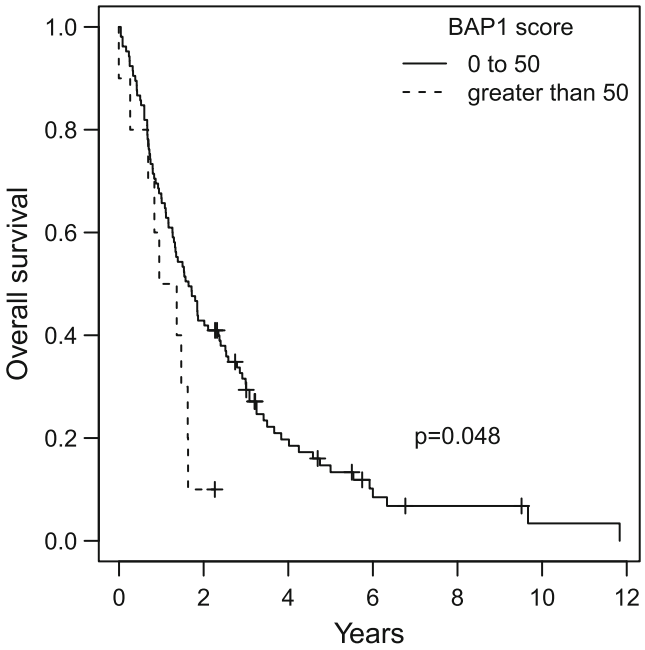


**Fig. 5** Dependence of overall survival time on BAP1 expression. Mortality is lower at higher BAP1 expression ($p=0.0014$). In order to visualize this association, survival curves for high and low BAP values were shown separately. The corresponding log rank test yielded $p=0.048$

Fig. 5). We have chosen this cutoff to make sure that only samples with an intense staining and an average or high percentage of stained cells are assessed as positive. Survival times differed significantly between the two groups ($p=0.048$). The higher the BAP1 expression and therefore the expression of non-mutated BAP1, the shorter the overall survival.

## Discussion

Malignant pleural mesothelioma is an aggressive tumor that is resistant to current therapies [23] and due to its anatomical localization, it is a tumor in which early diagnosis is difficult and therefore MPM is often diagnosed in advanced stages when patients have a median survival of 6–12 months. But when patients are diagnosed at Stage IA, survival time of five or more years is not uncommon [21, 24]. There is an urgent need to know more about prognostic factors of outcome and driving forces, because of an increase of MPM worldwide [2–5]. In recent years, *BAP1* has been identified to be mutated in a wide variety of human tumors and it was stated that these germline mutations predispose to e.g. melanocytic tumors, meningioma, lung adenocarcinoma and malignant mesothelioma [14, 19, 20]. The existence of a *BAP1*-related cancer syndrome was reported, which is characterized by mesothelioma, uveal melanoma and possibly other cancer types [14]. Wiesner et al. identified 19 *BAP1* germline mutation carriers in the three families they were investigating and they state that these mutation carriers are predisposed to the development of melanocytic skin lesions, uveal and cutaneous melanoma and mesothelioma with varying degrees of penetrance—only two patients with *BAP1* mutation were diagnosed with pleural mesothelioma [22]. It was reported that around 40 % of MPM patients have a *BAP1* loss, a *BAP1* mutation or both and 32 different *BAP1* mutations have been identified so far [25–27]. Genomic alterations are seen frequently in MPM and *BAP1* gene inactivation occurs at very high frequency in patients with malignant epithelioid mesothelioma and this fact could also be useful for diagnosis [27]. It was also hypothesized that in patients who have a *BAP1* mutation and who have been exposed to asbestos, malignant mesothelioma predominates even though *BAP1* mutation alone may be sufficient to cause mesothelioma [14].

While most of the published studies were dealing with the identification of specific mutations of the *BAP1* gene in MPM and other tumors and the consequences of mutant-BAP1 expression, our study was focused on the impact of asbestos exposure on BAP1 expression and BAP1 as a possible prognostic factor of outcome. Most of the MPM cases used for the present study were not positively stained by the anti-BAP1 antibody meaning that these cases most likely have a *BAP1* mutation. If this is equivalent to real *BAP1* mutations or caused by epigenetic downregulation of BAP1 still needs to be clarified in a subsequent study.

Asbestos exposure has no statistically significant effect on BAP1 expression in our 52 samples. This indicates that asbestos exposure is not responsible for *BAP1* mutations or BAP1 downregulation in MPM. Our results concur with previously published data which also did not detect a correlation between *BAP1* mutation and asbestos exposure [25].

Several groups described the role of BAP1 in cell cycle progression and its function as a tumor suppressor [15, 17, 28–30]. Growth suppression in vitro and in vivo was achieved when cells—normally expressing mutant BAP1—were re-expressing wild-type BAP1 [15, 17]. Therefore we expected that the expression of non-mutated BAP1 correlates with an increased overall survival and that mutations of this tumor suppressor gene lead to an earlier death. Surprisingly high BAP1 expression detected by immunohistochemistry correlates with shorter overall survival. That means that non-mutated *BAP1* resulting in BAP1 protein synthesis causes earlier death and therefore might cooperate with proteins causing progression and dismal outcome. In another study it was shown that there is no significant correlation between *BAP1* mutation and variables such as sex, histologic subtype and overall survival but a significant correlation of *BAP1* mutation and age was found—a mean age of 66.7 years in mutant *BAP1* compared to 58.6 years in wild-type *BAP1* [25]. These results are not confirmed by our study since we found a statistically significant correlation between BAP1 expression

and overall survival and patients with wild-type BAP1 expression die earlier than those with no BAP1 expression, which indicates possible mutations in the *BAP1* gene. In previous studies only small numbers of patients have been analyzed, whereas our study presents the largest cohort investigated so far.

*BAP1* mutations were found in familial cases of MPM with no history of asbestos exposure. All of them were long-term survivors and showed negative immunohistochemical staining for BAP1 [14, 22]. This fits to our data of improved survival in BAP1 negative MPM.

Only a few mutations common in malignant mesothelioma have been identified so far. Two frequent genetic alterations have been described: the most common are homozygous 9p21 deletions centered on *CDKN2A* found in up to 72 % of tumors and 80 % of MPM cell lines [31]. This deletion is predominantly found in short-term survivors and not in long-term survivors [32]. *NF2* loss through monosomy 22 or 22q deletions was described as another common event [33]. While several studies show that *BAP1* mutations frequently occur and even predispose to malignant mesothelioma [14, 21, 25–27], one group described that there are no significant associations between *CDKN2A* loss and loss or mutation of *BAP1* or *NF2* which indicates that these three genetic events happen independently [25].

To understand the cellular roles of BAP1, several groups have examined the proteins bound by BAP1 and possibly deubiquitinated by BAP1. Proteins identified include HCF1, ASXL1, ASXL2, FOXK1, FOXK2, ANKRD17, HAT1, UBE2O and AOF1 [34–36]. BAP1 nuclear deubiquitinase seems to be involved in maintaining an appropriate level of regulatory ubiquitination of target histones, the HCF1 transcriptional co-factor and maybe other transcriptional proteins [25, 29, 30]. It has been reported that BAP1 binds to and deubiquitinates HCF1 in several cell lines and even in MPM cell lines [25, 34, 35] and that common BAP1 inactivation causes transcriptional deregulation in the pathogenesis of this highly lethal cancer [25]. Previous studies have shown that BAP1-HCF1 interaction may be important for HCF1-mediated growth effects, in doing so HCF1 acts through modulation of transcription at E2F-responsive promoters [24]. Another research group looked specifically at the downstream targets of E2F—like Cyclin A2, E2F1, p107 and CDC25C—after BAP1 knockdown in MPM cell lines and they found that all the effectors were downregulated. These results go along with the possibility that BAP1 loss leads to post-translational inactivation of HCF1 and therefore downregulation of downstream E2F-responsive genes important for cell cycle progression [25].

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