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Expression and Function of Modulator of Apoptosis (MOAP-1) in Blood Cancers

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Running title: MOAP-1 in blood cancers

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ABSTRACT

Background: The function of the Ras association domain family 1 (RASSF1)/modulator of apoptosis 1 (MOAP-1) molecular tumor suppressor pathway is often perturbed in many solid and blood cancers by epigenetic loss of RASSF1A via promoter specific methylation. However, a detailed analysis of expression and stability of MOAP-1 as well as effect on cellular proliferation and cell death in blood cancers has not been explored.

Methods: Expression of MOAP-1 RASSF1A was performed by immunoblotting analysis, MOAP-1 effect on biology determined by cell proliferation, cell death and tumorigenicity assays. Lastly, proteomic analysis in MOAP-1 in form 1 and 2 expressing cells reveal new interacting partners to MOAP-1.

Results: The expression of MOAP-1 appears to be quite varied and exists as two forms, a p39 and p46 form in blood cancers. The higher MOAP-1 p46 form was mainly observed in acute myeloid leukemia (AML) and some acute lymphocytic leukemia (ALL) patients and cell lines. Furthermore, MOAP-1 p46 form can be reduced to the p39 form upon calf intestinal or lambda phosphatase treatment, suggesting post-translational phosphorylation likely produces the slower migrating form. Blood cancer cell lines containing the p46 form of MOAP-1 appears to be more resistant to cell death signals and have increased growth. In addition, we document *in vivo* protection from tumorigenesis of cells containing MOAP-1 following tail vein injection of cancer cells. Lastly, proteomic analysis revealed several interesting components of the MOAP-1 interactome in blood cancer cells that we validated.

Conclusions: MOAP-1 is a *bona fide* tumor suppressor in blood cancers with functions beyond apoptosis.

Keywords: MOAP-1, blood cancer, apoptosis, DNA damage, RASSF1A, tumor suppressor protein

Background

The Ras association domain family 1 (RASSF1)/modulator of apoptosis 1 (MOAP-1) molecular pathway has been demonstrated to modulate several aspects of homeostasis ranging from cell cycle control to inflammation [1, 2]. Numerous publications have solidified the role for RASSF1A/MOAP-1 tumor suppressor protein pathway in the solid cancers [3]. Others have documented detailed roles for MOAP-1 in liver homeostasis [4] and in ovarian cancer resistance to cisplatin [5]. In blood cancers, it is known that RASSF1A is epigenetically silenced in Hodgkin's lymphoma [1, 6] and *Rassf1a*^{-/-} mice have increased tumor incidence in the breast, lung and immune system by 12-16 months of age including B-cell related lymphomas to suggest importance in B and T cell signaling [7, 8]. RASSF1A can carry out its tumor suppressor properties by regulation of the anaphase promoting complex/cyclosome (APC/C)-cdc20 complex, the degradation of A and B cyclins [9-11] and regulation of proper sister chromatid segregation at the metaphase plate to avoid inheritable aneuploidy and unwanted malignancy.

The phenotype of *Moap-1*^{-/-} whole body knockout mice was reported by Tan et al. (2016) [4] and was characterized by a defect in Fas ligand induced cell death resulting in the survival of the mice and with no liver damage [4]. MOAP-1 is thus a robust trigger of death receptor induced

cell death in the liver upon Fas/Fas ligand engagement to promote liver damage. Mitochondrial linkage of MOAP-1 was critical for this observation and Tan et al. (2017) [4] identified several mitochondrial proteins that function with MOAP-1 to modulate liver apoptosis. MOAP-1 is a highly regulated protein with numerous ubiquitin-associated pathways regulating the stability of MOAP-1 [5, 12, 13] that ultimately govern the involvement in cellular homeostasis. We have also recently demonstrated how the scaffolding protein, receptor of activated protein C kinase (RACK)1, can aid in the regulation of MOAP-1 in solid cancers and that should also hold true in blood cancers. In this study, we detailed the expression status of MOAP-1 in blood cancers, demonstrated how it can inhibit metastatic expansion of blood cancer cells and carried out proteomic analysis of the MOAP-1 interactome in various blood cancer cell lines.

Methods

Antibodies

Antibodies were purchased from the following vendors: rabbit anti-MOAP-1 (QED Biosciences, #11009), rabbit anti-Erk1/2 (sc-292838), rabbit anti-ubiquitin (sc-47721), anti-tubulin (NEB#2125S), anti-GAPDH (NEB#2118), Anti-DDB1 (Pierce PA5-29884); anti-RAD50 (Pierce PA5-21282) and TKT (sc-390179). Mouse anti-RASSF1A (CLONE C10) was obtained in house from hybridoma cells obtained by Dr. Shairaz Baksh.

Cell culture and transfection

All cells were grown at 37°C and 5% CO₂ in RPMI containing 10% bovine growth serum (BGS) and 1% solution of 50 U/ml penicillin and 50 µg/ml streptomycin. BGS was purchased from ThermoFisher Scientific (USA). All leukemia cell lines were kindly obtained from Dr. Narendran

(University of Calgary, Edmonton, Canada) and all lymphoma cell lines were obtained from Dr. Luc Berthume (University of Alberta, Edmonton, Canada).

Cell lysis, immunoprecipitation and immunofluorescence

Cells were lysed in SB lysis buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM MgCl₂, 1.5 mM EDTA, 0.5% Triton X-100, 20 mM β -glycerolphosphate, 5 mM iodoacetamide, 100 mM NaF, 0.1 mM PMSF and immunoprecipitated (IP) as previously described [14] using protein G agarose beads. Protein complexes were resolved by SDS-PAGE and transferred to PVDF membrane for immunoblotting.

Mass Spectrometry Analysis

Cells were lysed in SB lysis buffer and immunoprecipitated with rabbit anti-MOAP-1 (QED antibody) and absorbed onto Protein A sepharose beads as previously described [14]. Cell lysates were resolved by SDS-PAGE, incubated in blue-Silver coomassie staining solution (0.67 M ammonium sulphate, 1.4 mM Coomassie Blue G-250, 10% phosphoric acid and 20% methanol) overnight with shaking and rinsed in water until clear. Protein bands of interest were excised and sent for in-gel digestion and protein identification by liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis at the Alberta Proteomics and Mass Spectrometry Facility (University of Alberta). For most of these protein IDs, we were able obtain about 10 - 20% peptide coverage to identify the band. Bands were selected based on uniqueness in the MOAP-1 immunoprecipitation lane.

108

109 **Phosphatase assays**

110 To evaluate the phosphorylation status of MOAP-1, we carried out phosphatase treatments with
111 both calf intestinal phosphatase (CIP) and lambda phosphatase according to manufacturer's
112 conditions. In short, CIP treatment was performed as follows; 50 µg of protein lysate from KG1
113 or SEM cells was added to NEB Buffer 3.1 containing 50 U of CIP in a 100 µl volume and
114 incubated at 37 °C for 4 hours. For lambda phosphatase, 50 µg of protein lysate from KG1 or SEM
115 cells was added to lambda phosphatase buffer (50 mM HEPES, pH 7.5, 100 mM NaCl, 2 mM
116 DTT, 5 mM MnCl₂) containing 50 U of CIP in a 100 µl volume and incubated at 37 °C for 4 hours.
117 For the combination of both phosphatases, NEB buffer 3.1 and 50 U of each was utilized for
118 enzymatic cleavage at 37 °C for 4 hours. Following cleavage, protein loading dye was added to
119 1X and proteins separated by SDS-PAGE, transferred to PVDF and immunoblotted as indicated.

120

121 **Use of animals**

122 All methods involving animals were performed in accordance with the relevant guidelines and
123 regulations approved by the University of Alberta Animal Care and Use Committee under protocol
124 #AUP000218. The *Moap-1*^{-/-} animals were a generous gift of Dr. Victor C. Yu, National University
125 of Singapore, Singapore.

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127

128

129 **Xenograph Assay**

130 THP-1 cells were transfected with CFP or CFP-MOAP-1 using the Amaxa machine as per
131 manufacturer's instructions. Stables were established and 2×10^6 cells were injected into male
132 nude mice (NCRNUMM, CRTAC:NCRFOXN1NU, from Taconic Biosciences) via tail vein
133 injection. Tumors were formed at the base of the neck region and in the inner lining of the colon
134 after 2 months and were excised at a size of < 2 cm. THP-1-CFP and THP-1-CFP-MOAP-1 cells
135 were injected into 3 mice each for this experiment.

136 **Statistical Analysis**

137 All experiments were carried out at least three times and statistical analysis was carried out using
138 Student's T test analysis.

139

140 **Results**

141 *Blood cancer cells have two forms of MOAP-1.* We previously demonstrated in various solid
142 cancers and in a limited amount of blood cancers that MOAP-1 can exist as two forms based on
143 the phosphorylation status – a p46 and p39 kDa form. It appears that form 1 (the cDNA predicted
144 39 kDa form) exists in all the solid cancers whereas form 2 (the slower migrating 46 kDa form) of
145 MOAP-1 is in AML and some ALL cells and patients. Form 1 also appears in most other categories
146 of leukemia/lymphoma (Fig. 1a). There does not appear to be a difference in the mRNA levels for
147 MOAP-1 in all leukemia/lymphoma cells lines explored indicating that the modified form 2 of

MOAP-1 is due to a post-translational event (data not shown). Interestingly, we can detect the ubiquitination status of MOAP-1 in most of the leukemia/lymphoma cell lines tested and it appears that MOAP-1 may be ubiquitinated mainly in the G0/G1 phases with some detection in G2/S and to a lesser extent in the M phase of the cell cycle (Fig. 1b). Furthermore, the p46 kDa form can be partially reduced to the p39 kDa form upon calf intestinal phosphatase (CIP) or lambda phosphatase treatment suggesting the involvement of phosphorylation contributing to the generation of the p46 kDa form (Fig. 1c). Because we can only observe a partial loss of phosphorylated MOAP-1 upon CIP or lambda phosphatase treatment, further testing with other phosphatases to induce complete loss and/or help in determining the modifications required to generate the presence of the p46 form. It is conceivable also that there exists a mixed population of a phosphorylated species of MOAP-1 combined with a monoubiquitinated form (Ub is 7 kDa).

Stability of MOAP-1 can be restored with MG132 Treatment. MOAP-1 is a highly regulated protein in solid cancers that is controlled by protein dependent ubiquitination involving TRAF2 [15], UBR5 [5], anaphase-promoting complex (APC/C[Cdh1]) [12] or TRIM39 [13]. These various E3 ligases ubiquitinate MOAP-1 in different cellular settings. However, to stabilize and detect MOAP-1, the proteasome inhibitor, MG132, can be applied over a 24 hour time period as show in Fig. 1d. It is quite clear that in cell lines with low MOAP-1 detection, such as THP-1 cells, MG132 can restore MOAP-1 proteins level to detectable levels (Fig. 1d, top panel). Conversely, if cells that have detectable expression of MOAP-1, such as J77 Jurkat cells, are treated with cyclohexamide (to prevent new MOAP-1 protein production) a rapid loss of MOAP-1 is observed within 1.5 hours as seen in Fig. 1d, bottom panel. This is comparable to what is observed in

epithelial cells and therefore by extension in most solid cancers [13]. Thus, in blood cancer cells, MOAP-1 is regulated in a similar manner to that observed in solid cancers.

Correlation of Cell Proliferation with MOAP-1 Expression. MOAP-1 is a robust driver of apoptosis via death receptors and intrinsic mitochondrial activation. We explored the difference in the growth rates of cells with detectable MOAP-1, with low to no detectable levels of MOAP-1 and those with the modified form 2 of MOAP-1. Surprisingly, cells lines with form 2 of MOAP-1 had similar growth rates to cell lines with no detectable MOAP-1 with both having a hyperproliferative phenotype (Fig. 2a). Cells with form 1 of MOAP-1 had relatively normal growth rates. Since we can detect robust expression of MOAP-1 mRNA in all cell lines, the context of “no detection” really refers to a higher turnover of MOAP-1 in those particular cells that may be attributed to a higher activity or expression of the E3 ligase regulating its stability. Interestingly, cells with form 2 of MOAP-1 or no detectable MOAP-1 have endogenous RASSF1A (Fig. 2c, AML and ALL) to suggest that, although normal levels of RASSF1A are found, the presence of form 1 MOAP-1 is needed for a functional growth controlled RASSF1A/MOAP-1 pathway. In most lymphomas (that generally have hyperproliferation such as Hodgkin’s lymphoma cell lines L428 and KMH2), RASSF1A expression is epigenetically lost whereas these cell lines retain expression of MOAP-1 (as form 1) (Fig. 1a and Fig. 2c). This suggests that form 1 of MOAP-1 does not compensate for the loss of RASSF1A in Hodgkin’s lymphoma. However, the BL2 and RAMOS Burkitt’s lymphoma cell lines appear to display normal proliferative rates despite the loss of RASSF1A and retention of MOAP-1 form 1 (Fig. 2a). Thus, the role of MOAP-1 in lymphomas appear rather complex. This could be attributed to the functions/activities of the interacting proteins found in Fig. 3 and 4 that may govern the biological outcome of MOAP-1 in different cellular settings. In support of these findings, splenocyte counts from *Moap-1*^{-/-} mice at 3 months

of age had > 2-fold higher cellularity when compared to wild type mice to suggest importance in regulating the growth rate of immune cells (Fig. 2b).

Analysis of Pro-apoptotic Characteristics of Blood Cancer Cells. It was recently published that cells from *Moap-1*^{-/-} mice have defective cell death properties [4], confirming its importance in apoptotic pathways. As such, cells missing MOAP-1 or having form 2 of MOAP-1 have a lack of activation of cell death as followed by poly ADP ribose polymerase (PARP) cleavage (Fig. 2d). Our data raises an intriguing question regarding the importance of form 2 MOAP-1 in AML or ALL. If indeed it is the unique phosphorylated form of MOAP-1, what is driving this phosphorylation in AML or ALL? We are currently addressing some of these questions.

MOAP-1 protects against metastatic dissemination in blood cancer cells. By definition, a tumor suppressor protein is expected to suppress hyperproliferative growth and/or prevent metastatic spread of cancer cells. We explored this concept using CFP loaded cells injected into the tail vein of nude mice that normally will not reject the cells. Once present in the animal, cells will spread to secondary lymph nodes leading to discomfort (Fig. 2e). THP-1 cells were selected for this analysis as a cell line with a lack of detectable expression due to high MOAP-1 protein turnover and ease of transfection via electroporation using an Amaxa machine. Once in the animal, these cells migrated to the lymph nodes in the base of the neck area and, surprisingly, to the colon to form colonic nodules as seen in Fig. 2f. On average, cells containing CFP MOAP-1 produced about 16 nodules in the colon while cells containing CFP-MOAP-1 produced 4 nodules. 4. Thus, MOAP-1 can interfere with the metastatic nature of cells and most likely by inducing cell death.

214

215 *MOAP-1 can associate with proteins involved in cell death, DNA damage control and metabolism.*

216 The majority of MOAP-1 interacting proteins to date are involved in cell death, ubiquitination,
217 mitochondrial biology and microtubule stability [2]. Using MOAP-1 antibody
218 immunoprecipitation, we explored MOAP-1 associated proteins in cells containing robust
219 expression of MOAP-1 (such as J77 Jurkat cells), cells containing form 2 of MOAP-1 (SEM and
220 KG1) and a lymphoma cell line (BL2) containing form 1 of MOAP-1. Unique bands to each cell
221 line (compared to a control IgG) were excised and identified by mass spectrometry (Fig. 3). In J77
222 Jurkat cells, we observed associations with DNA damage control proteins, cell death proteins and
223 a metabolic protein associated with a role in aerobic glycolysis and the Warburg effect. In the SEM
224 cell line, an RNA binding protein interleukin enhancer-binding factor 3 was identified to be a
225 MOAP-1 associated protein in addition to TKT, a protein associated with aerobic glycolysis and
226 the Warburg effect. In BL2, we identified two more protein interactions linked to DNA damage
227 control/repair and cell death [DNA repair protein RAD50 (RAD50) and DNA damage-binding
228 protein 1 (DDB1)], respectively. These associations are in line with the current molecular role for
229 MOAP-1 and the RASSF1A/MOAP-1 tumor suppressor family of proteins.

230 We validated the association of MOAP-1 with DDB1, Rad50 and TKT in HCT-116 cells
231 due to ease of transfection and to avoid form 1/form 2 confusion for association (a question beyond
232 the scope of this article). As can be observed in Fig. 4, DDB1 appears to associate with MOAP-1
233 in an inducible DNA damage dependent manner, whereas RAD50 associated with MOAP-1 in a
234 consistent constitutive manner. This may indicate a housekeeping role for MOAP-1 in somehow
235 modulating the functions of RAD50. TKT, a metabolic enzyme, robustly associated with MOAP-

1 upon serum deprivation and could indicated a function for MOAP-1 under low nutrient conditions.

Discussion

In this study, we explored the role for MOAP-1 in blood cancers and the molecular players utilized to modulate the growth rate of blood cancer cells. We can clearly observe two forms of MOAP-1 in blood cancers, a p39 form encoded by the mRNA and a p46 form that is post-translationally phosphorylated to have a slower migration. Interestingly, form 2 is mainly present in blood cancers (mainly AML and some ALL subtypes) and not solid cancers [3]. This is an interesting observation as epigenetic loss of RASSF1A is predominantly absent in the blood cancers. We have clearly demonstrated that the presence of form 2 of MOAP-1 may alter the growth rate towards hyper-proliferation in AML and ALL cells and interfere with the ability of MOAP-1 to function as an activator of cell death. What is driving the formation of this modification is unknown but is a question currently being explored. What we can conclude is that in AML and ALL patients, the RASSF1A/MOAP-1 molecular pathway may not be functional because MOAP-1 is modified.

What may give us clues about the interactome of form 2 of MOAP-1 is our mass spectrometry analysis in cells with form 2 MOAP-1 (Fig. 4, KG1 and SEM). Several hits are in line with what we know about the biological role for MOAP-1 but provide new insights. Ubiquitin-like modifier-activating enzyme 1 (UBA1) is the E1 component that catalyzes the first step in ubiquitin conjugation to target cellular proteins for degradation using NEDD8. It has been suggested to be involved in DNA damage control and associated with X-linked infantile Spinal Muscular Atrophy, a rare neurological disorder involving death of motor neurons of the spinal cord resulting in muscle wasting (atrophy) [16]. In addition, this E1 ligase has been demonstrated

to be involved in angiotension induced activation of dendritic cells and may serve as the potential therapeutic target for DC-mediated autoimmune diseases [17] and leukemia [18]. We speculate that MOAP-1 may be a molecular target of the UBA1/NEDD8 modification system that may regulate MOAP-1 stability in blood and neuronal cells.

PARP1 is a specific target of active caspases and cleavage of PARP1 occurs during mid- to late-apoptotic signaling and prior to nuclear condensation. PARP1 activity is predominantly involved in necroptosis, immunological responses, nucleotide base excision repair and other forms of DNA strand breaks [19]. The interactome for PARP1 includes XPA (Xeroderma pigmentosum compelmanation group A), SMAD4 and CDK7. Interestingly, XPA can also associate with DDB1, another identified MOAP-1 interacting protein in J77 cells, and with RASSF1A [20] to suggest a role in XPA signaling for both RASSF1A and MOAP-1.

Three other molecular targets identified in our immunoprecipitation with MOAP-1 including DDB1 (validated in Fig. 4), XRCC6 and RAD50 (validated in Fig. 4) suggest an unexplored role in DNA damage control. DDB1 has been demonstrated to regulate nucleotide excision repair and mutation or loss of DDB1 can lead to ineffective repair in patients with xeroderma pigmentosum complementation group E (XPE) - an autosomal recessive disorder leading to photosensitivity and early onset of carcinomas. RASSF1A has been demonstrated to have a functional site for ATM phosphorylation, a kinase involved in double strand breaks, apoptosis and genotoxic stress and can associate with XPA as mentioned above. XRCC6 is also involved in regulating double strand breaks while RAD50 can associate with ATM [21]. We have compelling evidence that the MOAP-1 interactome can influence the repair of damage DNA and

279 may aid or take the place of RASSF1A to ensure that DNA is repaired in a timely and accurate
280 manner.

281 The last set of interacting proteins ILF3, TKT and PDCD6-IP are involved in three
282 important aspects of what makes a cancer cell grow, proliferate and expand. This includes altered
283 immune function (ILF3), altered metabolism (TKT) and altered cell death (PDCD6-IP). MOAP-1
284 has already proven to modulate cell death, but has not been proven to influence metabolism and
285 we have compelling data to reveal its importance as a negative regulator of inflammation by
286 restricting the activation of NFκB (Volodko et al, unpublished results). Not much is known about
287 ILF3 other than its involvement in DNA gene transcription and mRNA stability. TKT has been
288 shown to be involved in the Warburg effect that allows a cancer cell to grow under aerobic
289 conditions with a high rate of glycolysis [22, 23]. TKT influences the pentose phosphate pathway,
290 carbon metabolism and glucose metabolism [24] to possibly influence malignant growth. The
291 exact role for MOAP-1 in influencing the Warburg effect is unknown but association with TKT
292 may tip the balance of the use of oxidative phosphorylation versus aerobic glycolysis and the
293 inhibition of the malignant state. PDCD6-IP is a multifunctional protein involved in endocytosis,
294 membrane repair, cytokinesis and apoptosis. One link to apoptotic signaling is association with
295 death associated protein kinase 1 (DAPK1), a TNFα stimulated pro-apoptotic protein kinase that
296 can also associate with RASSF1A (Baksh et al, unpublished results). Apart from the link to
297 DAPK1, really nothing is known about PDCD6-IP.

298 These identified associations with MOAP-1 are intriguing and are in line with what we
299 know about the biology of the RASSF1A/MOAP-1 tumor suppressor pathway that functions at

multiple levels to modulate growth and prevent malignant transformation. The associations in Fig. 4 appear to be unique to MOAP-1 and have not previously appeared on the RASSF1A interactome.

Conclusions

MOAP-1 is a *bona fide* tumor suppressor in blood cancers with functions beyond apoptosis. It will be interesting to further characterize the novel protein associations identified in blood cancers and explore in detail how form 1 and 2 of MOAP-1 contribute to the biology of MOAP-1 and overlap with RASSF1A associations.

Figures

Fig. 1. Expression of MOAP-1 in blood cancers. (A) Cell lines (lanes 1-13) were derived from the peripheral blood of pediatric acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML) patients. Anti-Erk1/2 was used as a loading control. Lanes 14-21, MOAP-1 protein expression in AML patients. Please note that data in lanes 1-4 appear in Law et al. (2015) [3]. (B) MOAP-1 ubiquitination in various exponentially growing blood cancer cell lines. Clarified cell lysates were obtained and immunoprecipitated and immunoblotted. In addition, J77 cells were subjected to various cell cycle arrest drugs and MOAP-1 immunoprecipitated and immunoblotted. HU = hydroxyurea. Erk1/2 was utilized as a loading control. (C) Treatment with calf intestinal and/or lambda phosphatase results in a partial reduction in mobility to a 39 kDa band that is most likely unmodified MOAP-1. (D) Immunoblotting (IB) for MOAP-1 in selected cell lines upon the

addition of the proteasome inhibitor MG132 (10 μ M) (top) or cyclohexamide (50 μ g/ml) (bottom) for the indicated times.

Fig. 2. Functional role of MOAP-1 in blood cancer cells. (A) Proliferation of leukemia cells in an MTT assay. (B) The spleen was isolated from 3 month old male wild type and *Moap-1*^{-/-} animals and number of cells counted. P value is < 0.0002 and n = 8 (from 8 different animals). (C) Expression of RASSF1A in select leukemia and lymphoma cell lines (BL, Burkitt's Lymphoma; HL, Hodgkin's Lymphoma). (D) PARP cleavage (a marker of late cell death) in leukemia cell lines with varied MOAP-1 expression (see Fig. 1A for expression of MOAP-1 in these cells). (E, F) Male nude mice were tail vein injected with THP-1 cells containing the indicated stable expression constructs. Two months later tumors were imaged close to the neck area and in the colon to reveal GFP staining. No tumors were formed in the CFP-MOAP1 animals. N = 3 for all genotypes. (E) Imaging of mice and tumors found in the neck region (left and middle panels). Right panel, expression of CFP and CFP-MOAP-1 in cells injected via tail vein. (F) Nodule formation in the colon following tail vein injection of CFP cells are circled. All images were taken using the Kodak In vivo Multispectral FX imager from Carestream in the laboratory of Dr. Catherine Chan, University of Alberta.

Fig. 3. Identification of MOAP-1 interacting proteins. Leukemia cells were lysed in SB lysis buffer, immunoprecipitated with the QED MOAP-1 antibody and associated protein complexes identified (circled bands). The circled bands were unique to that cell line and were cut out and identified by mass spectrometry analysis. No bands were excised out from the Molt-3 or KG1 lane

and were only included in this figure for comparison. Please note that Molt-3 cells have the p46 form of MOAP-1 (data not shown). Although not shown, controls for the MOAP-1 immunoprecipitation was immunoprecipitation with rabbit control IgG.

Fig. 4. Validation of MOAP-1 interacting proteins. Validation was carried out by overexpression of Myc-MOAP-1 in HCT-116 colon cancer cells followed by stimulation as indicated and immunoprecipitation using the indicated antibodies to DDB1 (A), Rad50 (B) and Transketolase (TKT) (C). We present data in HCT-116 cells due to ease of transfection and avoidance of the complication of MOAP-1 forms 1 and 2.

Declarations

Ethics approval and consent to participate

All methods involving animals were performed in accordance with the relevant guidelines and regulations approved by the University of Alberta Animal Care and Use Committee under protocol #AUP000218.

Consent for publication

Not applicable.

Availability of data and materials

The datasets analyzed during the current study are available from the corresponding author upon request.

363

364 **Competing interests**

365 None to declare

366

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369 Institute.

370

371 **Authors' contributions**

372 JL contributed to the design of many of the experiments outlined in this manuscript including Fig.
373 1A, 2 and part of 3. OS contributed to the Fig. 1C and 3. KF contributed to Fig. 1C and Fig. 2; SB
374 carried out Fig. 4. and SB and AN contributed to the patient samples and cell lines used in this
375 study; RF aided in the mass spectrometry analysis in Fig. 4. EK contributed to experiments in Fig.
376 1a, 1c and 2c and was supervised by BPH; SB supervised all of these experiments, drafted and SB
377 and BPH edited this manuscript.

378

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Please note that excerpts of this review have been extracted from the M.Sc. thesis of Jennifer Law entitled “MOAP-1: A Candidate Tumor Suppressor Protein” (Department of Biochemistry, Faculty of Medicine and Dentistry, University of Alberta, 2011).

Authors' information (optional)

Not applicable

Abbreviations

AML, acute myeloid leukemia; **ALL**, acute lymphocytic leukemia, **ATM**, Ataxia telangiectasia mutated; **GFP**, green fluorescent protein;; **MOAP-1**, modulator of apoptosis 1; **NFκB**, nuclear factor kappa B; **RASSF**, Ras association domain family; **SDS-PAGE**, sodium dodecyl sulphate polyacrylamide gel electrophoresis; **TNFα**, tumor necrosis factor α; **TNF-R1**, tumor necrosis factor receptor 1; **TRAF**, TNF receptor associated family.

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Figures

Figure 1

Expression of MOAP-1 in blood cancers. (A) Cell lines (lanes 1-13) were derived from the peripheral blood of pediatric acute lymphoblastic leukemia (ALL) and acute myleoid leukemia (AML) patients. Anti-Erk1/2 was used as a loading control. Lanes 14-21, MOAP-1 protein expression in AML patients. Please note that

data in lanes 1-4 appear in Law et al. (2015) [3]. (B) MOAP-1 ubiquitination in various exponentially growing blood cancer cell lines. Clarified cell lysates were obtained and immunoprecipitated and immunoblotted. In addition, J77 cells were subjected to various cell cycle arrest drugs and MOAP-1 immunoprecipitated and immunoblotted. HU = hydroxyurea. Erk1/2 was utilized as a loading control. (C) Treatment with calf intestinal and/or lambda phosphatase results in a partial reduction in mobility to a 39 kDa band that is most likely unmodified MOAP-1. (D) Immunoblotting (IB) for MOAP-1 in selected cell lines upon the addition of the proteasome inhibitor MG132 (10 μ M) (top) or cyclohexamide (320 50 μ g/ml) (bottom) for the indicated times.

Figure 2

Functional role of MOAP-1 in blood cancer cells. (A) Proliferation of leukemia cells in an MTT assay. (B) The spleen was isolated from 3 month old male wild type and Moap-1^{-/-} animals and number of cells counted. P value is < 0.0002 and n = 8 (from 8 different animals). (C) Expression of RASSF1A in select leukemia and lymphoma cell lines (BL, Burkitt's Lymphoma; HL, Hodgkin's Lymphoma). (D) PARP cleavage (a marker of late cell death) in leukemia cell lines with varied MOAP-1 expression (see Fig. 1A

for expression of MOAP-1 in these cells). (E, F) Male nude mice were tail vein injected with THP-1 cells containing the indicated stable expression constructs. Two months later tumors were imaged close to the neck area and in the colon to reveal GFP staining. No tumors were formed in the CFP-MOAP1 animals. N = 3 for all genotypes. (E) Imaging of mice and tumors found in the neck region (left and middle panels). Right panel, expression of CFP and CFP-MOAP-1 in cells injected via tail vein. (F) Nodule formation in the colon following tail vein injection of CFP cells are circled. All images were taken using the Kodak In vivo Multispectral FX imager from Carestream in the laboratory of Dr. Catherine Chan, University of Alberta.

Bands Identified by Mass Spectrometry:

Cell	Band, MW (kDa)	ID	Function
J77	(1) 117	Ubiquitin-like modifier-activating enzyme 1 (UAB1) 9 peptides identified/13 % coverage	Activates ubiquitin by first adenylating its C-terminal glycine residue with ATP (an E1 ligase component)
J77	(1) 119	Poly [ADP-ribose] polymerase 1 (PARP1) 5 peptides identified/7 % coverage	Catalyzes the post-translational modification of proteins by the addition of multiple ADP-ribose moieties; biomarker of late cell death
J77	(1) 126	DNA damage-binding protein 1 (DDB1) 4 peptides identified/5 % coverage	Xeroderma pigmentosum complementation group E (XPE)-binding factor; functions in nucleotide-excision repair; may function as part of the DCX (DDB1-CUL4-X-box) E3 ubiquitin-protein ligase complexes
J77	(2) 70	X-ray repair cross-complementing protein 6 (XRCC6) 7 peptides identified/13 % coverage	Involved in DNA non-homologous end joining (NHEJ) required for double-strand break repair and V(D)J recombination complexes with other proteins.
SEM/J77	(2) 67	Transketolase (TKT) 4 peptides identified/11% coverage	Associated with aerobic glycolysis and Warburg effect
SEM	(3) 95	Interleukin enhancer-binding factor 3 (ILF3) 3 peptides identified/5 % coverage	A double-stranded RNA (dsRNA) binding protein that complexes with other proteins, dsRNAs, small noncoding RNAs, and mRNAs to regulate gene expression and stabilize mRNAs.
BL2	(4) 153	DNA repair protein RAD50 (RAD50) 13 peptides identified/13 % coverage	Involved in DNA double-strand break repair, non-homologous joining of DNA ends, involved in maintaining telomere integrity. Forms a complex with MRE11 and NBS1 and may associate with ATM.
BL2	(5) 95	Programmed cell death 6-interacting protein (PDCD6-IP) 4 peptides identified/6.5 % coverage	Interacts with DAPK1 (a RASSF1A kinase target); required for completion of cytokinesis; may localize to centrosomes

Figure 3

Identification of MOAP-1 interacting proteins. Leukemia cells were lysed in SB lysis buffer, immunoprecipitated with the QED MOAP-1 antibody and associated protein complexes identified (circled bands). The circled bands were unique to that cell line and were cut out and identified by mass spectrometry analysis. No bands were excised out from the Molt-3 or KG1 lane and were only included in this figure for comparison. Please note that Molt-3 342 cells have the p46 form of MOAP-1 (data not shown). Although not shown, controls for the MOAP-1 immunoprecipitation was immunoprecipitation with rabbit control IgG.

Figure 4

Validation of MOAP-1 interacting proteins. Validation was carried out by overexpression of Myc-MOAP-1 in HCT-116 colon cancer cells followed by stimulation as indicated and immunoprecipitation using the indicated antibodies to DDB1 (A), Rad50 (B) and Transketolase (TKT) (C). We present data in HCT-116 cells due to ease of transfection and avoidance of the complication of MOAP-1 forms 1 and 2.

Supplementary Files

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