Modulator of Apoptosis 1 (MOAP-1) is a tumor suppressor protein linked to RASSF1A

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Running title: MOAP-1 is a tumor suppressor protein

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Capsule

Background: MOAP-1 is a pro-apoptotic protein.

Results: MOAP-1 expression is reduced in cancers and high expression correlated with increased patient survival. MOAP-1 can associate with tubulin and modulate tubulin stability. Furthermore, loss of the BH3 domain of MOAP-1 resulted in lack of tumor suppressor function.

Conclusion: MOAP-1 is a highly regulated tumor suppressor protein.

Significance: The loss of MOAP-1 loss may significantly contribute to tumorigenesis.

Abstract

Modulator of apoptosis 1 (MOAP-1) is a BH3-like protein that plays key roles in cell death or apoptosis. It is an integral partner to the tumor suppressor protein, Ras association domain family 1A (RASSF1A), and functions to activate the Bcl-2 family pro-apoptotic protein, Bax. Although RASSF1A is now considered a bona fide tumor suppressor protein, the role of MOAP-1 as a tumor suppressor protein is yet to be determined. In this study, we present several lines of evidence from cancer databases, immunoblotting of cancer cells, proliferation and xenograft assays as well as DNA microarray analysis to demonstrate the role of MOAP-1 as a tumor suppressor protein. Frequent loss of MOAP-1 expression, in at least some cancers, appears to be attributed to mRNA downregulation and the rapid proteasomal degradation of MOAP-1 that could be reversed utilizing the proteasome inhibitor, MG132. Overexpression of MOAP-1 in several cancer cell lines resulted in reduced tumorigenesis and upregulation of genes involved in cancer regulatory pathways that includes apoptosis (p53, Fas and MST1), DNA damage control (PARP and ATM), those within the cell metabolism (IR-α, IR-β and AMPK) and a stablizing effect on microtubules. The loss of RASSF1A (an upstream regulator of MOAP-1) is one of the earliest detectable epigenetically silenced tumor suppressor proteins in cancer and we speculate that the additional loss of function of MOAP-1 may be a second hit to functionally compromise the RASSF1A/MOAP-1 death receptor dependent pathway and drive tumorigenesis.

Key words: RASSF1A, MOAP-1, tumor suppressor gene, tubulin, apoptosis, cancer, ubiquitination, xenograft assay, GWAS, Bax

Introduction

MOAP-1 was first identified as a novel Bax-associating protein in a yeast two-hybrid protein screen (1,2). It has been demonstrated to be involved in both the intrinsic and extrinsic pathways of cell death and required for inducing Bax conformational change and mitochondrial localization in order to promote subsequent cell death (2,3). MOAP-1 can also selectively associate with the anti-apoptotic proteins Bcl-2 and Bcl-X_L (1). We and others have demonstrated that MOAP-1 knockdown cells are resistant to a variety of

apoptotic stimuli including staurosporine, serum withdrawal, UV irradiation, tumor necrosis factor (TNF) α and TNF-related apoptosis inducing ligand (TRAIL) (2-4). In non-apoptotic cells, MOAP-1 is an unstable protein (half-life ~25 minutes) that is constantly turned over via the proteasome (5). However, during apoptotic stimulation, MOAP-1 expression is up-regulated in response to serum withdrawal, etoposide, TRAIL or endoplasmic reticulum stress inducer thapsigargin (5) MOAP-1 up-regulation under apoptotic stress can occur as a result of tripartite motif containing 39 (TRIM39)-mediated inhibition of anaphase-promoting complex (APC/C^{Cdh1}) E3 ubiquitin ligase-dependent polyubiquitination of MOAP-1 (5-7). Although it has been shown that MOAP-1 is degraded in a cell cycle-specific manner requiring the cell cycle regulator APC ^{Cdh1} E3 ubiquitin complex, it remains unknown how this may be important for the control of apoptosis or cell proliferation (5,6).

We and others have demonstrated association of the tumor suppressor, RASSF1A, with MOAP-1 and requirement of RASSF1A/MOAP-1 for TNF-R1-driven apoptosis (2,4,8). The loss of RASSF1A by small interfering RNA (siRNA) knockdown or genetic knockout results in a significant loss of death receptor dependent apoptosis with only minor effects on the intrinsic pathway of cell death (2). RASSF1 is involved in apoptosis, cell cycle control, regulation of microtubule stability and tumor suppression and is epigenetically silenced in human cancers. RASSF1A can associate with MOAP-1 independent of the presence of K-Ras (2) in contrast to some reports (4). Similarly, RASSF6 has also been demonstrated to associate with MOAP-1 and the Hippo kinase, MST2, can interfere with this association to prevent RASSF6/MOAP-1 directed apoptosis. The importance of these

observations physiologically is still unknown but association between RASSF6 and MOAP-1 does not require the presence of activated K-Ras (9,10).

In this study we demonstrate that ectopic expression of MOAP-1 resulted in reduced formation of foci on soft agar and reduced proliferative capacity of H1299 cells. Furthermore, overexpressed MOAP-1 in several cancer cell-lines resulted in reduced formation of tumors in a xenograft model in athymic nude mice to suggest a universal role as a tumor suppressor protein. Mechanistically, we can demonstrate that MOAP-1 may carry out its tumor suppressor function by the involvement in apoptosis (2,8) and associations with tubulin isoforms to stabilize tubulin (this study). This will aid in sister chromatid separation in a similar manner to RASSF1A (11,12). In addition, we provide evidence for involvement in DNA damage control and cell metabolism that may also influence control of tumor formation. These studies illustrate important links to RASSF1A (a bona fide tumor suppressor protein) and suggests that MOAP-1 synergizes with RASSF1A to inhibit tumorigenesis.

Materials and Methods

Antibodies and reagents

Antibodies were purchased from the following vendors: p53 and Aurora B from AbCAM; mouse anti-ubiquitin (sc-8017), STAT3 (sc-482), STAT5a (sc-1081), GNB2L1/RACK1 (sc-17754), IRα (sc-710), β (sc-371), rabbit anti- γ-tubulin (sc-10732), mouse anti-α-tubulin (sc-8035) were from Santa Cruz Biotechnology; anti-RASSF1A (M304) was a gift from Dr. Gerd Pfiefer; PTEN (#9552) and PARP (#9542S) PKM2 (Y105, 3827S and total, #4053S), GS3K-

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3β (S9 [#9336S and total [#9315S]), and AMPK (Thr-172, #2531S; total, #2532) were from Cell Signaling; MAP1S (kindly provided by Dr. Leyuan Liu, Texas A&M Health Science Center); mouse anti-β-tubulin was from Sigma-Aldrich (T-5201), mouse anti-acetylated α-tubulin from was from Sigma-Aldrich (T-6793) and ECL detection (GE Amersham ECL RPN2106). SYBR Green SuperMix (Applied Biosystems, Foster City, CA).

Cell culture and transfection. Cells were grown and transfected with PEI as described previously (11,13). Cells were lysed in SB lysis buffer [50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM $MgCl_2$, 1.5 mM EDTA, 0.5% Triton X-100, 20 mM β-glycerolphosphate, 100 mM NaF, 0.1 mM PMSF] (11) or in standard RIPA buffer (8) as indicated. Apoptotic assays were carried as previously described (2). The cell lines utilized in this study included: HEMa-LP cells and melanoma cell lines from Dr. Sujata Persad (University of Alberta); breast cancer cell lines from Dr. Ing Swie Goping (University of Alberta); pediatric leukemia cell lines (Dr. Aru Narendran, University of Calgary); colon cancer cell lines (Dr. Eytan Wine, University of Alberta); ovarian cancer cell lines (Dr. YangXin Fu, University of Alberta), and neuroblastoma cell lines form Dr. Roseline Godbout (University of Alberta), All ubiquitination experiments were carried out in SB lysis buffer + 5 mM iodoacetamide. HCT116 cells (containing endogenous RASSF1A and MOAP-1) were utilized for our xenograft assays as they transfect to > 40%, maintain the expression of transiently transfected HA-RASSF1A for up to 10 days in culture and produce tumors within 30 days (11). For most xenograft assays, the growth path is determined within the first 5-10 days. As such, even if expression is reduced, the growth will continue. For those experiments

whereby we do use stables, they are pools and not single clones expression MOAP-1.

Reverse Transcriptase (RT) PCR. 1 μg of RNA was treated with DNase I (Invitrogen) according to manufacturer's instructions. RNA was then converted into cDNA with an Applied Biosystems high-capacity cDNA Reverse Transcription Kit according to manufacturer's instructions. After reverse transcription cDNA was diluted 10 times with RNase-free water and 5ul was used in PCR reactions using NEB Taq DNA polymerase with standard Taq buffer. PCR parameters were: denaturation 94ºC for 2 min (1 cycle), 94ºC for 1 min, 54ºC or 58ºC (for MOAP1 or GAPDH) for 1 min and 68ºC for 1 min (35 cycles) followed by a final extension at 68ºC for 10 min. PCR products were analyzed on a 2 % agarose gel and visualized with ethidium bromide. The following primers were used: MOAP1 forward 5'-ACATGAAAATGGCTCCTTAGAC-3', MOAP1 reverse 5'-GACACGAATAACATCAAGTGCT-3', GAPDH forward 5'-CATGACAACTTTGGTATCGTG-3', GAPDH reverse 5'-GTGTCGCTGTTGAAGTCAGA-3'.

Genome-wide association study (GWAS) of human tumor xenografts. HCT116 human tumor xenografts were established in athymic nude mice. At day 35, tumors were excised, stored in RNAlater solution (Qiagen) and used for RNA isolation according to manufacturer's protocol (Qiagen RNeasy kits). RNA samples with RNA integrity numbers (RIN) > 7.0 were used for gene expression analysis as previously described (14). Data normalization and analysis was performed using GeneSpring GX 11.5.1 (Agilent Technologies). Normalized data were $\log_2$ transformed and averaged over three independent tumor replicates per construct

(GEO accession number GSE43990 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE43990).

Analysis of MOAP-1 expression in breast cancer database. Breast cancer microarray data was generated as previously described, and provided to us through the courtesy of Dr. John Mackey (14). Significance of data were evaluated by Student's t-tests (two-tailed) and receiver operating characteristic (ROC) curve analysis was performed to establish the MOAP-1 expression cut-off.

Canonical pathway and biological function analysis of GWAS expression changes. Post-analysis of gene expression changes was performed using Ingenuity Pathway Analysis software (IPA; Ingenuity systems, www.ingenuity.com). Functional analysis was performed to identify the biological functions most significant to the disregulated molecules in our dataset with p-values calculated by right-tailed Fisher's exact test. Canonical pathway analysis was employed to identify the pathways from the IPA library that were most significant to our dataset.

Oncomine meta-analysis of human cancer microarrays. Differential expression analysis was performed for MOAP-1 in the normal versus cancer category using Oncomine cancer microarray database, version 4.4 Research Edition (Compendia Bioscience, Ann Arbor, MI). We assigned a cut threshold of fold change ≥ 1.5 and p ≤ 0.05 for our meta-analysis and only included MOAP-1 expression data for studies that met this significance. Results were grouped based on cancer type and re-plotted as fold changes in MOAP-1 mRNA levels relative to normal tissue.

Kaplan-Meier survival analysis. Kaplan-Meier survival curves for MOAP-1 and

RASSF1A expression in 88 neuroblastoma patients was kindly generated for us by Dr. Rogier Versteeg (University of Amsterdam, Netherlands). mRNA levels of MOAP-1 and RASSF1A were quantified from 88 neuroblastoma patients and Kaplan-Meier survival curves generated to demonstrate the correlation between expression levels of MOAP-1 and RASSF1A with overall patient survival probability from neuroblastoma.

Statistical Analysis. All experiments were carried out at least three times. Significance of data was evaluated by performing a Student's t-test (two-tailed) as indicated.

Results

Oncomine database reveals reduced MOAP-1 expression in human cancers

A meta-analysis of microarray data was performed using the online cancer microarray database Oncomine (Compendia Bioscience, Ann Arbor, MI) (15). Differential expression analysis of MOAP-1 was carried out in samples from normal versus malignant tissues with data subsequently compiled from microarray studies meeting the threshold fold-change ≥ 1.5 and p value ≤ 0.05. The results obtained from this meta-analysis indicate that MOAP-1 expression is markedly reduced in multiple types of human cancers (Figure 1A), especially in the brain, breast, blood, skin and lung suggesting that loss of MOAP-1 is important for tumorigenesis to occur. MOAP-1 may possess a potential tumor suppressor function in specific cell types. Interestingly, parathyroid, myeloma, prostate/ovarian/cervical (that is, the reproductive organs) and gastric datasets revealed elevated expression of MOAP-1 to suggest that MOAP-1 expression can vary widely and may have differential function in

numerous tissues. Please note that plots for "sarcoma" classification includes the following: Dedifferentiated Liposarcoma, Myxofibrosarcoma, Round Cell Liposarcoma and Fibrosarcoma. Similarly for "leukemia": B-Cell Childhood Acute Lymphoblastic Leukemia, T-cell prolymphocytic leukemina (all underexpressed) and Hairy cell leukemia (overexpressed); and "lymphoma": anaplastic Large Cell Lymphoma (ALK-Positive), primary cutaneous anaplastic large cell lymphoma, classical Hodgkin's lymphoma, angioimmunoblastic T-Cell lymphoma (all underexpressed) and mantle cell lymphoma (overexpressed).

Low MOAP-1 expression in neuroblastoma patients correlates with decreased survival probability

Neuroblastoma is predominantly a pediatric form of cancer that arises from progenitor cells of the sympathetic nervous system and is the most common solid tumor in childhood (16). Remarkably, neuroblastomas display the highest rate of spontaneous regression among all human cancers possibly due to delayed activation of normal apoptotic signaling pathways (17). Since MOAP-1 is frequently downregulated in brain cancer it may therefore have a role in inhibiting malignancy formation in brain tissues. We explored MOAP-1 mRNA expression in 88 neuroblastoma patients in collaboration with Dr. Rogier Versteeg (University of Amsterdam, Netherlands). Kaplan-Meier survival analysis demonstrated that patients with high expression of either *MOAP-1* or *RASSF1A* experienced greater survival rates versus those with low expression of either gene (Figure 1B and 1C), thereby suggesting that both MOAP-1 and RASSF1A may behave as tumor suppressor proteins in the

pathogenesis of neuroblastoma. We confirmed our database results by real time quantitative PCR (qPCR) in neuroblastoma cell lines (Figure 1D) and by protein immunoblotting (Figure 1E). Several neuroblastoma cell lines revealed 80-90% reduction of MOAP-1 - Be(2)c [bone marrow disease derived from SK-N-Be(2)], GoTo (derived from Stage IV disease), IMR-32 (derived from the abdominal mass metastatic site), SMS-KAN (from primary pelvic tumor) (Figure 1E), Nub7 (MycN amplified) and SK-NAS (bone metastasis in origin)] (data not shown) when compared to levels in a normal human epithelial cells (NHBE). RASSF1A expression by qPCR was virtually absent due to epigenetic silencing [data not shown and (12,18). These data reinforce previous findings demonstrating frequent inactivation of RASSF1A and reduced expression of MOAP-1 to result in poor disease outcome (19,20). Considering that both MOAP-1 and RASSF1A are essential components of death receptor-mediated apoptosis (2,8), these observations suggest that the reduction of MOAP-1 and RASSF1A expression levels in neuroblastoma cells contributes significantly to poor patient prognosis and decreased survival.

Decreased MOAP-1 expression in breast cancer patients correlates with increased cancer aggressiveness

Currently, four major breast cancer subtypes can be distinguished based on the expressions of estrogen receptor (ER), progesterone receptor (PR) and Her2/Neu and are classified as the following in the order of increasing cancer aggressiveness: luminal A (ER+, PR+, Her2-); luminal B (ER+, PR-/+, Her2+); Her2-amplified (ER-, PR-, Her2+); and triple negative or basal-like (ER-, PR-, Her2-) (21). Luminal A breast cancers are the most common subtype that respond well to adjuvant hormone therapy and have the best

overall prognosis. In contrast, there are currently no targeted-therapies available for the treatment of triple-negative breast cancers that are associated with the worst overall and disease-free survival rates (21,22).

Breast cancer microarray expression data generated from 176 primary, treatment-naive breast cancer samples and 10 normal breast tissue samples (14) revealed that MOAP-1 expression was reduced five-fold in breast tumor samples (Figure 2A). Detailed analysis revealed a steady and significant decrease in MOAP-1 expression that correlated with increasing breast cancer aggressiveness in luminal B, Her2-amplified and triple-negative subtypes (Figure 2B). These changes were confirmed by qPCR for MOAP-1 on patient tumor samples obtain from the Alberta Cancer Research Biobank (Figure 2C) and from representative cell lines that reflect the categories in Figure 2B (Figure 2D). MOAP-1 expression was readily detected in the normal primary lung epithelial line, NHBE, in the immortalized breast epithelial line, hTERT (breast epithelial cells immortalized with human telomerase), in the non-invasive cell line derived from a patient with fibrocystic disease of the breast, MCF-10A (Figure 2D). In contrast, expression is reduced or not detectable in several breast cancer cell line sub-types as indicated in Figure 2D.

All samples in Figure 2C and 2D contain > 40% of their RASSF1A promoter CpG island regions epigenetic silenced by methylation. Epigenetic silencing of RASSF1A in breast cancer is well documented and is one of the earliest detectable changes in breast cancer progression (12). These results suggest that MOAP-1 expression is down-regulated during breast cancer progression, may parallel the loss of RASSF1A and subsequently contribute to the poor patient

outcomes associated with more aggressive breast cancer subtypes. Although we did not observe an association between levels of MOAP-1 expression and breast cancer family history, recurrence, death or Her2-amplification, we did discover a significantly higher proportion of patients expressing low MOAP-1 with ER negative and PR negative breast tumors (Figure 2E [Her2+ amplified and TNBC] and Table 1). Hormone receptor negative breast cancers carry a less favorable prognosis than tumors with hormone receptors, and do not benefit from endocrine therapies (23). Therefore, the loss of the pro-apoptotic MOAP-1 protein may contribute significantly to the less favorable prognosis than tumors with hormone receptors.

Reduced expression of MOAP-1 in cultured cancer cells

In addition to detectable expression of MOAP-1 in NHBE, hTERT (immortalized with human telomerase) and MCF-10A, we can also detect MOAP-1 in other non-transformed cell lines such as HEMA-LP and PNT1A (Figure 3A - C). MOAP-1 protein was reduced or absent in many cancer cells as shown in Figure 2D and Figure 3. Noticeably, MOAP-1 expression was detected in the p53-positive cell lines (U2OS, HCT116, ZR-75, IMR-32, PANC1) but was reduced/absent in in p53-null cells (SAOS-2 and SKOV3), p53 frameshift mutation or rearrangement cell lines (Caco-2, CAPAN-2) and p53-mutant cell lines (SKBR3, MDA-MB-468, MDA MB-231,BT-549, BT-20 [breast cancer cells, Figure 2D] and OVCAR-3 [ovarian cancer]. However, the expression of MOAP-1 did not always correlate with the status of p53 as SW480 (p53-null) has detectable MOAP-1 while the p53 wild type cell lines, A549 and majority of the neuroblastoma cell lines, do not have robust detection of MOAP-1. Of the

cell lines are $p53^{+/+}MOAP\text{-}1^{-/-}$ all have epigenetic silencing of RASSF1A [please see references (12)]. Therefore, we suspect that RASSF1A may influence the expression of MOAP-1 and we are currently exploring this unexpected observation. The dual loss of MOAP-1 and RASSF1A expression may contribute to malignant cell growth in the solid cancers.

In contrast to the solid cancers, MOAP-1 in several blood cancer cell lines revealed two distinct forms as detected by two independent antibodies (Figure 3B, right side). The slower migrating 46 kDa form (Form 2) of MOAP-1 can be found in cancers originating from the blood as ALL or AML subtypes (Figure 3B and unpublished observations). We are currently investigating why MOAP-1 appears as a slower migrating band in blood cancers but we suspect that it may involve phosphorylation of MOAP-1 by an unidentified kinase.

Differential MOAP-1 expression in cancer cells may also be controlled by post-translational ubiquitination

Exon sequencing revealed no mutations in MOAP-1 in several cancer lines investigated including HCT116 colon cancer cells, H1299 small cell lung cancer and MDA-MB231 breast cancer cells. Quantitative PCR revealed decreased mRNA production for MOAP-1 in several cancer cells and patient tumor tissues (Figure 1D and 2C). It is known that MOAP-1 appears to be highly turned over every 25 minutes via the ubiquitin-mediated proteasomal degradation (5) and thus may explain lack of detection.   If MOAP-1 is regulated by ubiquitin-directed degradation, then the proteasome inhibitor, MG-132, should inhibit the degradation of MOAP-1 and stabilize its expression. Indeed,

we can observe this with MG-132 treatment of several cell lines that initially did not reveal detectable MOAP-1 expression including MDA-MB-468, MDA-MB-231, MCF-7, A549 and A2058 (Figure 4). For the most part, MOAP-1 ubiquitination can be detected in most of the cell lines in Figure 4 and are in lines with the observations of Lee et al. (2009) (5). This would suggest that ubiquitin directed modulation of MOAP-1 expression can occur in several cancers. Curiously, under MG-132 treatment, MOAP-1 expression in SKOV3 cells is barely detectable for unknown reasons. These experiments reveal the complexity of MOAP-1 regulation. We are currently investigating the importance of ubiquitination for MOAP-1 biology, the mechanism by which this occurs, characterizing the importance of two identified E3 ligase interacting proteins with MOAP-1 and how they may control the biology of MOAP-1.

MOAP-1 inhibits cell proliferation in culture and promotes cell death in cancer cells

A fundamental property that is shared by many tumor suppressor proteins is the ability to negatively regulate cell growth and proliferation. H1299 non-small cell lung carcinoma cells stably expressing Myc-MOAP-1 and HCT116 colon cancer cells transiently expressing shRNA to MOAP-1 were generated. Stable expression in H1299 cells was obtained using G418-resistant pools of cells expressing MOAP-1 in order to avoid effects of clonal variation. Using the MTT colorimetric assay for cell proliferation (24), we observed that cells expressing Myc-MOAP-1 were capable of inhibiting cell proliferation while cells with reduced MOAP-1 expression promoted cell proliferation (Figure 5A and B). Colony formation assay also revealed the growth suppressive function of MOAP-1 (Figure

5C). This would suggest importance in inhibiting tumor formation.

Further analysis of several MOAP-1 negative and positive cells verified the important role of MOAP-1 in growth suppression by promoting apoptosis (Figure 6). Cells having robust expression of MOAP-1 (HCT116 and HT-29 colon cancer cells) had robust cleavage of poly (ADP-ribose) polymerase (PARP) upon TNFα/CHX (extrinsic pathway) stimulation while cells with reduced or absent endogenous MOAP-1 expression (MDA-MB-468, SKOV3 and H1299 cells) displayed very limited PARP cleavage upon TNFα/CHX stimulation (Figure 6A - B). Upon stable re-expression of MOAP-1 in H1299 cells, PARP cleavage was significantly enhanced and the p85 cleaved form of PARP was detected upon TNFα/CHX addition that appeared to be augmented by the presence of stably expressed RASSF1A and MOAP-1 (Figure 68C). Furthermore, staurosporine (intrinsic apoptotic pathway stimulation) was also more robust in the presence of stably expressed MOAP-1 in H1299 cells (Figure 6D). Annexin V staining confirmed our PARP results to illustrate that both early (annexin V staining) and late (PARP cleavage) markers of apoptosis were generated in the presence of MOAP-1 (Figure 6E). Similar results were obtained for A549 lung cancer cells and SKOV3 ovarian cancer cells (data not shown). Together, these results demonstrate tumor suppressor properties of MOAP1 via repressing cell proliferation and promoting cell death in cancer cell lines.

MOAP-1 inhibits tumor formation in vivo via its pro-apoptotic function

To directly validate the tumor suppressor function of MOAP-1 *in vivo*, xenograft tumor assays were performed in athymic nude mice lacking a functioning immune system (25). To carry out these assays we selected cancer cell lines with reduced or absent MOAP-1 expression (DAOY medulloblastoma cells, SKOV3 and H1299) or with a detectable amount of MOAP-1 (HCT116 cells). Myc-MOAP-1 was transiently expressed in DAOY medulloblastoma cells, SKOV3 (stable expression, ovarian cancer) (Figure 7A-B) or H1299 (stable expression, lung cancer, Figure 7C-D) and transiently in HCT116 colon cancer cells (Figure 8A-B). Following subcutaneous injection of these cells into the left and right flanks of athymic mice, a significant difference in the growth of tumors containing overexpressed Myc-MOAP-1 emerged for all cell lines tested when compared to tumors containing a control vector. This suggested a role for MOAP-1 in growth suppression. Interestingly, the loss of the function of the BH3 domain of MOAP-1 (either as a deletion or mutation) resulted in the inability to suppress tumor formation to suggest a significant dependence of MOAP-1 on its pro-apoptotic function to carry out tumor suppression (Figure 7C – D). Complementary to our overexpressed cells, shRNA knockdown of MOAP-1 resulted in a significant increase in tumor formation following subcutaneous injection of HCT116 cells containing shRNA to MOAP-1 (Figure 8C). Not surprisingly, tumor formation was additionally reduced in the presence of HA-RASSF1A (for DAOY and H1299 cells, Figure 7B-C) suggesting a requirement for the RASSF1A/MOAP-1 apoptotic pathway in inhibiting tumor formation in these cell lines.

MOAP-1 can associate with tubulin to possibly inhibit tumor formation

RASSF1A can physically associate with microtubules and with α, β and γ-tubulin (11,26) and the lack of microtubule association promotes tumor formation (11,27). It has been demonstrated to co-localize with α, β and γ-tubulin and influence the stability of α-tubulin in several cell types in the *Rassf1a*^{-/-} knockout MEFs.(11,26,28) RASSF1A was also found to stabilize γ-tubulin at the metaphase plate and serve as an important component of sister chromatid separation (28,29) and the appearance of aneuploidy. Thus associations with tubulin is an important aspect of the tumor suppressor role for RASSF1A and possibly MOAP-1 due to the intimate connection between these two pro-apoptotic proteins. We thus explored if MOAP-1 can associate with α, β and γ-tubulin in the absence and presence of RASSF1A. SW480 and H1299 cells were utilized as they are RASSF1A positive and negative cell lines, respectively. Associations of MOAP-1 with α and γ-tubulin was readily detected in asynchronous cells as well as nocodazole and taxol treated cells (albeit at a much lower stoichiometry, Figure 9A). In comparison, RASSF1A can associate equally with tubulin isoforms (11). Furthermore, the association with γ-tubulin was independent on the presence of RASSF1A (Figure 9B). Using deletion constructs to MOAP-1, we were able to identify residues 1-115 as important for γ-tubulin association whereas residues important for α-tubulin association were indeterminate. MOAP-1 expression constructs 1-115, 1-160 and 1-190 can associate with α-tubulin whereas 250-351 cannot (Figure 9C). It may well be that α-tubulin docks to multiple regions on MOAP-1. Interestingly, the MOAP-1 mutants in the BH3 domain, the M1 mutant (that lacks RASSF1A association (2) or the mutant M6 (that lacks TNF-R1 association) do not interfere with either α-tubulin or γ-tubulin associations (Figure 9C). The association of MOAP-1 with α-tubulin resulted in greater stability of α-tubulin in asynchronous H1299 cells that have a low detectable level of MOAP-1 (Figure 9D). Furthermore, the presence of overexpressed MOAP-1 or RASSF1A resulted in greater stabilization of tubulin by taxol, resistance to nocodazole or colchicine destabilization of tubulin (as monitored by its acetylation status, Figure 9D, bottom graph). Thus, similar to the ability of RASSF1A to promote the stability of tubulin, MOAP-1 can also achieve this to suggest the importance of both RASSF1A and MOAP-1 to tubulin stability, organization of spindle formation at the metaphase plate and to the effective separation of sister chromatids during mitosis in order to reduce the incidences of aneuploidy and fulfill its function as a tumor suppressor protein.

Transcriptome analysis of MOAP-1 overexpressing tumors reveals novel involvement in several molecular pathways

The role for MOAP-1 in cell death is well documented. MOAP-1 is known to partner with TNF-R1, RASSF1A, Bax to promote apoptosis (1,2) and partner with TRIM39 for protein stabilization (5). Although cell death and microtubule stability are important aspects of MOAP-1 biology, other possible mechanisms may exist to explain how MOAP-1 inhibits cell proliferation and tumor formation. To gain insight into these other mechanisms of tumor suppression, gene expression profiling was performed on MOAP-1 overexpressing xenograft tumors in nude mice. All experiments were carried out by transient transfections in HCT116 cells as previously described (11). Using this method, we are able to obtain > 60% expression by day 2 that is sustained until day 10 after transfection in tissue culture (11). Since most of the tumor growth initiates between 7 - 14 days (as seen in Figure 8B-C), we postulate

that > 60% of the HCT116 cells expressing Myc-MOAP-1 will be sufficient to direct a growth path towards tumor suppression. RNA was extracted from resulting tumors and subjected to genome wide association study (GWAS) using the Agilent platform. GWAS revealed a total of 1434 differentially expressed genes by overexpression of MOAP-1 or, most likely, as a consequence of the biological ability of MOAP-1 to induce cell death and other physiological processes (Figure 10A). Using the "core analysis" function in Ingenuity Pathway Analysis (IPA, Ingenuity systems) (30) we were able to interpret gene expression changes in the context of biological functions and signaling pathways. Table 2 and Figure 10B display several biological functions and signaling pathways identified as being most significant to the dataset of vector versus wild type MOAP-1 based on Fisher's exact test. Table 3 represents a selection of differentially expressed genes whose expression is modulated as a consequence of MOAP-1 overexpression and enhanced pro-apoptotic function. Some of which encode potential growth regulatory or tumor suppressor functions. Cell death (805 molecules), cell growth and proliferation (625 molecules) and gene expression (545 molecules) were among the top biological functions associated with the disregulated molecules (Figure 10B).

Of particular interest, *TP53* encoding the tumor suppressor protein p53, was found to be up-regulated by over three-fold in the presence of wild type MOAP-1 relative to vector (Table 3 and validated in Figure 10C). Several molecules involved in p53-dependent signaling pathways were also present at elevated levels (Table 3). These include cyclin-dependent kinase 8 (CDK8) (31), cell division cycle 14 homolog B (CDC14B) (32) and transforming growth factor beta regulator 1 (TBRG1/NIAM) (33) p53 transcriptional targets, such as proliferating cell nuclear antigen (PCNA) (34), Fas (35) and epidermal growth factor receptor (EGFR) (36) were also found to be differentially expressed (Table 3). Together, these gene expression changes may be involved in the maintenance of genomic integrity and protection against uncontrolled cell proliferation. In addition, other molecules from p53-dependent signaling pathways (not directly related to proliferation and cell death) were also found to be elevated with wild type MOAP-1 over-expression. These include hypoxia-inducible factor 1 α subunit (HIF1α) and the G2/M DNA damage checkpoint kinase Ataxia telangiectasia mutated (ATM, Table 3). Whether or not MOAP-1 may be involved in the cellular responses to hypoxia or DNA damage warrants further investigation.

The over-expression of MOAP-1 also resulted in the up-regulation of several pro-apoptotic kinases connected to RASSF1A, including mammalian ste20-like kinase 1 (MST1) (mammalian *Hippo*) and aurora kinase B (Table 3 and validated in Figure 10C). The activity of MST1 is initiated by association with RASSF1A (37) while aurora kinases can directly phosphorylate RASSF1A to promote its degradation during mitosis via the anaphase promoting complex/cyclosome (APC/C) (38,39). Currently, the influence of MOAP-1 in Hippo pathway regulation or APC/C function remains unknown but our GWAS study would suggest this and thus warrants further investigation.

Lastly, modulators of gene expression were also differentially regulated in the presence of overexpressed MOAP-1. These include serum response factor (SRF), E-26 like protein 1 (ELK1) transcription factor and signal transducer and activator of transcription 3 (STAT3, validated in Figure 12C). We were also able to validate the

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increase in both STAT3 and STAT5a, GNB2L1/RACK1, PTEN and PARP (all validated in Figure 10C) in xenograft-derived tumor lysates overexpressing MOAP-1 while the receptor for the STATs, Janus kinase 1 (JAK1), did not appear to be up-regulated (confirmed in Figure 10C). Interestingly, the overexpression of MOAP-1 also resulted in modulation of key elements linked to metabolism and the Warburg effect (40-42). These include insulin receptor β, AMPK and pyruvate kinase M2 (PKM2) (validated in Figure 12C). MOAP-1 may thus have a role in modulating the appearance of biomarkers of the Warburg effect and in modulating metabolic pathways. Overall, our GWAS results suggest that MOAP-1 functions as more than just a pro-apoptotic protein and may be involved in numerous other aspects of homeostatic regulation in colon epithelial cells (Figure 11).

Discussion

Our current data supports the role of MOAP-1 as a tumor suppressor involved in cell death, tubulin stability and in several unexplored biological functions important for its ability to mediate growth suppression. MOAP-1 expression levels appear to be regulated by mRNA control, ubiquitination (Figure 4) (3) and, possibly by post-translational phosphorylation (Figure 2B, lanes 6 – 8, slower migrating band). Analysis using Scan Site (http://scansite.mit.edu/) reveals the presence of several internal sequences that may be phosphorylated by aurora kinase A, glycogen synthase kinase 3β, Akt, polo-like kinase, protein kinase C isoforms μ, ε and ζ or the DNA damage kinase ATM, a kinase that can also phosphorylate the ATM site on RASSF1A (43). Evaluation of the role of these kinases is underway.

Immunostaining for MOAP-1 in several cancer cell lines illustrated that, under normal physiological conditions, the intracellular abundance of MOAP-1 is maintained at low levels as a result of its constitutive degradation by the ubiquitin-proteasome system (6) with APC/C^{cdh1} (7) similar to APC/C^{cdc20}-directed ubiquitination of RASSF1A during mitosis (38,44). It is possible that MOAP-1 ubiquitination is controlled in a cell cycle specific manner by the APC/C^{cdh1} (7) pathway. This complex regulation may reflect the need to tightly control a pro-apoptotic molecule bridging both the extrinsic and intrinsic death receptor pathways.

Adding further evidence for its tumor suppressor function, our xenograft assays suggest that MOAP-1 can be additive to the tumor suppressor property of the RASSF1A (Figure 7B) and that the pro-apoptotic function of MOAP-1 may govern the tumor suppressor function (Figure 7C - D). In addition, the important role of RASSF1A in modulating tubulin stability may be shared with its association with MOAP-1 to further strengthen the role of the RASSF1A/MOAP-1 tumor suppressor pathway. Utilizing splenocytes from a *Moap-1$^{-/-}$* mouse (courtesy of Victor Yu, National University, Singapore), we can observe > 2 fold increased cellularity in the *Moap-1$^{-/-}$* versus wild type mice to suggest importance in growth control (unpublished observation). Furthermore, we have reconstituted *Moap-1* into *Moap-1$^{-/-}$* splenocytes and can observe reconstitution of cell death sensitivity upon intrinsic and extrinsic stimulation of *Moap-1$^{-/-}$* splenocytes (unpublished observation). This strongly suggests a role for MOAP-1 in several modes of cell death and the mechanism of how it may control growth and behave as a tumor suppressor.

Our GWAS analysis clearly reveal elements that have been demonstrated to also influence or be influenced by RASSF1A, such as DNA damage control (ATM), Hippo pathway signaling (MST1) (12), cell cycle control (p53 and Aurora Kinases) (45), cell death (Fas and MAP1S) (37,46), and cell signaling (Rap1) (47) to mention a few. The role for MOAP-1 in influencing the aforementioned elements is currently underway and will be informative. Our GWAS study also revealed the possible role for MOAP-1 in influencing pathways modulated by the Warburg effect. These include pyruvate kinase M2 [PKM2] (48) and AMPK (49) as described in Table 2 and 3. Modulation of PKM2 and AMPK will influence how tumors survive in an environment of limited nutrient supply. We are currently exploring changes in metabolic parameters in the presence of overexpressed and unexpressed MOAP-1 expression.

A potentially interesting connection for MOAP-1-dependent tumor suppression is the link to DNA damage control by expression changes in PARP1 and PARP3 (key players involved in the detection and repair of DNA double strand breaks), ATM (a DNA damage checkpoint kinase), ATR interacting protein and the catalytic subunit of DNA activated protein kinase (DNA-PK, a DNA damage sensor) (Table 2 and 3). ATM can promote growth arrest and DNA repair through several different pathways involving hypoxia inducible factor 1α (HIF1α) (50) an element 3.7 fold increased in MOAP-1 overexpressing tumors (data not shown). Since defects in DNA repair pathways can lead to genomic instability and thereby promote carcinogenesis, it is imperative that we understand how MOAP-1 may influence DNA damage control. It is already known that RASSF1A can be modulated by ATM levels (43,51) and associates with the nucleotide excision repair enzyme, XPA

(52,53). It will be interesting to explore how intimately connected MOAP-1 is to RASSF1A modulated DNA damage control (or other biological processes) in order to better understand MOAP-1/RASSF1A-dependent and independent functions.

Preliminary evidence does suggest that there is a strong positive correlation between RASSF1A and MOAP-1 expression in cancer cells. Both have now been demonstrated to be involved in TNF-R1 dependent cell death (8), tubulin stability [this study and (11)] and tumor suppressor function [this study and (2)]. Furthermore, we can observe associations of both RASSF1A and MOAP-1 with Toll receptor (TLR) family of pattern both recognition receptors to affect NFκB activity [(54) and unpublished observations]. Thus, the RASSF1A/MOAP-1 molecular pathway may cooperate together to as a tumor suppressor pathway.

The results of our microarray (summarized in Figure 13) reveals that MOAP-1 may be a tumor suppressor protein with multiple functions in physiology, influencing several molecular pathways connected to numerous aspects of biology. The MOAP-1 interactome needs to be better defined, how RASSF1A influences this interactome needs to be explored and is the loss or reduced expression of both RASSF1A and MOAP-1 found in other diseases. Some of these questions are being currently explored as well as the links outlined in Figure 13.

Acknowledgments

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

JL contributed to > 60% of the data for the manuscript (including the writing and editing). MS contributed to Figure 1D and 2C. AZ contributed to Figure 1E and 2D. YW contributed to early versions of Figure 2D and to maintaining most of the cells in this manuscript. LL contributed to the majority of Figure 10. NV contributed to the editing of the manuscript and parts of Figure 7 and 8. OS contributed to Figure 6. KF contributed to parts of Figure 5. JL contributed to parts of Figure 6. MS contributed to parts of Figure 10. JRB contributed reagents and time to parts of Figure 10. CTT provided MOAP-1 reagents for the study and contributed to the editing of the manuscript. Y-CS contributed to the editing of the manuscript. VC-Yu contributed to the editing of the manuscript and reagents for MOAP-1. JM contributed to Figure 2A and 2B (mainly the access to the breast cancer database used in Figure 2). SB is the corresponding author overseeing this project and manuscript production.

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Legends

Tables

Table 1: MOAP-1 expression is significantly reduced in ER-negative and PR-negative breast cancers. Breast cancer patient data was analyzed based on levels of MOAP-1 expression with relation to various clinical and pathological features of interest as shown. ROC (region of choice) curve analysis calculated MOAP-1 expression cut-off at 0.1124 (low MOAP-1 expression ≤ 0.1124, high MOAP-1 expression > 0.1124). ER-negative breast cancers: n = 64, p value = 1.2E-06; PR-negative breast cancers: n = 82, p value = 7.5E-05.

Table 2. Potential canonical pathways dysregulated by overexpressed Myc-MOAP-1 relative to vector are shown.

Table 3. A select list of genes differentially expressed by overexpressed Myc-MOAP-1 relative to vector control.

Figures

Figure 1. Evidence of reduced MOAP-1 expression from cancer databases and in neuroblastoma. (A) Differential expression analysis of MOAP-1 in normal versus cancer tissues from the microarray database Oncomine (www.oncomine.org). Results from individual microarray studies are each represented by a single point on this plot (n = 48 within each study). Y-axis depicts the fold change in MOAP-1 mRNA levels relative to normal tissue for each of the multiple cancer types for which MOAP-1 expression data were available shown on the x-axis. Horizontal bars represent the average MOAP-1 mRNA fold-change within that cancer type. All fold-changes are greater than or equal to 1.5 and p values less than or equal to 0.05. (B - C) Kaplan-Meier survival curves for neuroblastoma patients demonstrating a correlation between expression levels of (B) MOAP-1 (n high expression = 72, n low expression = 16; expression cut-off: 700.1 for high expression versus low expression for MOAP-1 [arbitrary mRNA units indicated]) and (C) RASSF1A (n high expression = 61, n low expression = 27; expression cut-off: 101.7 [same comparison as for MOAP-1]) with overall patient survival probability from neuroblastoma. (D) mRNA expression of MOAP-1 by qPCR 1 in normal (normal human bronchoepithelial, breast and colon epithelial cells data was pooled) versus neuroblastoma cell lines [Bec (2) C, GoTo, LAN-1, KAN, Nub7, SKNAS and SH-SY5Y]. (E) Protein expression of MOAP-1 in neuroblastoma cell lines as indicated.

Figure 2. Expression of MOAP-1 in breast cancer. (A) A comparison of MOAP-1 mRNA levels in cancerous versus normal breast tissue (n = 10) and breast cancer (n = 176); p value = 2.15E-09. (B) Average MOAP-1 expression is significantly reduced in 3 out of 4 major breast cancer subtypes relative to normal breast tissue. (Luminal A: p value = 0.34, n = 75; Luminal B: p value = 0.0018, n = 19; HER2-amplified: p value = 0.0067, n = 8; Triple negative: p value < 0.0001, n = 56; Normal: n = 10. Note that 18 patients were excluded from this assessment since they could not be classified. Note that "normal" values were plotted from results using normal bronchial,

breast and colon epithelial cells (C) mRNA expression of MOAP- 1 by qPCR in breast cancer patient subtypes as indicated (n = 6). (D) Protein expression of MOAP-1 in the indicated cell lines classified by breast cancer subtypes. (E) MOAP-1 expression is significantly reduced in ER-negative and PR-negative breast cancers. Breast cancer patient data was analyzed based on levels of MOAP-1 expression with relation to various clinical and pathological features of interest as shown. ROC curve analysis calculated MOAP-1 expression cut-off at 0.1124 (low MOAP-1 expression ≤ 0.1124, high MOAP-1 expression > 0.1124). ER-negative breast cancers: n = 64, p value = 1.2E-06; PR-negative breast cancers: n = 82, p = 7.5E-05.

Figure 3. Protein expression of MOAP-1 in the indicated cancer cell lines. Erk 1/2 is shown as a loading control for the lanes and all lanes marked with an "*" are a normal cells. Two forms of MOAP-1 can be observed blood cancers that migrate at ~ 46 kDa instead of the normal 39 kDa. This result has been confirmed using the rabbit anti-MOAP-1 (from QED Biosciences) and rabbit anti-MOAP-1 from Sigma as indicated in panel (B, right side).

Figure 4. MOAP-1 is rapidly turned over in several human cancer cell lines. Immunoblotting (IB) for MOAP-1 was performed in selected cell lines by the addition of the proteosome inhibitor, MG132, at a concentration of 10 μM for the indicated times.

Figure 5. MOAP-1 can inhibit cell proliferation *in vitro*. (A - B) MOAP-1 suppresses H1299 cell growth in culture. H1299 cells stably expressing vector or Myc-MOAP-1 (A) or MOAP-1 shRNA (B) were seeded at 4000 cells per well in a 96 well plate and an MTT assay was carried out as outlined in our experimental methods. Inset represents immunoblot to confirm the expression of Myc-MOAP-1 with Erk1/2 as a loading control. For (A) P value = 0.0006, n = 16 and (B) P value = 0.0022, n = 8. Right of (B), immunoblot to confirm shRNA knockdown of endogenous MOAP-1. (C) Colony formation was carried out in H1299 stable cells expressing the indicated constructs. P value = 0.0361 and n = 4 -5 for both. H1299 cells were seeded at 600 cells per plate and allowed to grow for 11 days prior to staining with crystal violet. Number of cell colonies was counted by visual examination expression of Myc-MOAP-1 can be detected in these cells for > 30 days after stables are formed (see Figure 9C for stable expression of MOAP-1 in H1299).

Figure 6. Defective cell death in the absence of endogenous MOAP-1. (A - C) PARP cleavage was analyzed in response to TNFα/CHX in the indicated cancer cells.. In addition, (D) cell death was also analyzed by staurosporine treatment of H1299 cells. Stable pools of Myc-MOAP-1 expressing H1299 cells were established for (D and E). (E) Annexin-V staining in H1299 stable cells with the indicated expression constructs. All proteins were expressed at similar levels (data now shown). N = 3, p values as indicated.

Figure 7. MOAP-1 can inhibit tumor formation *in vivo*. Xenograft assays were carried out in DAOY medulloblastoma and SKVO3 ovarian cancer cells (A-B), in H1299 lung cancer cells (C-D). Stable cells were utilized for H1299 and SKOV3 cells while DAOY cells contained transiently transfected expression vectors. For SKOV3, the anti-GFP antibody recognized both CFP-MOAP-1 and GFP-1A. For all, cells were harvested 48 hours post transient transfection, resuspended in matrigel and subcutaneously injected into male athymic mice. Immunoblots beside the graphs represent stable or transient expression of the indicated proteins. Athymic nude mice (Taconic Laboratories #NCRNU-M, CrTac:NCr-FoxN1Nu) were utilized for this assay as previously described.(55) For (A) DAOY cells, p value between vector and HA-RASSF1A is 0.0003; p value

between vector and Myc-MOAP-1 or vector and Myc-MOAP-1 plus HA-RASSF1A is 0.0023 and 0.0065 respectively; n = 6 – 10 for all. For right of (A), expressions of the indicated constructs are shown. For SKOV3, p-value between vector and HA-RASSF1A and vector and Myc-MOAP-1 was 0.009 and 0.004 respectively; n = 6 for all. For (C) p value between tumors formed in vector vs MOAP-1 cells was 0.0075, vector and RASSF1A was 0.0214 and vector and RASSF1A/MOAP-1 was 0.0049 (n = 8 - 10 for all). P value between MOAP-1 wild type and δBH3 or BH3 mutant is < 0.027 and < 0.0327, respectively.

Figure 8. MOAP-1 can inhibit tumor formation driven by HCT116 colon cancer cells *in vivo*. HCT116 colon cancer cells with overexpressed proteins (A-B) or with shRNA to MOAP-1 (C) were generated by transient transfection with the indicated expression vectors. Cells were prepared and injected into nude mice as in Figure 9. For HCT116 cells, p value between vector and HA-RASSF1A and vector and Myc-MOAP-1 was 0.0026 and 0.0043 respectively; n = 10 - 16 for all. Middle of (B), expression of the indicated constructs are shown as well as mouse and tumor images and far right of (B), representative tumors at day 35 of tumor growth. For (A), tumour images were captured using a multispectral FX instrument from Carestream to support the use of stable expression of GFP constructs for this assay. MOAP-1 shRNA in (C) resulting in > 70% loss of MOAP-1 expression as presented.

Figure 9. MOAP-1 can associate robustly with tubulin isoforms, may not require RASSF1A for this association and can stabilize microtubules. (A) Association of MOAP-1 with tubulin isoforms in SW40 cells, in (B) H1299 cells and (C) in SW480 cells using previously published mutants of MOAP-1 as indicated. (D) The acetylation status of α-tubulin -/+ Myc-MOAP-1 was determined with an anti-acetyl-α-tubulin antibody as indicated and quantified in the bottom panel. Bottom panel, total lysates of H1299 cells -/+ MOAP-1 or 1A were immunoblotted for acetylated-tubulin followed by reprobing with an α-tubulin antibody to total tubulin content. Densitometry was carried on the immunoblots using the ImageJ software and plotted as shown. Expression of Myc-MOAP-1 and HA-1A was comparable (data not shown) and n = 3 for each point independently carried out.

Figure 10. Genes differentially modulated by the transient overexpression of Myc-MOAP-1. (A) Volcano plot of the distribution of genes differentially regulated by Myc-MOAP-1 overexpression. (B) Histogram plot of the total number of gene expression changes associated with the indicated biological function in MOAP-1 overexpressing HCT 116 tumor cells. (C) Validation of a few selected genes from Table 3.

Figure 11. Summary of genes modulated by the loss of MOAP-1. Green names indicate down-regulation of expression; Red names indicate up-regulation of expression.

Table 1. MOAP-1 expression is significantly reduced in ER-negative and PR-negative breast cancers.

	No. patients with low MOAP-1 (log2 mRNA≤0.1124)	No. patients with high MOAP-1 (log2 mRNA>0.1124)	P value
Total # patients	**104**	**72**	
Cancer recurrence	49	39	0.36071
Death	30	27	0.23622
Negative ER status	52	12	1.2E-06
Negative PR status	61	21	7.5E-05
HER2 amplified	18	12	0.9119
Breast cancer family history	45	32	0.85813

Table 2. Potential canonical pathways disregulated by overexpressed Myc-MOAP-1 (relative to vector)

Ingenuity Canonical Pathways[b]	-log (p-value)[c]	Ratio[d]	Molecules[e]
HIF1α Signaling	3.3	0.15	TP53, MAPK6, PIK3R5, KRAS, HIF1A, SLC2A3, LDHB, ARNT, PIK3R3, CUL2, NCOA1, PIK3CB, APEX1, MMP17, LDHA, ATM
EGF Signaling	2.8	0.17	PIK3R3, JAK1, SRF, PIK3R5, PIK3CB, STAT3, ELK1, ATM, EGFR
ILK Signaling	2.3	0.11	RELA, TMSL3, MYL6, CFL1, PPP2R2A, ACTB, ILK, PIK3R5, HIF1A, ATF2, PTEN, PIK3R3, MYL9, PPP2CB, RHOA, ARHGEF6, ILKAP, PIK3CB, RPS6KA5, ITGB6, ATM
PI3K/AKT Signaling	2.2	0.11	TP53, RELA, JAK1, PPP2R2A, TYK2, ILK, KRAS, PTEN, YWHAQ, PIK3R3, PPP2CB, HSP90AB1, GAB1, PIK3CB, MCL1
FAK Signaling	2.2	0.12	PIK3R3, ASAP1, ACTB, ARHGEF6, PIK3R5, CRK, TLN1, KRAS, PIK3CB, PTEN, ATM, EGFR
mTOR Signaling	2.1	0.11	MAPKAP1, PRKAB1, PPP2R2A, PIK3R5, FKBP1A, KRAS, HIF1A, PRKAG1, PIK3R3, PPP2CB, EIF4G2, RHOA, EIF4A1, PIK3CB, RPS6KA5, ATM, EIF4B
Notch Signaling	2.1	0.16	MAML1, ADAM17, HES5, LFNG, NOTCH1, APH1A, PSEN1
DNA Double-Strand Break Repair by NHEJ	2.1	0.21	PRKDC, DCLRE1C, PARP1, ATM
Myc Mediated Apoptosis Signaling	2.0	0.15	YWHAQ, PIK3R3, TP53, APAF1, PIK3R5, KRAS, PIK3CB, FAS, ATM
p53 Signaling	1.9	0.13	PIK3R3, TP53, PRKDC, TP53AIP1, PCNA, APAF1, PIK3R5, PIK3CB, FAS, CDK2, PTEN, ATM
JAK/Stat Signaling	1.9	0.14	PIK3R3, JAK1, CISH, TYK2, PIK3R5, KRAS, PIK3CB, STAT3, ATM
SAPK/JNK Signaling	1.9	0.12	MAP4K3, PIK3R3, TP53, LCK, GAB1, PIK3R5, CRK, KRAS, PIK3CB, ELK1, ATM, ATF2
AMPK Signaling	1.5	0.08	CAB39, PRKAB1, PPP2R2A, PIK3R5, PFKL, PFKFB2, PRKAG1, PIK3R3, PPP2CB, SMARCA2, PIK3CB, PPAT, PRKAR1A, ATM
EIF2 Signaling	1.5	0.10	PIK3R3, EIF2S2, EIF2B4, EIF4G2, EIF4A1, EIF2B5, PIK3R5, KRAS, PIK3CB, ATM
Apoptosis Signaling	1.3	0.10	TP53, RELA, APAF1, KRAS, DIABLO, LOC100510692/NAIP, FAS, DFFA, MCL1, PARP1
Cell Cycle: G2/M DNA Damage Checkpoint	1.3	0.12	YWHAQ, TP53, PRKDC, CKS1B, TOP2A, ATM
PTEN Signaling	1.2	0.09	PIK3R3, RELA, BMPR1B, BMPR1A, PIK3R5, ILK, FGFR2, KRAS, PIK3CB, PTEN, EGFR
NF-κB Signaling	1.0	0.09	RELA, TNFSF11, PIK3R5, FGFR2, KRAS, NGF, PIK3R3, LCK, BMPR1B, BMPR1A, TLR6, TGFA, PIK3CB, EGFR, ATM
Integrin Signaling	1.0	0.08	TSPAN7, ASAP1, ACTB, ILK, PIK3R5, CRK, TLN1, KRAS, PTEN, PIK3R3, RHOA, ARPC4, ILKAP, PIK3CB, ITGB6, NEDD9, ATM
TGF-β Signaling	0.9	0.09	SMAD2, NKX2-5, ZNF423, BMPR1B, AMH, BMPR1A, ACVR1, KRAS
Death Receptor Signaling	0.8	0.09	RELA, APAF1, CFLAR, DIABLO, LOC100510692/NAIP, FAS
Wnt/β-catenin Signaling	0.8	0.08	TP53, SOX4, AXIN2, SOX1, PPP2R2A, WNT2B, ACVR1, ILK, KREMEN1, PPP2CB, SOX3, GNAO1, TLE3, SOX18
TNFR1 Signaling	0.2	0.06	RELA, APAF1, LOC100510692/NAIP

[a,b]Ingenuity canonical signaling pathways that were most significant to the WT MOAP-1 versus vector control HCT 116 xenografts dataset were assigned from the IPA library of canonical pathways.
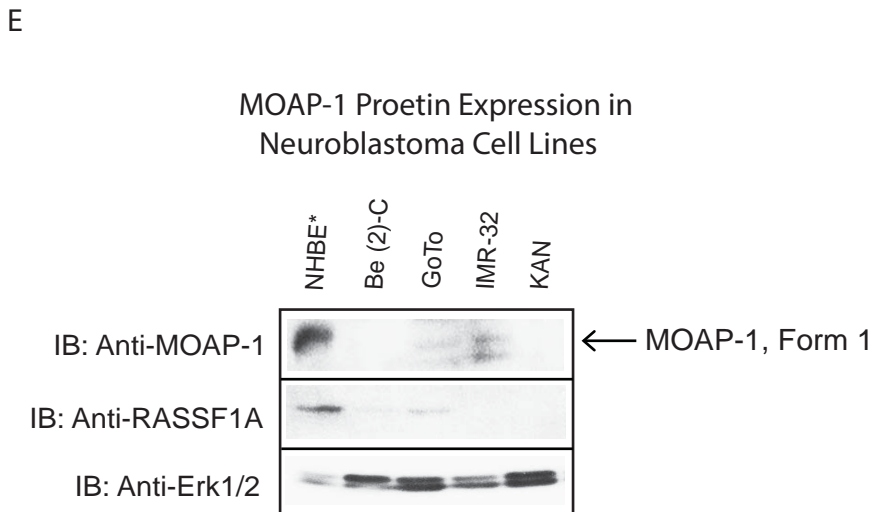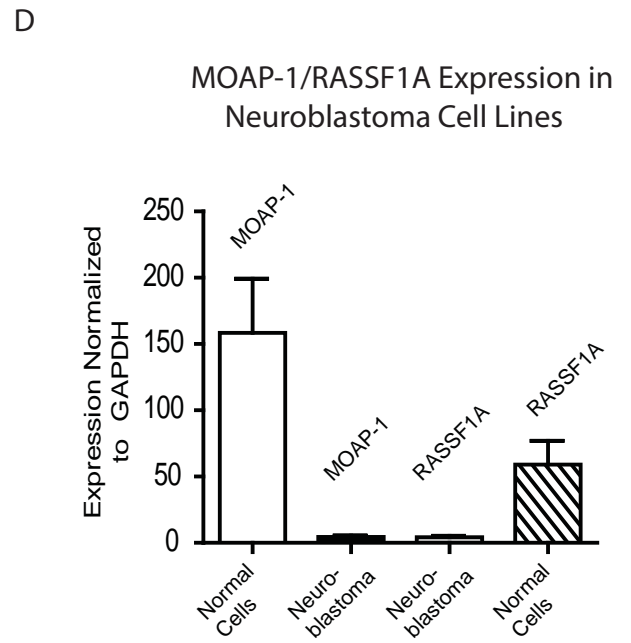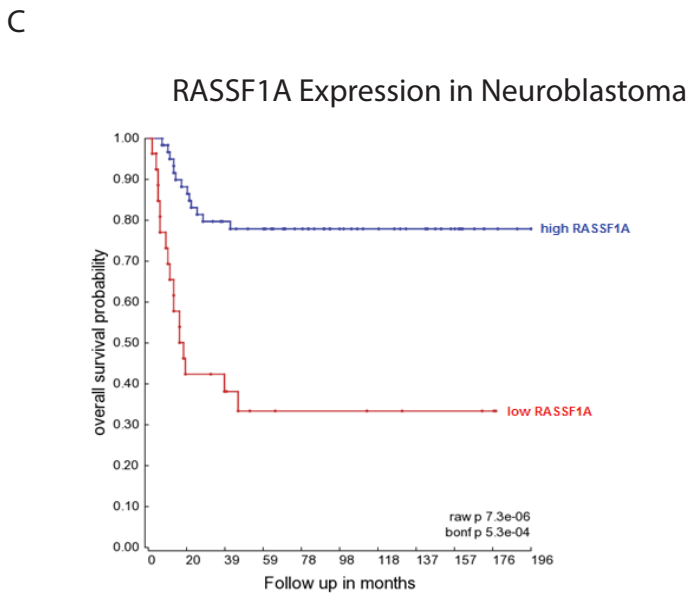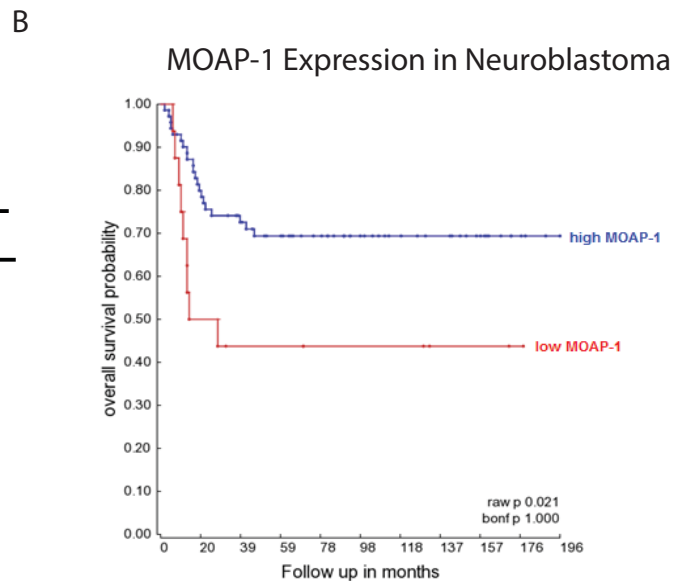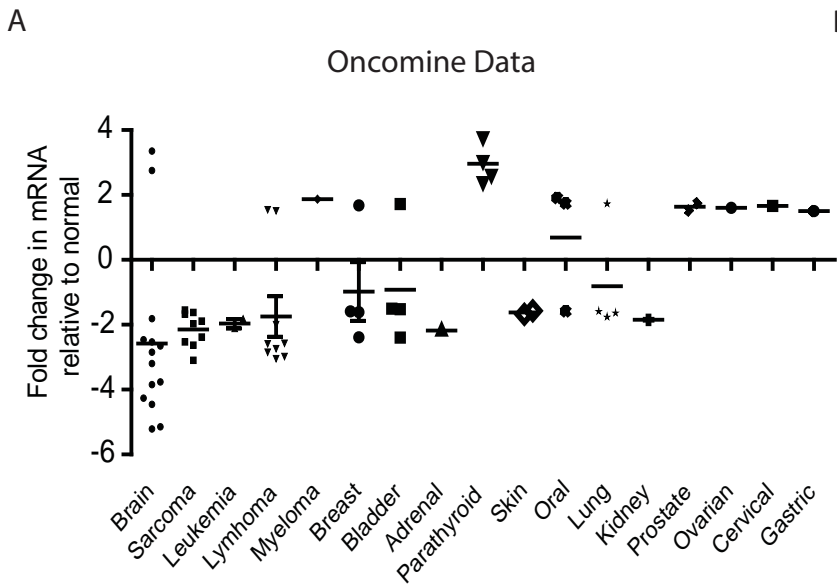
[c]-log(p-value) ≥1.3 is equivalent to p-value ≤0.05

[d]Ratio gives the number of dataset molecules that meet cut criteria in a given pathway divided by the total number of known molecules in that pathway.

[e]Molecules from the dataset that meet cut criteria and are involved in the corresponding signaling pathway.
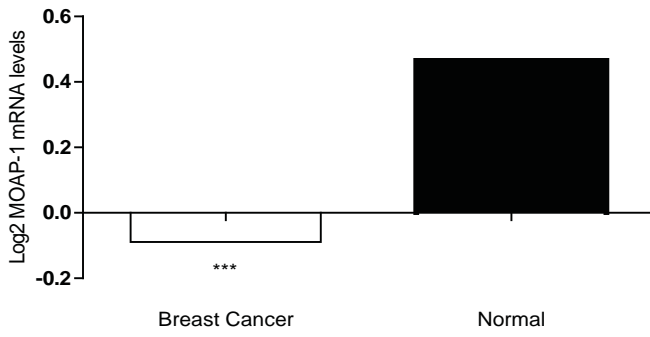
Table 3: Selected genes differentially expressed by overexpressed Myc-MOAP-1 (relative to vector control)

Biological association	GenBank accession no.	Probe Name	Gene Symbol	Description	Avg. fold change	p-value
Cell cycle	NM_032811	A_24_P21752	TBRG1	transforming growth factor beta regulator 1	3.8	1.4E-03
	NM_000546	A_23_P26810	TP53	tumor protein p53	3.2	4.3E-03
	NM_033331	A_23_P216679	CDC14B	CDC14 cell division cycle 14 homolog B (S. cerevisiae)	2.5	1.5E-02
	NM_001260	A_23_P139958	CDK8	cyclin-dependent kinase 8	2.5	1.8E-04
	NM_004217	A_23_P130182	AURKB	aurora kinase B	2.3	5.6E-03
	NM_000051	A_23_P35916	ATM	ataxia telangiectasia mutated	2.2	2.6E-03
	NM_001798	A_23_P98898	CDK2	cyclin-dependent kinase 2	2.0	7.2E-05
	NM_001826	A_32_P206698	CKS1B	CDC28 protein kinase regulatory subunit 1B	2.0	5.3E-03
Cell death	NM_003374	A_32_P163169	VDAC1	voltage-dependent anion channel 1	3.7	1.6E-03
	NM_021960	A_24_P336759	MCL1	myeloid cell leukemia sequence 1 (BCL2-related)	3.2	9.5E-03
	NM_006282	A_24_P94054	STK4	serine/threonine kinase 4	3.0	4.6E-03
	NM_020313	A_32_P204381	CIAPIN1	cytokine induced apoptosis inhibitor 1	2.8	9.6E-03
	NM_007350	A_24_P915692	PHLDA1	pleckstrin homology-like domain, family A, member 1	2.7	8.0E-04
	NM_000043	A_33_P3332112	FAS	Fas (TNF receptor superfamily, member 6)	2.1	6.3E-04
	NM_181861	A_23_P36611	APAF1	apoptotic peptidase activating factor 1	2.1	1.9E-03
	NM_022112	A_23_P340171	TP53AIP1	tumor protein p53 regulated apoptosis inducing protein 1	2.1	9.3E-04
	NM_006098	A_23_P41716	GNB2L1	guanine nucleotide binding protein (G protein), beta polypeptide 2-like 1	2.0	2.3E-02
	NM_138929	A_33_P3330125	Smac/DIABLO	diablo homolog (Drosophila), nuclear gene encoding mitochondrial protein	-2.2	6.5E-03
	NM_018174	A_33_P3343090	MAP1S	microtubule-associated protein 1S	-2.3	1.0E-02
Gene expression	NM_003131	A_24_P337657	SRF	serum response factor (c-fos serum response element-binding transcription factor)	4.9	1.1E-02
	NM_006565	A_24_P347704	CTCF	CCCTC-binding factor (zinc finger protein)	3.8	2.1E-03
	NM_013449	A_23_P203841	BAZ2A	bromodomain adjacent to zinc finger domain, 2A	3.7	4.8E-03
	NM_181054	A_33_P3231277	HIF1A	hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)	3.7	1.8E-03
	NM_018416	A_24_P21985	FOXJ2	forkhead box J2	3.6	3.9E-03
	NM_001418	A_33_P3306545	EIF4G2	eukaryotic translation initiation factor 4 gamma, 2	3.5	2.1E-04
	NM_031314	A_24_P178423	HNRNPC	heterogeneous nuclear ribonucleoprotein C (C1/C2)	3.2	4.9E-04
	NM_001003652	A_32_P12580	SMAD2	SMAD family member 2	2.9	1.2E-04
	NM_001880	A_24_P128145	ATF2	activating transcription factor 2	2.7	1.2E-03
	NM_001040619	A_24_P33895	ATF3	activating transcription factor 3	2.7	6.2E-03
	NM_006015	A_24_P92952	ARID1A	AT rich interactive domain 1A (SWI-like)	2.6	4.2E-03
	NM_213662	A_23_P100795	STAT3	signal transducer and activator of transcription 3 (acute-phase response factor)	2.4	1.9E-02
	NM_001034116	A_23_P154058	EIF2B4	eukaryotic translation initiation factor 2B, subunit 4 delta, 67kDa	2.3	2.0E-02
	NM_022490	A_23_P9458	POLR1E	polymerase (RNA) I polypeptide E, 53kDa	2.3	3.1E-02
	NM_139045	A_23_P60354	SMARCA2	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 2	2.2	3.0E-03
	NM_005229	A_23_P171054	ELK1	ELK1, member of ETS oncogene family	2.2	1.9E-02
	NM_015832	A_24_P119201	MBD2	methyl-CpG binding domain protein 2	2.2	3.5E-02
	NM_003907	A_23_P110062	EIF2B5	eukaryotic translation initiation factor 2B, subunit 5 epsilon, 82kDa	2.2	1.5E-02
	NM_000938	A_23_P144311	POLR2B	polymerase (RNA) II (DNA directed) polypeptide B, 140kDa	2.2	1.7E-04
	NM_001012426	A_33_P3284019	FOXP4	forkhead box P4	-2.2	7.0E-03
	NM_130439	A_33_P3383029	MXI1	MAX interactor 1	-2.3	3.4E-04
	NM_002357	A_23_P408094	MXD1	MAX dimerization protein 1	-2.5	1.7E-03
Cell growth and proliferation	NM_002748	A_24_P333663	MAPK6	mitogen-activated protein kinase 6	4.5	9.3E-04
	NM_002227	A_33_P3784283	JAK1	Janus kinase 1	3.3	3.0E-04
	NM_004655	A_23_P148015	AXIN2	axin 2	3.1	9.8E-04
	NM_001001924	A_23_P347169	MTUS1	microtubule associated tumor suppressor 1	3.1	2.4E-04
	NM_153000	A_23_P337262	APCDD1	adenomatosis polyposis coli down-regulated 1	3.0	2.2E-03
	NM_207123	A_23_P335239	GAB1	GRB2-associated binding protein 1	2.4	1.4E-02
	NM_152858	A_32_P219368	WTAP	Wilms tumor 1 associated protein	2.2	5.7E-04
	NM_003107	A_24_P911676	SOX4	SRY (sex determining region Y)-box 4	2.2	3.4E-03
	NM_000314	A_24_P913115	PTEN	Phosphatase and tensin homolog	2.2	2.3E-03
	NM_025208	A_24_P124349	PDGFD	platelet derived growth factor D	-2.0	6.0E-03
	NM_201283	A_33_P3351944	EGFR	epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian)	-2.2	3.1E-03
	NM_017617	A_33_P3370424	NOTCH1	Notch homolog 1, translocation-associated (Drosophila)	-2.2	3.4E-02
DNA replication, recombination, and repair	NM_001003716	A_23_P324989	RECQL5	RecQ protein-like 5	4.5	9.5E-03
	NM_006904	A_23_P9603	PRKDC	protein kinase, DNA-activated, catalytic polypeptide	4.5	8.9E-05
	NM_003211	A_33_P3357445	TDG	thymine-DNA glycosylase	4.2	2.0E-04
	NM_001067	A_23_P118834	TOP2A	topoisomerase (DNA) II alpha 170kDa	3.6	4.1E-05
	NM_003286	A_23_P305507	TOP1	topoisomerase (DNA) I	3.6	1.1E-04
	NM_080649	A_23_P151653	APEX1	APEX nuclease (multifunctional DNA repair enzyme) 1	2.6	5.4E-03
	NM_139178	A_33_P3225250	ALKBH3	alkB, alkylation repair homolog 3 (E. coli)	2.4	9.5E-03
	NM_001618	A_33_P3236921	PARP1	poly (ADP-ribose) polymerase 1	2.4	1.1E-03
	NM_000122	A_23_P5325	ERCC3	excision repair cross-complementing rodent repair deficiency, complementation group 3	2.2	9.0E-03
	NM_024782	A_24_P251381	NHEJ1	nonhomologous end-joining factor 1	2.2	6.3E-03
	NM_001033858	A_33_P3386219	DCLRE1C	DNA cross-link repair 1C (PSO2 homolog, S. cerevisiae)	2.2	3.2E-02
	NM_004284	A_23_P45831	CHD1L	chromodomain helicase DNA binding protein 1-like	2.1	1.8E-02
	NM_005485	A_24_P402779	PARP3	poly (ADP-ribose) polymerase family, member 3	2.1	1.8E-02
	NM_002592	A_33_P3258612	PCNA	proliferating cell nuclear antigen	2.0	6.8E-03
	NM_032166	A_24_P371425	ATRIP	ATR interacting protein	-2.1	3.5E-03
Cell-to-cell signaling and interaction	NM_003247	A_33_P3365735	THBS2	thrombospondin 2	10.4	3.5E-02
	NM_000093	A_23_P83818	COL5A1	collagen, type V, alpha 1	9.0	4.7E-02
	NM_006403	A_23_P344555	NEDD9	neural precursor cell expressed, developmentally down-regulated 9	2.4	1.2E-02
	NM_018891	A_23_P160968	LAMC2	laminin, gamma 2	2.3	4.8E-03
	NM_000426	A_23_P70719	LAMA2	laminin, alpha 2	2.2	2.1E-03
	NM_001943	A_23_P141730	DSG2	desmoglein 2	2.1	2.7E-02
	NM_001014795	A_23_P105066	ILK	integrin-linked kinase	2.1	1.3E-02
	NM_003183	A_23_P143120	ADAM17	ADAM metallopeptidase domain 17	2.1	1.2E-03
	NM_002870	A_23_P46369	RAB13	RAB13, member RAS oncogene family	2.1	2.9E-02
	NM_006105	A_23_P151307	RAPGEF3	Rap guanine nucleotide exchange factor (GEF) 3	2.0	4.2E-02
	NM_001085461	A_33_P3209716	CTNND1	catenin (cadherin-associated protein), delta 1	-2.6	8.0E-04
	NM_005602	A_33_P3255404	CLDN11	claudin 11	-2.9	3.8E-02
	NM_002885	A_24_P36890	RAP1GAP	RAP1 GTPase activating protein	-3.7	5.3E-04

A

Oncomine Data

B

MOAP-1 Expression in Neuroblastoma

raw p 0.021
bonf p 1.000

C

RASSF1A Expression in Neuroblastoma

raw p 7.3e-06
bonf p 5.3e-04

D

MOAP-1/RASSF1A Expression in Neuroblastoma Cell Lines

E

MOAP-1 Proetin Expression in Neuroblastoma Cell Lines

IB: Anti-MOAP-1 → MOAP-1, Form 1

IB: Anti-RASSF1A

IB: Anti-Erk1/2

Law et al, Figure 1

A

MOAP-1 Expression in Breast Cancer

Log2 MOAP-1 mRNA levels

0.6
0.4
0.2
0.0
-0.2

***

Breast Cancer   Normal

B

A comparison of MOAP-1 expression in four major breast cancer subtypes

Increasing cancer aggressivness

Log2 MOAP-1 mRNA levels

0.5
0.0
-0.5
-1.0

Normal   Luminal A   Luminal B   Her2-amplified   Triple-negative

**
**
***

C

MOAP-1 mRNA Expression in Breast Cancer Patients

Expression of MOAP-1 (Relative to GAPDH)

300
250
200
150
100
50
0

Normal   Luminal A   Luminal B   Her2+ Amplified   TNBC
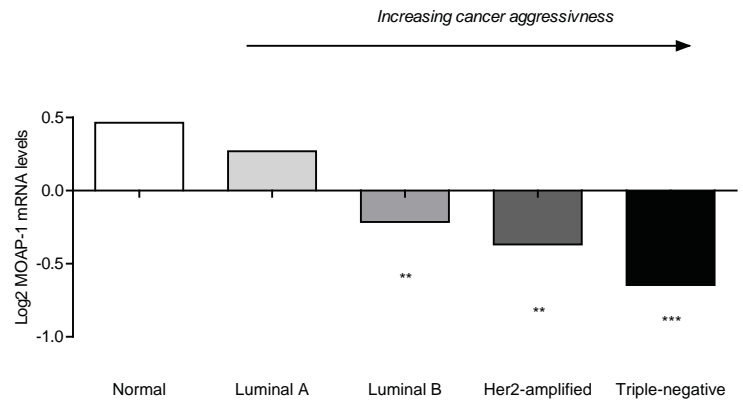
D

MOAP-1 Protein Expression in Breast Cancer Cells

NHBE* hTERT# MCF10A T47D (Luminal A) BT-474 ZR-75 SKBR3 MDA-MB-453 MDA-MB-468 MDA-MB-231 BT549 BT20

IB: Anti-MOAP-1

IB: Anti-Erk1/2

Lum. B   Her2+ Ampl.   TNBC Cells

* Normal human broncho-epithelial cells

hTERT immoralized Normal human breast epithelial cells

E

HER2 Amplified
PR Negative
ER Negative
Death
Recurrence

☐ MOAP-1 > 0.1124
■ MOAP-1 ≤ 0.1124

***
***

0   20   40   60   80

Percentage of patients

Law et al, Figure 2

A

Melanoma | Bone | Lung | Liver

HEMa-Lp* | A2058 | WM35 | WM793 | SAOS-2 | U2OS | H1299 | A549 | HepG2 | Hep3B

IB: Anti-MOAP-1

IB: Anti-Erk1/2

← MOAP-1, Form 1 (39 kDa)

* Primary non transformed cell line

B

Colon | Leukemia | Leukemia

HCT116 | HT-29 | SW480 | T84 | Caco-2 | J77 (T-ALL) | KG1 (AML) | C1 (pre B-ALL) | SEM (MLL) | Hep3B (Liver) | HT-29 (Colon) | KG1 (AML) | C1 (pre B-ALL)

IB: Anti-MOAP-1

IB: Anti-Erk1/2

← Form 2 (46 kDa) →
← Form 1 (39 kDa) →

IB: Anti-MOAP-1 (Sigma)

IB: Anti-Erk1/2

* Primary non transformed cell line

C

Ovary | Pancreas | Prostate

SKOV3 | OVCAR-3 | OVCAR-29 | PANC1 | CAPAN2 | PNT1A* | DU145

MOAP-1, Form 1

IB: Anti-Erk1/2

Ovary

SKOV3 | OVCAR-3 | OVCAR-29

IB: Anti-RASSF1A

IB: Anti-Erk1/2

*Primary Prostate Epithelial Cell Line

Law et al, Figure 3

0 2 4 6   (h) 10 μM MG132

IB: Anti-
MOAP-1

T47D (Luminal A)

MCF-7 (Luminal A)

SKBR3 (Her2+ Amplified)

MDA-MB-468 (TNBC)

MDA-MB-231 (TNBC)

Breast
Cancer

A2058 (Melanoma)

A549 (Lu ng Cancer)

SKOV3 (Ovarian Cancer)

IB: Anti-
Erk1/2

A549

Law et al, Figure 4

A

MTT Absorbance at 560 nm

Control Vector
Myc-MOAP-1

IB: Anti-Myc
IB: Anti-Erk1/2

Vector

+ Myc-MOAP-1

Days Elaspsed Since Replating

B

HCT116 Cells
(MTT absorbance at day 8)

MTT Absorbance at 560 nm

Control
Vector

MOAP-1
shRNA

Control Vector
MOAP-1 shRNA

IB: Anti-MOAP-1
IB: Anti-Erk1/2

C

H1299 Stables

Colonies Formed (#Crystal Violet Positive Cells)

Vector

+ Myc
MOAP-1

Vector   + Myc
         MOAP-1

Law et al, Figure 5

Law et al, Figure 6

A Xenograft Assay in Nude Mice

DAOY Cells SKOV3 Cells

IB: Anti-Myc
IB: Anti-HA — DAOY Cells
IB: Anti-ERK 1/2

IB: Anti-GFP — SKOV3 Cells
IB: Anti-ERK 1/2

B DAOY Cells SKOV3 Stable Cells

Vector | HA-RASSF1A | Myc-MOAP-1 | Myc-MOAP-1 + HA-RASSF1A

Excised tumor

Vector | HA-RASSF1A | Myc-MOAP-1

No tumors formed

Excised tumor | GFP Detection
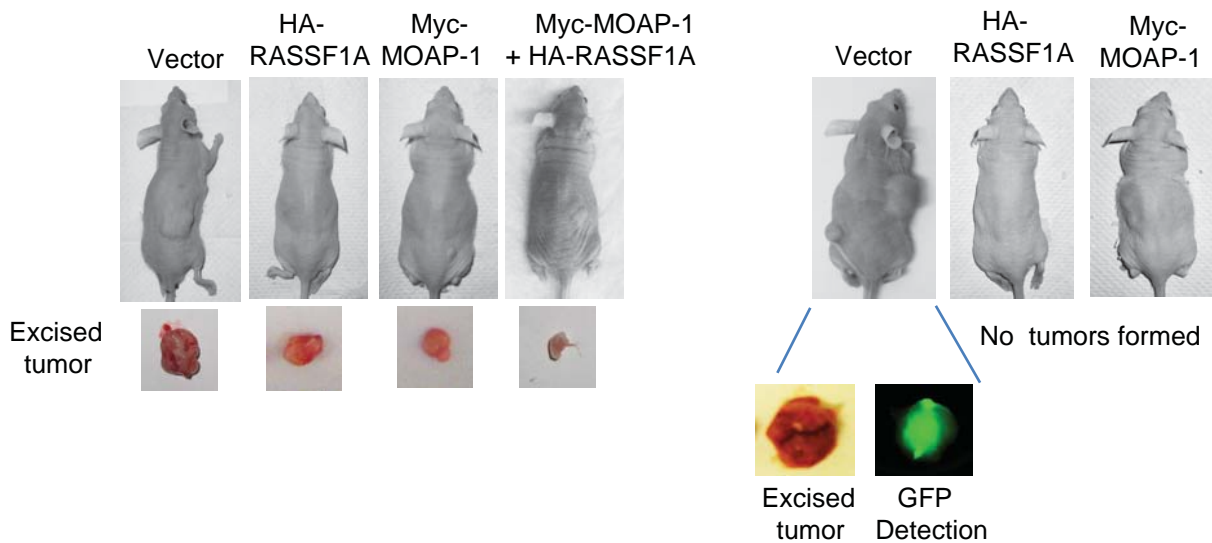
C Xenograft Assay in Nude Mice
(Day 35, H1299 Cells)

IB: Anti-Myc
IB: Anti-HA

D H1299 Stable Cells

Vector | MOAP-1 + 1A | MOAP-1 + BH3 Mutant

Excised tumor

Law et al, Figure 7

A

HCT116 Cells

GFP
(Tumor)

GFP-
RASSF1A
(Tumor)

CFP-
MOAP-1
(Tumor)

pCDNA3
Vector
(GFP negative
cells)

Immunofluoresence on Day 35

kDa

70 -

40 -

35 -

GFP

CFP-MOAP-1

GFP-RASSF1A

IB: Anti-GFP

B

Xenograft Assay in Nude Mice
(HCT116 Cells)

Tumor Volume (mm^3)

Days after s.c. injection

Vector

Myc-MOAP-1

HA-RASSF1A

Vector

Myc-MOAP-1

HA-1A

IB: Anti-Myc

IB: Anti-HA

Vector   + MOAP-1   + RASSF1A

Excised Tumour at Day 35

C

HCT116 Cells

* p value = 0.004, n = 6

MOAP-1 shRNA

Vector

Tumor Volume (mm^3)

Days after s.c. injection

Law et al, Figure 8

A

Asssociation of MOAP-1
with Tubulin in SW480
(*Rassf1a positive* cells)

B

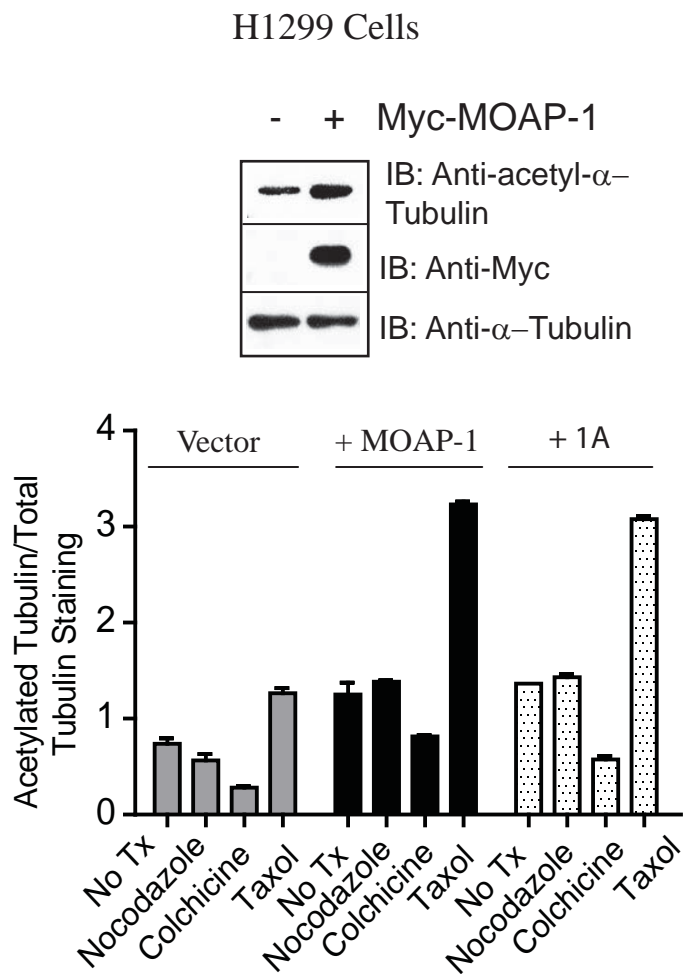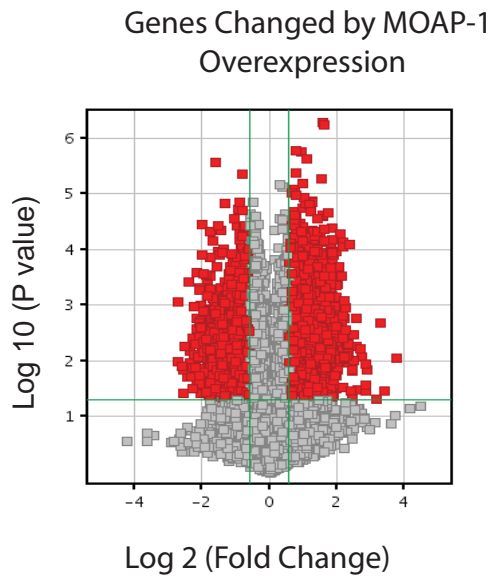Asssociation with γ–Tubulin
in H1299 Cells
(*Rassf1a negative* cells)

C

Residues on MOAP-1 for:
α-tubulin - 190-250; 1-115
γ-tubulin - 1-115
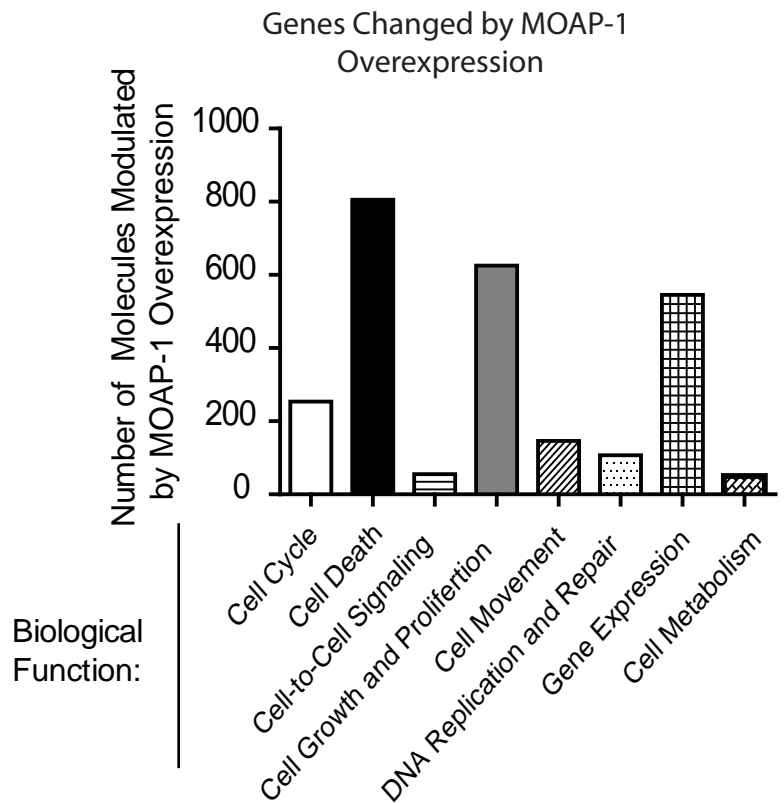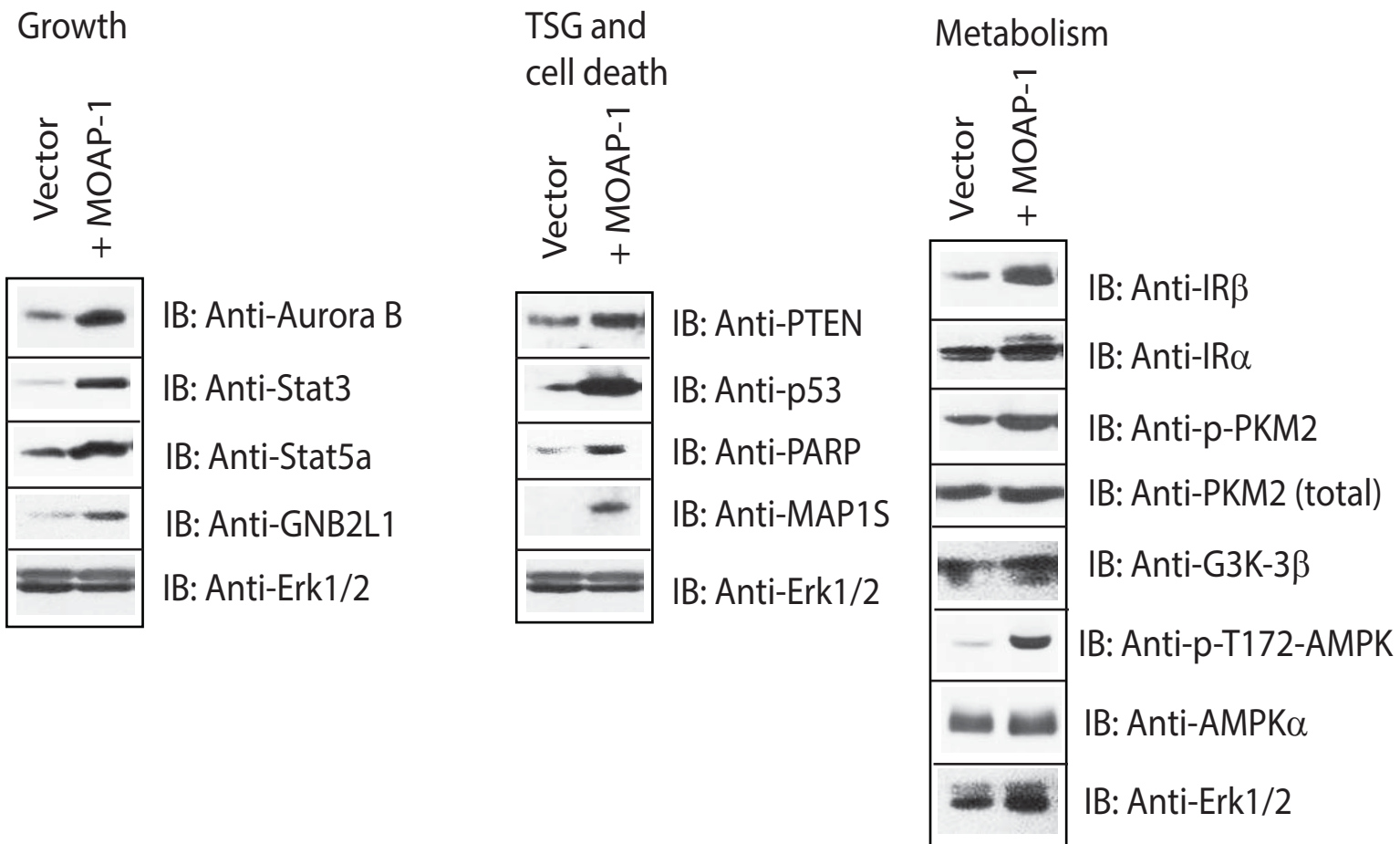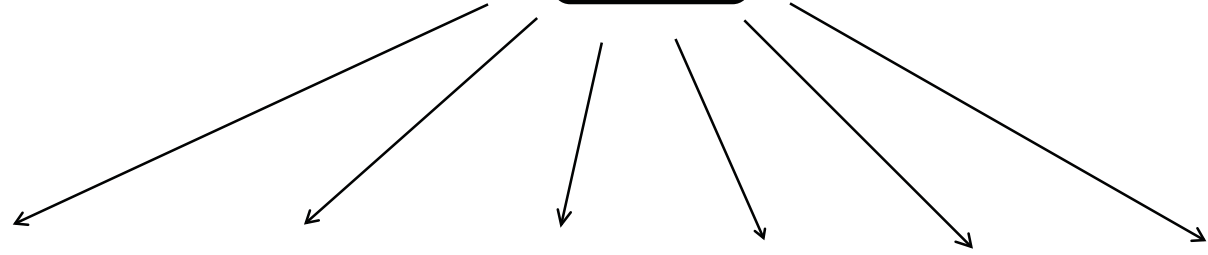
D

H1299 Cells

Law et al, Figure 9

A Genes Changed by MOAP-1 Overexpression

B Genes Changed by MOAP-1 Overexpression

C Validation of Microarray targets in Xenograft-Derived Tumor Lysates

Growth

- IB: Anti-Aurora B
- IB: Anti-Stat3
- IB: Anti-Stat5a
- IB: Anti-GNB2L1
- IB: Anti-Erk1/2

TSG and cell death

- IB: Anti-PTEN
- IB: Anti-p53
- IB: Anti-PARP
- IB: Anti-MAP1S
- IB: Anti-Erk1/2

Metabolism

- IB: Anti-IRβ
- IB: Anti-IRα
- IB: Anti-p-PKM2
- IB: Anti-PKM2 (total)
- IB: Anti-G3K-3β
- IB: Anti-p-T172-AMPK
- IB: Anti-AMPKα
- IB: Anti-Erk1/2

Law et al, Figure 10

Law et al, Figure 11