University of Alberta

MOAP-1: A Candidate Tumor Suppressor Protein

by

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ABSTRACT

Modulator of apoptosis 1 (MOAP-1) is a BH3-like protein that plays a key role in death receptor-dependent apoptosis and cooperates with the tumor suppressor protein Ras association domain family 1A (RASSF1A) to promote Bax activation during cell death. Although loss of RASSF1A expression is frequently observed in human cancers, it is currently unknown if MOAP-1 expression may also be affected during carcinogenesis to result in uncontrolled malignant growth. Therefore, we sought to investigate the role of MOAP-1 in cancer development. Here, we demonstrate that MOAP-1 can effectively inhibit cell proliferation both *in vitro* and *in vivo* and undergoes frequent loss of expression during carcinogenesis. The ability of MOAP-1 to suppress tumor formation requires protein kinase C (PKC)-dependent non-degradative ubiquitination at lysine residue K278 and may involve the novel MOAP-1-interacting protein receptor for activated C-kinase 1 (RACK1). Our data indicate that MOAP-1 is a candidate tumor suppressor protein regulated by PKC-dependent ubiquitination.

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LIST OF ABBREVIATIONS AND SYMBOLS

1A – RASSF1A
A – alanine
ACVR1C – activin A receptor, type 1C
ADP – adenosine diphosphate
AIDS – acquired immune deficiency syndrome
AIF – apoptosis-inducing factor
AKAP12 – A kinase anchor protein 12
ALL – acute lymphocytic leukemia
AML – acute myelocytic leukemia
ANOVA – analysis of variance
Apaf-1 – apoptotic protease activating factor 1
APC – anaphase-promoting complex
ARF – alternate reading frame
ARF1 – ADP-ribosylation factor 1
ARID1A – AT rich interactive domain 1A
Asp – aspartic acid
ATF – activating transcription factor
ATM – ataxia telangiectasia mutated
ATP – adenosine triphosphate
Bad – Bcl-2 antagonist of cell death
Bak – Bcl-2 antagonist/killer
Bax – Bcl-2-associated x protein
Bcl-2 – B-cell lymphoma-2
Bcl-2A1 – Bcl-2-like protein A1
Bcl-B – BCL2-like 10
Bcl-xL – Bcl-2-like protein x large isoform
Bcl-w – Bcl-2-like protein w
BDNF – brain-derived neurotrophic factor
BH – Bcl-2 homology
Bid – BH3-interaction domain death agonist
Bik – Bcl-2-interacting killer
Bim – Bcl-2-interacting mediator of cell death
Bmf – Bcl-2 modifying factor
BMPR1B – bone morphogenetic protein receptor, type IB
Bok – Bcl-2-related ovarian killer
C – carboxy
C1 – protein kinase C conserved region 1
C2 – protein kinase C conserved region 2
CAD – Caspase-activated DNAse
cAMP - cyclic adenosine monophosphate
CARD – caspase recruitment domain
CASP10 – caspase 10
CAT-1 – cationic amino transporter 1
CDC – cell division cycle

CDK – cyclin-dependent kinase
CEBPα – CCAAT-enhancer binding protein α
CHO – Chinese hamster ovary
CHOP – CEBP homologous protein
CHX - cycloheximide
CIP – calf intestinal alkaline phosphatase
CIS – cytokine-inducible Src homology 2 domain-containing protein
CISH – cytokine inducible SH2-containing protein
CLDN11 – claudin 11
CLL – chronic lymphocytic leukemia
CNK1 – connector enhancer of kinase suppressor of Ras 1
CO_2 – carbon dioxide
COL5A1 – collagen, type V, alpha 1
CREB – cyclic adenosine monophosphate response element binding
CTNND1 – catenin (cadherin-associated protein), delta 1
CXCL11 – chemokine (C-X-C motif) ligand 11
CYCS – cytochrome C
D – aspartic acid
Da – dalton
DAG – diacylglycerol
DAPI – 4'6-diamino-2-phenylindole
DD – death domain
DDX – DEAD-box protein
DED – death effector domain
DIABLO – Direct IAP-binding protein with low pI
DISC – death-inducing signaling complex
DMP1 – dentin matrix protein 1
DMSO – dimethylsulfoxide
DMTF1 – cyclin D binding myb-like protein 1
DNA – deoxyribonucleic acid
DR4 – death receptor 4
DR5 – death receptor 5
E – glutamic acid
E1 – ubiquitin activating enzyme
E1A – early region 1A
E2 – ubiquitin conjugating enzyme
E3 – ubiquitin ligase
E4 – ubiquitin chain elongation or assembly factors
E7 – early region 7
ECL – enhanced chemiluminescence
EDTA – ethylenediaminetetraacetic acid
EGF – epidermal growth factor
EGFR – epidermal growth factor receptor
eIF – eukaryotic initiation factor
EPLIN – epithelial protein lost in neoplasm
ER – estrogen receptor

ERCC3 – excision repair cross-complementing rodent repair deficiency, complementation group 3
Erk – extracellular signal-regulated kinase
F – phenylalanine
FACS – fluorescence-activated cell sorting
FADD – Fas-associated death domain
Fas (CD95/Apo-1) – apoptosis-stimulating fragment
FOXJ2 – forkhead box J2
FOXO3 – forkhead box O3
g – gram
G1 – Gap 1
G2 – Gap 2
GFP – green fluorescent protein
GNB2L1 – guanine nucleotide binding protein, beta polypeptide 2-like 1
GST – glutathione S-transferase
GTP – guanosine-5'-triphosphate
h – hour
H2A – histone 2A
H2B – histone 2B
H3 – histone 3
HA – haemagglutinin
HECT – homologous to the E6-AP carboxyl terminus
HER2/neu – human epidermal growth factor receptor 2
Hic1 – hypermethylated in cancer 1
HIF – hypoxia-inducible factor
HIST1H1B – histone cluster 1, H1b
HIST1H1T – histone cluster 1, H1t
HRAS (H-Ras) – v-Ha-ras Harvey rat sarcoma viral oncogene homolog
Hrk – Harikiri/Death protein-5
HRP – horseradish peroxidase
HtrA2/Omi – high temperature requirement protein A2
IAP – inhibitor of apoptosis
IB – immunoblot
ICAM3 – intercellular adhesion molecule 3
IFNE – interferon epsilon
IGFBP7 – insulin-like growth factor binding protein 7
IgG – immunoglobulin G
INK4A – inhibitor of cyclin-dependent kinase 4A
IP – immunoprecipitate
IPA – Ingenuity Pathway Analysis
IQSEC3 – IQ motif and Sec7 domain 3
ITB2 – integrin beta-2 precursor
k – kilo-
K – lysine
kb – kilobase
K-Ras – v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog

KSR – kinase suppressor of Ras
l – litre
L – leucine
LAMA2 – laminin alpha 2
LAMC2 – laminin gamma 2
LATS1 – large tumor suppressor, homolog 1
LC-MS/MS – liquid chromatography with tandem mass spectrometry
LIMA1 – LIM (Lin-11, Isl-1 and Mec-3) domain and actin binding protein 1
LRPPRC – leucine-rich PPR-motif containing protein
μ – micro-
m – milli-
mA – milliamp
mm – millimetre
M- molar
M phase – Mitosis
MAP1S – microtubule-associated protein 1S
MAPK – mitogen activated protein kinase
MDa - megadalton
MAP – microtubule-associated protein
Mcl-1 – myeloid cell leukaemia sequence 1
Mdm2 – mouse double minute 2
MMTV – mouse mammary tumor virus
MOAP-1 – modulator of apoptosis 1
MOMP – mitochondrial outer membrane permeabilization
mRNA – messenger ribonucleic acid
MST – mammalian Sterile 20-like
MTT – 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MTUS1 – microtubule associated tumor suppressor
MURF1 – Muscle-specific RING finger protein 1
N – amino
NAD – nicotinamide adenine dinucleotide
NEDD8 – neural precursor cell expressed, developmentally downregulated 8
NF-κB – nuclear factor kappa-B
NHEJ1 – nonhomologous end-joining factor 1
NIAM – nuclear interactor of ARF and Mdm2
NK – natural killer
NORE1 – novel ras effector 1
N-Ras – neuroblastoma Ras
ODC – ornithine decarboxylase
p – short arm of chromosome
P – proline
π - pi
p53R2 – p53-inducible ribonucleotide reductase small subunit 2-like protein
p53AIP1 – p53-regulated apoptosis-inducing protein 1
P-CIP1 – PAM COOH-terminal interactor protein-1
PAGE – polyacrylamide gel electrophoresis

PAM – peptidylglycine alpha-amidating monooxygenase
PARP – Poly(ADP-ribose) polymerase
Pax5 – paired box 5
PBS – phosphate buffered saline
PCDH – protocadherin
PCNA – proliferating cell nuclear antigen
PCR – polymerase chain reaction
PDCD4 – programmed cell death protein 4
PDE4D – phosphodiesterase-4D
PEI – polyethyleneimine
PEST – proline/glutamic acid/serine/threonine
pH – power of hydrogen
PI – propidium iodide
PI3K – phosphoinositide-3 kinase
PKA – protein kinase A
PKC – protein kinase C
PMA – phorbol 12-myristate 13-acetate
PMSF – phenylmethylsulfonyl fluoride
PND – paraneoplastic neurological disorder
PNMA – paraneoplastic antigen Ma
PPP2R1B – protein phosphatase 2, regulatory subunit A, beta
PR – progesterone receptor
PTEN – phosphatase and tensin homolog
PUMA – p53 up-regulated modulator of apoptosis
PVDF – polyvinylidene fluoride
q – long arm of chromosome
Q – glutamine
r – radius
R – arginine
R-Ras – related RAS viral (r-ras) oncogene homolog
RA – Ras association
RACK1 – receptor for activated C-kinase 1
RAPGEF5 – Rap guanine nucleotide exchange factor 5
RASSF – Ras association domain family
RING – really interesting new gene
RIP – receptor-interacting protein
RIPA – radio immunoprecipitation assay
RB (pRb) – retinoblastoma 1
RNA – ribonucleic acid
ROC – receiver operating characteristic
RP – ribosomal protein
Runx – runt-related transcription factor
S – serine
S phase – synthesis phase
SARAH – Salvador/RASSF/Hippo
s.c – subcutaneous

Scp160 – *Saccharomyces cerevisiae* protein 160
SDS – sodium dodecyl sulphate
SFRP – secreted frizzled-related protein
SMAC – second mitochondrial-derived activator of caspases
SMARCA2 – SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 2
SOX6 – sex determining region Y-box 6
SRE – serum response element
SRF – serum response factor
STAT – signal transducer and activator of transcription
STK4 – serine/threonine kinase 4
SUMO – small ubiquitin-like modifier
SV40 – simian virus 40
t – translocation
T – threonine
tBid – truncated Bid
TBRG1 – transforming growth factor beta regulator 1
TBS-T – Tris buffered saline plus Tween-20
TCL – T-cell leukemia
TGF-β – transforming growth factor beta
THBS2 – thrombospondin 2
TMEM – transmembrane protein
TNFα – tumor necrosis factor alpha
TNFR – tumor necrosis factor receptor
TP53 (p53) – tumor protein p53
TP53AIP1 – p53-regulated apoptosis-inducing protein 1
TP53RK – p53-related kinase
TP73 (p73) – tumor protein 73
TRADD – TNFR-associated death domain
TRAF2 – TNF receptor-associated factor 2
TRAIL (Apo2L) – TNF-related apoptosis-inducing ligand
TRIM - tripartite motif
Trk – tyrosine kinase receptor
Trp – tryptophan
Ub – ubiquitin
Ubc – ubiquitin conjugating
UPS – ubiquitin-proteasome system
UV – ultraviolet
V – volt
V5 – protein kinase C variable region 5
VDAC1 – voltage-dependent anion channel 1
VEGF – vascular endothelial growth factor
VHL – von Hippel-Lindau
W – tryptophan
WCL – whole cell lysate
WD40 – tryptophan-aspartic acid 40

Wnt – wingless type
WT – wild type
Y – tyrosine
YAP1 – yes-associated protein 1

CHAPTER 1

INTRODUCTION

1.1. TUMOR SUPPRESSOR PROTEINS

Cancer is a disease of uncontrolled cell proliferation that is responsible for approximately 29% of deaths each year in Canada (Canadian Cancer Statistics 2010). The abnormal proliferation of cells during cancer development results from a multistep process involving the deregulation of genes that promote cell growth (oncogenes) and those that normally function to restrain it (tumor suppressors). As a consequence of these deregulations, normal cells are transformed into malignant derivatives that can freely divide uninhibited. Interestingly, approximately 90% of the genes that are associated with cancer development have now been identified as being tumor suppressors [1].

1.1.1. Oncogenes versus tumor suppressors

Somatic mutations in over 350 genes comprising ~1.6% of the human genome are causally implicated in cancer development [2]. These cancer-associated genes can be broadly divided into two main classes referred to as oncogenes or tumor suppressors. By definition, an oncogene is any gene that has the ability to transform cells in culture or to induce tumor formation in animals [3]. The first somatic point mutation to be identified in human cancer resulting in a glycine to valine amino acid substitution led to the discovery of the now well-known oncogene, *HRAS* [4, 5]. Oncogenes are altered derivatives of their normal cellular counterparts, the proto-oncogenes, and have acquired enhanced activity due to increased expression or activating ("gain-of-function") mutations. With respect to *HRAS*, a substitution of valine at position 12 results in a constitutively active form

of the GTPase that can mediate aberrant cell growth and survival signaling. In general, proto-oncogenes encode proteins that are involved in cell growth and differentiation and if transformed into an oncogene, can allow for increased cell proliferation thus promoting tumor growth or formation.

On the other hand, tumor suppressor genes generally encode proteins that are involved in DNA maintenance and repair or inhibiting cell proliferation and can contribute to cancer development as a result of decreased expression or "loss-of-function" mutations [6]. Classic tumor suppressor genes, such as *TP53* and *RB*, are known to possess three defining properties. These include: the requirement for biallelic inactivation in tumors, the ability of a single mutant allele to predispose individuals to cancer development when inherited, and lastly, that the same gene can be inactivated in both sporadic cancers as well as inherited forms of the disease [7].

It is often believed that human cancer results from an accumulation of mutations with the approximate number of genes involved ranging from three to seven [8]. However, the evolution of cancer has also been cited as requiring up to 20 different mutations in cancer-associated genes [9]. Therefore, the number of genetic changes involved in the development of cancer likely varies based on a number of different factors including the cancer type. Evidence suggests that cancer progression involves mutations in both oncogenes and tumor suppressors. The following sections will focus on the latter class of proteins and their role in neoplasia.

1.1.2. A brief history on tumor suppressor proteins

The initial discovery of dominantly acting oncogenes in the 1970s was followed several years later by the identification of a new set of cancer-associated genes that functioned in a distinctly different manner. The existence of these newly identified genes was uncovered over the course of somatic cell hybrid studies demonstrating how genetic elements in normal, healthy cells could suppress the tumorigenic phenotype of malignant cells when fused into a hybrid [10]. These genetic elements later became known as tumor suppressors. Further studies involving somatic cell hybrid experiments suggested that tumor suppressor proteins were likely involved in negatively regulating malignant growth and furthermore, that their loss of function was involved the development of cancer. However, at this time, the functions, locations and specific cancer associations of tumor suppressor genes were still unknown. To provide functional evidence of tumor suppressors and also to help identify the chromosomal location of some of these genes, monochromosome transfer studies were carried out whereby specific single chromosomes were transferred to cancer cells known to have deletions or losses of heterozygosity in their chromosomes [11]. The ability of a newly introduced chromosome to suppress the tumorigenic potential of a recipient cancer cell strongly suggested the presence of a mutant tumor suppressor gene that could be further localized to a specific gene locus using other approaches. Finally, the molecular cloning of tumor suppressor genes began in the mid-1980s employing techniques such as reverse genetics and chromosome walking. This led to the cloning of the first tumor suppressor gene in 1986 [12].

1.1.3. Classic tumor suppressor proteins pRb and p53

The first tumor suppressor gene to be identified encodes the retinoblastoma protein (pRb) that is involved in the development of the childhood retinal cancer retinoblastoma [12]. As a prototypical tumor suppressor, pRb is a nuclear phosphoprotein whose gene undergoes biallelic inactivation in both sporadic and familial cases of human cancer. Recently, roles for pRb in the regulation of both apoptosis and differentiation has been uncovered [13]. Nonetheless, this protein is still most widely recognized for its originally identified function of controlling cell cycle progression. When hypophosphorylated, pRb functions as a key regulator of the cell cycle where it is involved in repressing the transcription of multiple cell cycle regulatory genes required for transition from G1 to S phase [14]. Inhibition of gene expression is achieved through the interaction of pRb with members of the E2F family of transcription factors which, together as a complex, bind promoter regions and repress transcription of target genes [15, 16]. Once phosphorylated, however, pRb can no longer associate with E2F proteins and subsequently permits the expression of E2F target genes. Phosphorylation of pRb is regulated by cyclin-dependent kinase (CDK) complexes cyclin D-CDK4/CDK6 and cyclin E-CDK2 which become active during late G1 phase and phosphorylate pRb on specific amino acid residues resulting in its inactivation [17, 18].

Malignancy associated with disregulated pRb-E2F gene regulation can result from mutations in pRb; however, these mutations are usually observed in only a subset of cancers [19]. More commonly, mutations occur in genes encoding

proteins that are responsible for regulating the phosphorylation and thus activity of pRb and which may permit continuous entry into the cell cycle when mutated. Such types of genetic alterations are observed in the CDK inhibitor $p16^{INK4A}$ that helps to maintain the hypophosphorylated state of pRb [20, 21]; CDK4 and CDK6 which can acquire mutations that decrease their ability to bind their inhibitors [22, 23]; as well as cyclins that are overexpressed [24]. Furthermore, pRb is also susceptible to inactivation by a number of different viral oncoproteins including adenoviral E1A, papillomavirus E7 and polyoma virus large T antigen [25].

A second well-known classic tumor suppressor gene encodes the protein p53 which is often described as the "guardian of the genome". p53 was originally identified in its mutant form from cells that were transformed by simian virus 40 (SV40) and consequently was thought to be an oncogene rather than a bona fide tumor suppressor [26]. However, subsequent studies later identified p53 in its wild type form and discovered its true function as an inhibitor of cell growth [27]. Today, it is widely recognized that mutation of p53 occurs in over 50% of human tumors and that the vast majority of these mutations occur within its DNA-binding domain [28]. p53 functions as a tetrameric transcription factor that is involved in the transactivation of genes necessary for the induction of cell cycle arrest or apoptosis. Among its target genes, p53 regulates the transcription of the G1 cell cycle arrest protein $p21^{WAF1}$ [29], DNA repair and G2/M arrest protein p53R2 [30] and proapoptotic molecules Bax, p53AIP1, Noxa and PUMA [31]. Interestingly, p53 mutants can display a dominant-negative behaviour towards its wild type form due to heterodimerization of both wild type and mutant p53 resulting in impaired

transcription factor binding [32, 33]. Therefore, the sequestration of wild type p53 by mutant p53 impedes the normal function of this protein.

Under normal cellular conditions, p53 is maintained at low levels due to its constitutive Mdm2-mediated proteasomal degradation [34]. However, cellular stresses such as DNA damage, hypoxia, oxidative damage, or nucleotide imbalance result in a rapid accumulation of p53 within the nucleus where it can activate the transcription of target genes required for the appropriate cellular response leading to DNA repair, cell cycle arrest and/or apoptosis. Nuclear accumulation of p53 results from stabilization of this protein and requires post-translational modifications including phosphorylation and acetylation. It is worth noting that the p53-mediated apoptotic response not only involves the transcriptional activation of pro-apoptotic genes, but also the direct involvement of p53 at the mitochondria where it is involved in permeabilization of the outer mitochondrial membrane and cytochrome c release [35].

Evidence suggests that the antiproliferative functions of pRb and p53 are linked through a third tumor suppressor protein known as $p14^{ARF}$ [36]. In the absence of functional pRb, the E2F-1 transcription factor can induce the expression of $p14^{ARF}$ which has been shown to bind the E3 ubiquitin ligase Mdm2 and thus prevent p53 degradation [37]. Therefore, activation of E2F-1 in the absence of pRb results in a p53-mediated cellular response that can function as an intrinsic back-up mechanism of tumor suppression.

1.1.4. Knudson's two-hit hypothesis

Due to the recessive nature of most tumor suppressor mutations, the loss of function in a tumor suppressor protein typically requires the inactivation of both alleles of its gene. This is in contrast to mutations that arise in proto-oncogenes which in general are dominantly acting and need only affect one gene copy. The first piece of evidence demonstrating the recessivity of tumor suppressor gene mutations came from work carried out by the geneticist Alfred Knudson [38]. Over the course of study on retinoblastoma patients from 1944-1969, Knudson came to the conclusion that retinoblastoma was caused by both somatic and germ-line mutations and a statistical analysis of patient data was consistent with a model in which a mutation occurring in each of two gene copies was required for cancer development. In sporadic cases of retinoblastoma, a child would acquire two separate mutations in the same gene (now known to be *RB1*) early on in life. However, familial cases of the cancer were due to inheritance of a mutant gene copy followed by a sporadic mutation in the other allele. The idea that the function in both tumor suppressor gene copies must be affected in order to promote malignant growth was later proposed by Knudson in 1971 and is now known as the "two-hit" hypothesis of gene mutation [38]. Importantly, while mutations in *RB1* are associated with most cases of retinoblastoma, it is now known that other genetic alterations may also contribute to the development of this pediatric cancer [39-41].

1.1.5. Loss of heterozygosity

The inheritance of a mutant allele or somatic mutation of a single copy of a tumor suppressor gene results in a heterozygous gene locus where the second allele is still presumably wild type. In a heterozygous state, most tumor suppressor proteins can still carry out their normal functions due to the recessive nature of most loss-of-function mutations. However, "loss of heterozygosity" refers to an event leading to the loss or inactivation of the second allele resulting in a tumor suppressor gene that is hemizygous or homozygous for a mutation. The mechanisms underlying loss of heterozygosity are believed to differ from the initial events leading to inactivation of the first allele due to the unlikelihood of eliminating both gene copies through mutational events [3, 42]. Loss of heterozygosity is often attributed to chromosomal breakage or translocation, gene conversion, homologous recombination or chromosome missegregation (also known as nondisjunction).

1.1.6. Haploinsufficiency

Certain tumor suppressor genes do not conform to the notion that biallelic gene inactivation is a prerequisite for cancer progression. In some cases (known as "haploinsufficiency"), mutation of a single gene copy is sufficient for promoting neoplasia even in the presence of a wild type allele. This has been observed for a few different tumor suppressors including p53 [43], TGF-β1 [44], p27Kip1 [45] and DMP1 [46] in experiments demonstrating that genetically deficient heterozygous mice are prone to tumor development. Importantly, these

predispositions to tumor formation do not involve the loss of the remaining wild type allele.

Haploinsufficiency of tumor suppressor genes can be explained through several different possibilities [47]. First, haploid levels of a tumor suppressor protein may be insufficient for execution of its normal function. This is commonly observed for tumor suppressor proteins that function as inhibitors and thus need to be present at a stoichiometric amount in relation to other proteins and at high enough levels so as to properly regulate downstream events. A second possibility to account for haploinsufficiency occurs when mutations in a tumor suppressor produce a dominant-negative effect towards its wild type form. Lastly, haploinsufficiency may also be attributed to changes in the expression of a wild type allele not due to mutations in itself but as a result of cooperating mutations in other proteins. This may involve epistasis. Therefore, the progression towards cancer development may involve loss of tumor suppressor function owing to either one (haploinsufficiency) or two (Knudson's hypothesis) "hits" of its gene.

1.2. RAS ASSOCIATION DOMAIN FAMILY OF PROTEINS

The Ras association domain family (RASSF) of proteins is comprised of ten different members known as RASSF1-10 that each share the presence of a Ras association (RA) domain within their structures [48-51]. Depending on the location of their RA domain, RASSF proteins are often typified as either C-terminal (RASSF1-6) or N-terminal (RASSF7-10) family members. RASSF1 serves as the founding member of this protein family and is also the most thoroughly

characterized and studied thus far. However, growing research on the other RASSF members reveals that many of these proteins share similar functions with its original member, RASSF1, and are involved in apoptosis, cell cycle control, and regulation of microtubule stability. Furthermore, several RASSF proteins have been shown to function as tumors suppressors and loss of their expressions have been implicated in a wide range of human cancers.

1.2.1. RASSF1

The identification of *RASSF1* originates from a long-standing and well-known history of cancer association with its genetic locus. Homozygous deletions and allelic loss in the short arm of chromosome 3 are one of the most frequent and earliest molecular changes detected in multiple cancer types including tumors of the lung, kidney, breast and gastrointestinal tract [52]. Although several different regions in 3p have been identified as potential areas for tumor suppressor genes, 3p21.3 is a particularly interesting site given that it houses a large number of genes [53] and is the site of multiple homozygous deletions present in several cancer cell lines and lung primary tumors [52, 54-56]. A minimal critical deletion region of 120 kb containing 8 different genes was subsequently identified through deletion mapping. Among these genes is *RASSF1* which, to date, has been shown to function as a bona fide tumor suppressor protein through regulation of several biological processes and is the most extensively studied gene from the 3p21.3 chromosomal region.

RASSF1 was first cloned and characterized by Dammann and colleagues in 2000 upon the isolation of its cDNA in a yeast two-hybrid screen [57]. Transcription of *RASSF1* generates seven different transcripts (*RASSF1A-G*) as a result of alternative splicing and differential promoter usage [58]. The two major isoforms, *RASSF1A* and *RASSF1C*, are transcribed from two different CpG island-containing promoters producing a unique n-terminal region for RASSF1A that is different from RASSF1C and which also contains a diacylglycerol (DAG)-binding domain/protein kinase C conserved region 1 (C1) domain. Both RASSF1A and RASSF1C contain an ataxia telangiectasia mutated (ATM) kinase phosphorylation consensus sequence as well as a SARAH (Salvador/RASSF/Hippo) binding domain that is involved in mediating protein-protein interactions [59]. Given the important role of RASSF1 in human biology, it is not surprising that this gene is also highly conserved in other species including the: chimpanzee (*Pan troglodytes*), mouse (*Mus musculus*), rat (*Rattus norvegicus*) and zebrafish (*Danio rerio*). RASSF1 homologs are also present in both fruit flies (*Drosophila melanogaster)* and worms *(Caenorhabdtis elegans)* but are less highly conserved with the human version of this gene.

Interestingly, a loss or decrease in RASSF1A expression is observed in a wide range of human cancers while RASSF1C expression remains largely unaffected. The primary mechanism underlying *RASSF1A* inactivation is due to methylation of its promoter CpG islands resulting in epigenetic transcriptional silencing [60]. On the other hand, RASSF1A somatic mutations are rare but have been identified in nasopharyngeal, lung, breast and kidney cancers and often map

to functional domains within the protein [61-63]. Frequent methylation-associated loss of RASSF1A expression is observed in cancers of the lung, kidney, breast, bladder, brain, liver and multiple other organs thus rendering RASSF1A one of the most frequently inactivated genes described in cancer thus far [58, 64, 65]. Given the high frequency and specificity of *RASSF1A* methylation in cancer, this gene is often thought to have the potential to serve as an ideal cancer biomarker and, accordingly, has already been patented for use in the detection of ovarian cancer in the United States (U.S. patent 6596488). The preferential loss of RASSF1A in cancer over its similar isoform RASSF1C reflects its unique role in several important growth regulatory pathways.

Emerging evidence suggests that RASSF1C may also function as a tumor suppressor protein in at least some cells types [66]. Unlike *RASSF1A*, *RASSF1C* has not been found to be epigenetically silenced in cancer [64]. Induced expression of *RASSF1C* in prostate and renal cancer cell lines LNCaP and KRC/Y, respectively, results in growth suppression both *in vivo* and *in vitro* [66]. However, RASSF1C had no effect on the growth of U2020 small-cell lung cancer cells**.** In contrast to its purported tumor suppressor function reported by Li et al. [66], Reeves and colleagues have found that RASSF1C actually promotes the migration and survival of breast cancer cells [67]. A recent study has also suggested a possible pathogenic role for RASSF1C in cancer after discovering that its expression was more than eleven-fold greater in pancreatic endocrine tumors than in normal tissue [68]. Thus, the ability of RASSF1C to function as a tumor suppressor remains to be further investigated and confirmed.

1.2.2. Biological functions of RASSF1A

RASSF1A contains 340 amino acids and has a molecular weight of approximately 39 kDa. When ectopically expressed in cancer cells, RASSF1A suppresses anchorage-independent growth and colony formation while inhibiting tumor development *in vivo* [58]. Genetic knockout of *Rassf1a* in mice produces offspring that are healthy and fertile but which develop spontaneous tumors later in life, including lung adenomas, lymphomas and breast adenocarcinomas, thereby signifying the importance of *Rassf1a* in the prevention of neoplasia [69]. The tumor suppressive functions of RASSF1A involve its role in several different biological processes that converge on its ability to regulate microtubule dynamics, mitosis and apoptosis.

RASSF1A localizes to microtubules and is involved in the control of their stability and polymerization through associations with cdc20 and the two microtubule-associated proteins (MAPs) MAP1B and C19ORF5/MAP1S [70-72]. The ability of RASSF1A to associate with and regulate microtubules is essential for its ability to inhibit growth and has also implicated RASSF1A in the maintenance of genomic stability [71]. Additionally, RASSF1A is also involved in controlling cell migration through its influence on microtubule dynamics [70]. Overexpression of RASSF1A results in decreased cell motility and increased cell-cell adhesion while knockdown of its expression increases both cell migration and invasion.

During mitosis, RASSF1A localizes to the centrosome and mitotic spindle where it helps to regulate the cell cycle [71, 73]. The importance of RASSF1A's

microtubule regulatory functions for its growth inhibitory effect is underscored by the observation that a RASSF1A mutant lacking its microtubule association domain is defective in its ability to promote cell cycle arrest [74]. Through its interaction with cdc20, an activator of the anaphase-promoting complex (APC), RASSF1A is capable of inhibiting the APC prior to the spindle checkpoint and thus regulate cell cycle progression [75]. The ability of RASSF1A to inhibit APC-cdc20 activity during mitosis is dependent on C19ORF5/MAP1S which recruits RASSF1A to spindle poles and also enhances the interaction between RASSF1A and cdc20 [73]. C19ORF5/MAP1S also helps to regulate the timing and progression of mitosis by inducing the stabilization of mitotic cyclins in a RASSF1A-dependent manner. Recently, C19ORF5/MAP1S has also been shown to associate with a component of the autophagosome, LC3, and the mitochondria-associated leucine-rich PPR-motif containing protein (LRPPRC) thus suggesting that it may serve as a potential link between autophagic cell death, mitochondria and microtubules [76].

An additional role for RASSF1A during the cell cycle occurs at the G1/S phase transition where it interacts with the transcriptional regulator $p120^{E4F}$ and inhibits passage through the G1 stage of mitosis [77]. $p120^{E4F}$ is involved in inhibiting the transcription of cyclin A which associates with CDK2 and is required for progression through S phase. RASSF1A cooperates with $p120^{E4F}$ to repress cyclin A expression by enhancing its binding at the promoter region [78]. Additionally, RASSF1A can engage the Rb family cell cycle checkpoint by post-

transcriptionally inhibiting the accumulation of cyclin D1 that is required for CDK4/6 activation, thus restricting G1/S transition by a second mechanism [79].

A third major function of RASSF1A is in the regulation of apoptosis which involves its interaction with pro-apoptotic proteins modulator of apoptosis 1 (MOAP-1) and the serine/threonine kinases mammalian sterile 20-like (MST) 1 and 2. Currently, the evidence for RASSF1A in MST1-mediated cell death is based on conflicting results. In one study, RASSF1A was shown to promote Fas-induced apoptosis through activation of MST1 [80]. However, in a separate study, Praskova et al. demonstrated that auto-activation of MST1 is inhibited in the presence of overexpressed RASSF1A [81]. Moreover, RASSF1A also forms a constitutive complex with MST1 and another RASSF family member, RASSF5/novel ras effector 1 (NORE1), and together they cooperate in responding to pro-apoptotic signals initiated by Ras signaling [82]. RASSF1A can also induce apoptosis through an MST2 pathway by releasing it from its inhibitor, Raf1, and allowing for LATS1-mediated activation of the transcriptional regulator YAP1 [83]. In turn, YAP1 can translocate to nucleus and associate with the p73 transcription factor in order to induce the transcription of proapoptotic *PUMA*. In response to death receptor signaling involving tumor necrosis factor alpha (TNFα) or TNF-related apoptosis-inducing ligand (TRAIL), RASSF1A is able to bind the Bcl2-associated x protein (Bax)-interacting molecule MOAP-1 [84, 85]. Association of RASSF1A with MOAP-1 promotes Bax translocation and integration into the mitochondrial membrane, resulting in the release of cytochrome c. Consequently, RASSF1A can initiate apoptosis through at least three different pathways.

Although RASSF1A contains a RA domain within its structure, whether or not it can directly associate with Ras to function as an effector of the protein remains uncertain. In 2000, Vos et al. demonstrated that RASSF1A can directly bind Ras *in vitro* [86]. However, results from other research groups indicate that the association between RASSF1A and Ras occurs only indirectly though interactions involving RASSF5A/NORE1A or the scaffold protein connector enhancer of kinase suppressor of Ras 1 (CNK1) [87, 88]. Moreover, research from our lab has failed to demonstrate RASSF1A association with N-, H-, K- or R-Ras (Baksh et al., unpublished observations) and thus we support the model of an indirect interaction between these two proteins [89].

1.2.3. Tumor suppressor and Ras binding properties of additional RASSF family members

Activated forms of Ras are often associated with cell growth and survival but it is important to note that Ras can also have a growth inhibitory effect by inducing apoptosis in cells [90]. In addition to RASSF1A, the inactivation of several other RASSF family members by promoter CpG island methylation or other mechanisms have also been documented. Moreover, a number of RASSF family members - in addition to RASSF1A - are thought to be candidate tumor suppressors and in several cases, the growth suppressive properties of these proteins have been shown to be mediated through Ras [86, 91-93]. The remainder of this section addresses some of the tumor suppressor and Ras binding properties of several RASSF proteins beginning with RASSF2.

RASSF2 has been described as a novel tumor suppressor gene in colorectal cancer [94]. Promoter methylation resulting in a decrease or loss of expression of the *RASSF2A* isoform is an early event during the development of colorectal carcinomas in up to 70% of cases [95] and is also implicated in the development of both gastric and nasopharyngeal cancers [96, 97]. Inactivation of *RASSF2* is correlated with Ras activation in tumor cells [95] and is known to associate with K-Ras but only weakly with H-Ras [91]. The tumor suppressor functions of RASSF2 involve its role in apoptosis and the induction of cell cycle arrest [91, 94].

RASSF3 is a ubiquitously expressed protein that is present in all normal tissues and is detectable in each cancer cell line that has been tested thus far [98]. Although the biological functions of this protein still remain unclear, *Rassf3* is overexpressed in the mammary gland of tumor-resistant mouse mammary tumor virus (MMTV)/neu transgenic mice and can inhibit the cell proliferation of human and mouse breast cancer cell lines [99]. Consequently, it is hypothesized that RASSF3 may function as a potential tumor suppressor protein.

Expression of *RASSF4*, also known as *AD037*, is downregulated by aberrant promoter methylation in multiple transformed cell lines and primary tumors [93, 100]. RASSF4 directly binds to activated K-Ras and overexpression of this RASSF member induces Ras-dependent apoptosis and inhibits the growth of cancer cells in a Ras-dependent manner [93].

RASSF5/NORE1 is the second most well-characterized member of the family after RASSF1A and was also the first RASSF protein to be cloned [101]. It is most commonly referred to by its original name novel ras effector 1 (NORE1).

Translocation breakpoints in *RASSF5/NORE1* are linked to the development of familial clear cell renal cell carcinomas [102]. The major isoform of RASSF5/NORE1, RASSF5A/NORE1A, is known to directly associate with Ras and also induces apoptosis in a Ras-dependent manner [82, 92]. RASSF5A/NORE1A is also believed to regulate the proapoptotic kinase MST1 [81] and is involved in mediating TNFα and TRAIL-induced apoptosis [103]. RASSF5A/NORE1A expression is lost or decreased in cancer as a result of promoter methylation and, similar to RASSF1A, mutations are rarely detected [104, 105]. NORE1 also produces a second splice variant known as RASSF5C/NORE1B/RAPL that associates with the Ras-related protein Rap and regulates lymphocyte adhesion [106]. RASSF5C/NORE1B/RAPL is a candidate tumor suppressor whose expression is epigenetically silenced in hepatocellular carcinoma [107].

RASSF6 expression is downregulated in primary tumors [108]. It is thought to function as a negative regulator of NF-κB activity and is able to bind activated Ras and also promote Ras-dependent apoptosis [108]. Loss of RASSF6 expression is thought to result from a combination of both epigenetic and genetic mechanisms [108, 109].

RASSF7 regulates microtubule dynamics and localizes to centrosomes during mitosis where it is required for spindle formation, activation of Aurora B and proper alignment of chromosomes at the metaphase plate [110, 111]. Evidence suggests that RASSF7 may possess oncogenic properties. Knockdown of RASSF7 was shown to inhibit cell growth in cancer cell lines and its expression

was also found to be 87-fold greater in pancreatic tumors relative to normal tissue [110, 112].

RASSF8 is a candidate lung tumor suppressor gene whose expression is decreased in lung adenocarcinomas and when re-expressed can inhibit anchorage-independent growth of transformed lung cancer cells in soft agar [113]. The mechanisms underlying its inactivation in cancer are currently unknown. Biochemically, RASSF8 is implicated in regulating both Wnt and NF-κB signaling pathways [114].

RASSF9 was originally identified as a peptidylglycine alpha-amidating monooxygenase (PAM)-interacting molecule and is also known as PAM COOH-terminal interactor protein-1 (P-CIP1) [115]. Research suggests that RASFF9/P-CIP1 may be involved in the endosomal trafficking of PAM [115]. More recently, however, RASSF9 has also been identified as a key protein involved in epidermal homeostasis wherein *Rassf9*-null mice display a phenotype that bears resemblance to human aging [116].

The last member of this family, RASSF10, is epigenetically silenced in thyroid cancer, childhood leukemias, and glioblastoma [109, 117, 118]. However, no biological functions have been assigned to this protein as of yet.

1.3. APOPTOSIS

By the mid to late 1800s, scientists had already recognized that cell death played a considerable role in the normal physiology of multicellular organisms [119]. However, it was not until was not until 1972 that the term “apoptosis” was

first introduced by Kerr et al. in order to describe the controlled and active process of self-destruction that is initiated by cells [120]. Today, we now know that multiple forms of cell death exist and that apoptosis refers to a specific mode of cell death that is defined by a key set of morphological features [121, 122]. Some of the structural changes that characterize apoptosis include: cell shrinkage, chromatin condensation, nuclear fragmentation, plasma membrane blebbing, and the formation of apoptotic bodies that are later engulfed by phagocytes. An important difference that distinguishes apoptosis from an unregulated form of cell death called necrosis is that apoptosis proceeds in an extremely controlled manner so as not to provoke an immune response or disturb and damage neighbouring cells [122].

The significance of apoptosis in metazoan biology is highlighted by the number of diseases that are associated with its deregulation including numerous neurodegenerative, ischaemic and autoimmune diseases, AIDS and cancer [123]. Apoptosis plays a critical role during the development of multicellular organisms and is also required for maintaining the homeostasis in adult tissues. Additionally, apoptosis is vital to the removal of damaged or dangerous cells. It has been estimated that in humans, approximately 100 000 cells undergo apoptosis each second [124]. Therefore, apoptosis is a highly efficient mechanism that requires precision and tight regulation.

1.3.1. Caspases

Caspases are cysteine aspartic acid-specific proteases that are centrally involved in apoptotic signaling and are activated in most cases of this form of cell

death [121]. The catalytic activities of these enzymes require the presence of a critical cysteine residue within their active sites and are known to cleave their substrates after Asp residues [125]. Although a number of different caspases are activated during apoptosis, not all caspases are involved in cell death [126]. There are currently 15 different mammalian caspases that have been identified and, in humans, it is caspases-2, -3, -6, -7, -8, -9 and -10 that are involved in apoptosis. Two sub-groupings have been defined in order to describe the roles of these enzymes during apoptotic signaling: initiator caspases (caspases-8, -9, and -10) are those that are actively involved in the outset and initiation of apoptosis whereas effector caspases (caspases-3, -6, and -7) are those that are normally activated by the initiator enzymes and which are also responsible for the execution of cell death. At this time, caspase-2 is thought to share functions of both an initiator and effector caspase [127].

In cells, caspases are initially synthesized as inactive zymogens called pro-caspases whose structures contain a prodomain at their n-termini followed by large and small catalytic subunits often separated by a linker region [125]. The prodomains of initiator caspases contain recruitment motifs recognizable by adaptor proteins and are over 100 amino acids in length while the prodomains present in effector caspases are significantly shorter averaging less than 30 residues in length [128].

In response to apoptotic signals, initiator caspases are activated by dimerization and this is facilitated by adaptor-mediated recruitment to activation complexes via their death effector domains (DED, in caspases-8 and -10) or

caspase recruitment domains (CARD, in caspases-2 and -9) [129]. The specific protein complexes involved in initiator caspase activation differ depending on the cell death stimulus and caspases involved. Death receptor signaling results in the formation of a death-inducing signaling complex (DISC) that recruits and activates caspases-8 and -10. On the other hand, caspase 9 is recruited and activated by the apoptosome during the mitochondrial pathway of apoptosis.

In contrast to initiator caspase activation, effector caspases exist within the cell as inactive dimers and are activated by proteolytic cleavage within the linker region between their small and large catalytic subunits [126, 129]. This cleavage process commonly requires initiator caspase activity but may also involve other proteases in different biological situations [128]. The cleavage of effector caspases allows for the formation of their mature, active forms composed of a tetramer containing two large and two small catalytic subunits [128].

During apoptosis, the activation of caspases results in the cleavage of key cellular substrates vital for normal cell functioning and is achieved through caspase-mediated activation or inactivation of select proteins that share a common set of functions [130]. A large number of caspase substrates include proteins that are involved in DNA repair and metabolism, cytoskeletal organization, cell cycle regulation, transcription and translation, or cell signaling. Due to the profound and irreversible cellular changes that occur in response to caspase activation, a number of intracellular caspase inhibitors exist in order to restrain their activation in the absence of apoptotic signals [131].

1.3.2. Pathways to apoptosis

There are two main pathways of apoptosis that can be initiated in response to intracellular or extracellular signals of cell death [132]. The intrinsic apoptotic signaling pathway is activated in response to a diverse set of signals originating from within cells in response to cellular stresses such as DNA damage, hypoxia, toxins or starvation [133]. In contrast, the extrinsic pathway of cell death is activated by the binding of death-inducing ligands to their receptors. Regardless of the pathway that is activated, both intrinsic and extrinsic apoptosis share the same set of execution events beginning with the cleavage and activation of the effector caspase-3.

1.3.3. Intrinsic cell death

1.3.3.1. Role of the mitochondria

Mitochondria play an important role in the induction of apoptosis. Activation of the intrinsic apoptotic signaling pathway causes changes within the inner mitochondrial membrane resulting in: the opening of the mitochondrial permeability transition pore, loss of mitochondrial membrane potential and the release of small apoptotic proteins [134]. Many of the proteins that are released from the mitochondria into the cytoplasm promote caspase activation and include cytochrome c, Smac/DIABLO and HtrA2/Omi [135-137]. While cytochrome c is required for apoptosome formation and activation of procaspase-9, Smac/DIABLO and HtrA2/Omi both inhibit the activities of the inhibitors of apoptosis proteins (IAPs) that normally prevent caspase activation. Additionally,

several other proteins that are released from the mitochondria translocate to the nucleus where they induce DNA fragmentation. These proteins include apoptosis-inducing factor (AIF), endonuclease G, and caspase-activated DNAse (CAD) [138-140]. Regulation of the mitochondrial events during apoptosis is controlled both directly and indirectly by proteins of the B-cell lymphoma-2 (Bcl-2) family [141].

1.3.3.2. Bcl-2 family of proteins

Proteins of the Bcl-2 family play a fundamental role in the intrinsic pathway of apoptosis by ultimately regulating the release of cytochrome c and other apoptotic factors from the mitochondria [141]. Interestingly, the first member of this family, *Bcl-2*, was originally discovered at the t(14;18) translocation breakpoint associated with B-cell follicular lymphoma and was later identified as the first oncogene to promote cell survival rather than cell proliferation [142-145]. Today, there are at least 25 different Bcl-2 relatives belonging to the family which has been divided into three different subgroups known as the antiapoptotic, multi-domain proapoptotic and BH3-only proteins that differ based on the number of Bcl-2 homology (BH) domains within their structures[133]..

1.3.3.2.1. Antiapoptotic Bcl-2 proteins

Antiapoptotic members of the Bcl-2 family contain a total of four BH domains (BH1-4) and have the primary function of inhibiting apoptosis [141, 146]. Members of this group include Bcl-2, Bcl-x_L, Mcl-1, Bcl-2A1, Bcl-w and Bcl-B that function by inhibiting the multi-domain proapoptotic proteins Bax and Bak

(Bcl-2 antagonist/killer-1). These antiapoptotic proteins contain a c-terminal hydrophobic domain and are localized at the endoplasmic reticulum, nuclear envelope and outer mitochondrial membrane [141].

1.3.3.2.2. BH3-only proteins

BH3-only proteins including Bid (BH3-interacting domain death agonist), Bad (Bcl-2 antagonist of cell death), Bim (Bcl-2-like 11), Bik (Bcl-2-interacting killer), BMF (Bcl-2 modifying factor), Noxa, Puma (p53 up-regulated modulator of apoptosis) and Hrk (Harikiri/Death protein-5) are characterized by the presence of a single BH domain within their structures that is required for both association with antiapoptotic Bcl-2 proteins and for promoting apoptosis [147, 148]. These proteins serve as the initial sensors of cell death and promote the activation of Bax and Bak by direct binding to antiapoptotic Bcl-2 family members resulting in their inhibition [149]. However, BH3-only proteins differ in their abilities to bind anti-apoptotic Bcl-2 family members. For instance, Puma and Bim are able to bind all anti-apoptotic Bcl-2 proteins whereas others such as Bad and Noxa can only interact with certain members. Evidence suggests that, in addition to inhibiting antiapoptotic Bcl-2 proteins, some BH3-only members may promote apoptosis by direct binding and activation of Bax and Bak [150, 151].

Members of the BH3-only subgroup are regulated in a number of distinct ways in response to apoptosis. Noxa and Puma are both transcriptionally upregulated by p53 in response to DNA damage [152, 153] while Bim expression can be induced by the forkhead box transcription factor FOXO3A, CEBPα (CCAAT-

enhancer binding protein α) or CHOP (CEBP homologous protein) [154, 155]. Alternatively, some BH3-only proteins can be regulated post-translationally and include Bid, which is activated by caspase-8 cleavage [156], and Bad which becomes dephosphorylated and released from 14-3-3 proteins upon activation [157]. The activity of BH3-only proteins above a threshold level is required in order to overcome the anti-apoptotic activity of Bcl2-family proteins.

1.3.3.2.3. Activation of multi-domain proapoptotic Bax and Bak

Members of the multi-domain Bcl-2 family contain three BH domains (BH1-3) and are directly responsible for the mitochondrial outer membrane permeabilization (MOMP) that occurs during apoptosis [141, 147]. Two members from this group are Bax and Bak, which research indicates the presence of at least one of these two proteins is required for apoptosis in cells [158].

Bax is a largely cytosolic protein with a minor fraction found at the outer mitochondrial membrane [159]. Within its structure, Bax contains a c-terminal hydrophobic membrane anchor that is normally sequestered inside its BH3-binding pocket. During apoptosis, Bax translocates to the mitochondria, undergoes a conformational change and utilizes its c-terminal membrane anchor in order to insert into the mitochondrial outer membrane as an integral membrane protein [160, 161].

In contrast, Bak is already present in nonapoptotic cells as an integral membrane protein at the outer mitochondrial membrane but is bound by

antiapoptotic proteins Mcl1 and Bcl-xL [162]. During apoptosis, Bak changes conformation in a Bid-inducible manner [163]

It is widely believed that Bax and Bak induce MOMP by the formation of channels in the outer mitochondrial membrane [164, 165]. However, a second hypothesis also suggests that Bax may interact with existing components of the mitochondrial permeability transition pore and subsequently create a larger channel to allow protein efflux including cytochrome c from the intermembrane space [166, 167]. Released cytochrome c subsequently binds to the protein apoptotic protease activating factor 1 (Apaf-1) in an ATP-dependent manner in order to form a heptameric apoptosome complex that triggers the activation of initiator procaspase-9 [137]. Following from this, activate caspase-9 can then cleave and activate downstream effector caspases, ultimately resulting in cell death [168].

In addition to Bax and Bak, a third less well-studied member of this group named Bok (Bcl-2-related ovarian killer) also exists. Similar to Bax, Bok also translocates from the cytosol to the mitochondria during apoptosis [169]. However, in contrast to Bax and Bak which are widely expressed, Bok expression is mainly restricted to reproductive tissues [170].

1.3.4. Extrinsic cell death

1.3.4.1. Death receptors and ligands

Activation of the extrinsic apoptotic signaling pathway occurs through cell surface death receptors belonging to the tumor necrosis factor receptor (TNFR) gene superfamily [171]. Examples of these receptors and their ligands include,

respectively: TNFR-1 and TNFα, Fas/CD95/Apo-1 and Fas ligand, as well as the TNF-related apoptosis inducing ligand (TRAIL) receptors DR4 and DR5 and their ligand Apo2L/TRAIL. Death receptors from the TNFR superfamily are characterized by a cysteine-rich extracellular domain involved in substrate recognition and are known to trimerize upon activation [172]. Additionally, these death receptors also contain a cytoplasmic death domain (DD) that functions as a platform for the recruitment of other DD-containing adaptor proteins such as TNFR-associated death domain (TRADD) or Fas-associated death domain (FADD) that form part of the DISC involved in procaspase-8 recruitment and activation.

Currently, the extrinsic apoptotic pathway has been best characterized for the FasL/FasR and TNFα/TNFR-1 model pathways. However, it is important to note that activation of TNFR-1 typically results in an inflammatory response unless there is a block in new protein synthesis, whereby induction of cell death then occurs [171]. Upon stimulation of cells with ligand, death receptors trimerize and recruit cytoplasmic adaptor proteins. While Fas signaling results in FADD binding, activation of TNFR-1 signaling results in the recruitment of the adapter TRADD followed by the binding of FADD and receptor-interacting protein (RIP) [173, 174]. The association between FADD and subsequently recruited procaspase-8 via their death effector domains results in the formation of a DISC that can promote procaspase-8 autoactivation which is required for the initiation of downstream signaling events.

1.3.4.2. Type I and type II cells

The classification of type I and type II cells was originally developed with respect to Fas activation and is based on the observation that two different signaling pathways can be activated downstream of a death receptor [175]. In type I cells, including lymphocytes, the levels of cleaved caspase-8 are high enough for the direct processing and activation of downstream effector caspases that can subsequently induce cell death through the cleavage of key cellular substrates.

On the other hand, type II cells - such as hepatocytes and pancreatic β-cells - are characterized by the activation of a mitochondrial-dependent amplification pathway downstream of death receptor activation due to levels of caspase signaling that are too weak to induce cell death on their own. Activation of this pathway involves caspase-8-mediated processing of BH3-interacting domain death agonist (Bid) to its truncated form (tBid) [176]. Generation of tBid allows for the translocation of this protein to the mitochondria where it can cooperate with the pro-apoptotic Bcl-2 family members Bax and Bak to stimulate the release of cytochrome c and other pro-apoptotic factors from the mitochondria. Following from this, apoptosome formation takes place leading to the processing of procaspase-9, the activation of downstream effector caspases, cleavage of cellular substrates and ultimately cell death.

1.3.5. Disposal of cells following apoptosis

The final event to occur following apoptosis involves the phagocytosis of dead cells [177]. The removal of apoptotic cells is an important event that requires

cells to be eliminated with their plasma membranes still intact in order to prevent the activation of an inflammatory or immune response. It is believed that the breakdown of relatively larger cells into smaller apoptotic bodies helps to facilitate their safe and efficient removal by phagocytes. Although phagocytes are normally also involved in the recognition and removal of foreign particles, the way in which they respond to apoptotic cells differs to a great extent and involves the inhibition of proinflammatory cytokine production [178].

As a consequence of apoptosis, a number of different cell surface markers become present on cells and subsequently signals for their uptake by phagocytes. The best characterized example involves the caspase-dependent translocation of phopshatidylserine from the inner to the outer leaflet of the plasma membrane during apoptosis [179-181]. However, a number of other potential phagocytic ligands also exist including oxidized low-density lipoprotein [182], the endoplasmic reticulum chaperone protein calreticulin [183], intercellular adhesion molecule 3 (ICAM3) [184] or annexin-1 [185]. Additionally, the release of certain chemoattractants by apoptotic cells has been shown to facilitate their engulfment by phagocytes [186].

1.4. MODULATOR OF APOPTOSIS-1 (MOAP-1)

Modulator of apoptosis-1 (MOAP-1) is a reported mitochondria-enriched 39.5 kDa molecule that was first identified as a novel Bax-associating protein in a yeast two-hybrid protein screen [187]. Located at genetic locus 14q32, MOAP-1 contains 351 amino acid residues in humans and is conserved in chimpanzee (*Pan trogolodytes*), rat (*Rattus norvegicus*) and mouse (*Mus musculus*). Interestingly, in

mouse and humans, the MOAP-1 coding sequence is contained in a single exon (NCBI GenBank). Since its discovery in 2001, research has established a central role for MOAP-1 in both mitochondrial and death receptor-mediated apoptosis [84, 189]. When overexpressed in mammalian cells, MOAP-1 induces caspase-dependent apoptosis. Moreover, MOAP-1 knockdown cells are resistant to a variety of apoptotic stimuli including staurosporine, serum withdrawal, UV irradiation, TNFα and TRAIL [189]. Decreased cell death is also observed in cells from *Moap-1*$^{-/-}$ animals (Baksh et al., unpublished observations). Altogether, these results demonstrate the importance of MOAP-1 in apoptosis. As a key effector of Bax conformational change and activation, MOAP-1 plays an essential role in the cell death process.

1.4.1. Interaction of MOAP-1 with Bcl-2 family members

MOAP-1 selectively interacts with members of the Bcl-2 protein family. In particular, its association with Bax requires the presence of a Bcl-2 homology 3 (BH3)-like domain that is present within MOAP-1 (amino acids 120-127) and which is also essential for mediating apoptosis [190]. Interestingly, the binding of MOAP-1 to Bax requires all three BH (BH1, BH2, BH3) domains of the latter protein and is thus in contrast to other known Bax-associating partners. Additionally, it is speculated that MOAP-1 may bind at the hydrophobic cleft of Bax since critical point mutations in any of the three BH domains in Bax results in a loss of MOAP-1 association. The interaction between MOAP-1 and Bax occurs upon induction of apoptosis in response to activators of both the intrinsic and

extrinsic cell death pathways and facilitates the release of cytochrome c from mitochondria [189].

In addition to Bax, MOAP-1 also associates with the prosurvival proteins Bcl-2 and Bcl-X_L [190]. However, MOAP-1 does not interact with Bid, BimL, Bak, Bad or Bcl-w under the same experimental conditions. Evidence suggests that its interactions with Bcl-2 and Bcl-X_L may function to restrain the pro-apoptotic activity of MOAP-1 since overexpression of Bcl-X_L is sufficient to block MOAP-1-mediated cell death. Surprisingly, MOAP-1 differs from most pro-apoptotic molecules in that its association with Bcl-X_L is not dependent on its BH3-like domain but rather involves both its N-and C-terminal regions.

1.4.2. Cooperation of MOAP-1 with RASSF1A in death receptor-mediated apoptosis

MOAP-1 is required for execution of both the intrinsic and extrinsic pathways of apoptosis where it promotes Bax conformational change and translocation from the cytosol to the mitochondria prior to the release of apoptogenic factors [84, 189]. Although the mechanistic details of its role in the intrinsic pathway are currently unknown, the death receptor-dependent pathway involving MOAP-1 has been delineated to a great extent [84, 85] (Fig. 1.1).

Under non-stimulated conditions, MOAP-1 is normally held in a “closed” conformation through an intra-electrostatic interaction involving regions ^{178}EEEF and ^{202}KRRR [85]. However, stimulation of cells with TNFα or TRAIL results in the

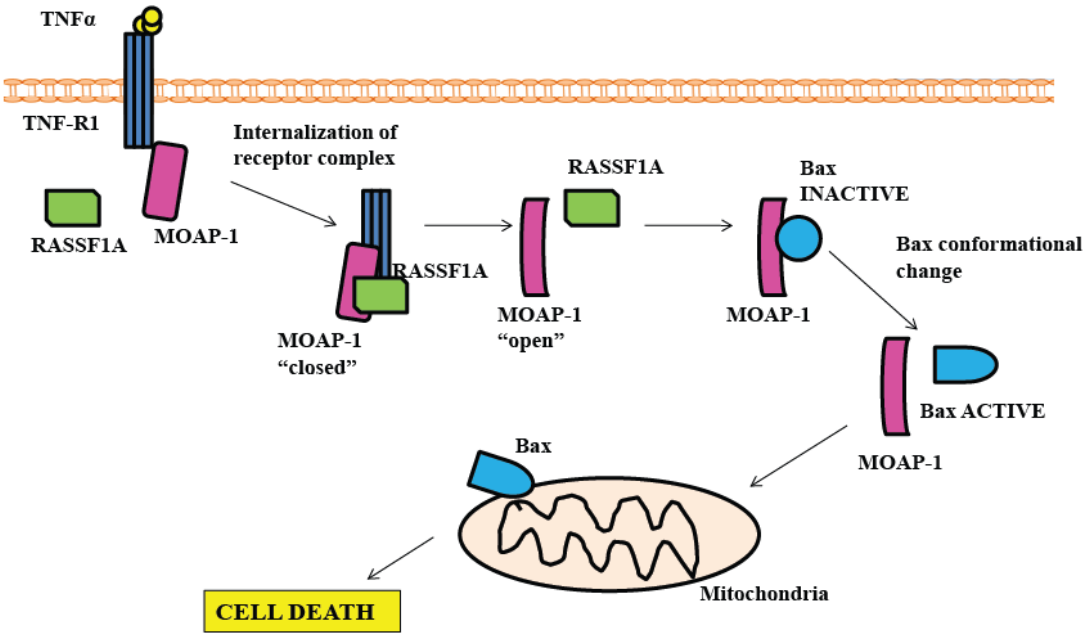

Figure 1.1. MOAP-1 cooperates with RASSF1A during death receptor-dependent apoptosis and promotes Bax function at the mitochondria. MOAP-1 is normally held inactive by intramolecular binding. In response to death receptor stimulation, MOAP-1 is first recruited to the receptor and then followed by RASSF1A association at the MOAP-1/receptor complex. The binding of MOAP-1 to RASSF1A promotes a conformational change in MOAP-1 that exposes its BH3-like domain required for Bax association. The subsequent interaction between MOAP-1 and Bax induces a conformational change in Bax that enables its translocation and insertion into the mitochondrial outer membrane resulting in the release of cytochrome c and other apoptogenic factors, leading to apoptosis.

recruitment of MOAP-1 to the receptor via a basic sequence (^{336}EEEEA) at its C-terminal end. A key protein involved in modulating MOAP-1 function during this signaling pathway is the tumor suppressor protein RASSF1A. Prior to death receptor association, RASSF1A loses its ability to homodimerize. Upon binding to the receptor through its N-terminal cysteine-rich (C1) domain, RASSF1A induces a conformational change in MOAP-1 to a more "open" state that exposes its BH3-like domain and allows it to bind and promote the activation of the pro-apoptotic protein Bax. The association of MOAP-1 with RASSF1A involves the sequence ^{202}KRRR in the former protein and ^{312}EEEE in the latter. Although activated K-Ras has been reported to be required for stabilization of the MOAP-1/RASSF1A complex [191], our lab is able to consistently detect robust associations between MOAP-1 and RASSF1A in experiments that do not require the presence of overexpressed active K-Ras [85]. Therefore, we are currently unable to explain or support the results of Vos and colleagues. MOAP-1-induced Bax conformational change enables Bax to translocate from the cytosol to the mitochondria where it can insert into the mitochondrial membrane and promote the release of cytochrome c as well as other apoptosis-inducing factors, resulting in cell death. Therefore, MOAP-1 functions alongside RASSF1A as a key component linking death receptor signaling to Bax activation and mitochondria-associated cell death. Moreover, this pathway exists as a separate, parallel signaling cascade that links the extrinsic and mitochondrial pathways of apoptosis independent of tBid [84].

1.4.3. Regulation of MOAP-1 stability by apoptotic signals

Under non-stimulated conditions, MOAP-1 is constitutively degraded by the ubiquitin-proteasome system and is normally a short-lived protein with a half-life of approximately 25 minutes [192]. However, evidence suggests that targeting of MOAP-1 to the proteasome may involve an unconventional mechanism given that no specific lysine residue can be identified as the site of polyubiquitination. Indeed, mutation of any individual lysine residue or combination of residues fails to abolish MOAP-1 ubiquitination. Thus, the process involved in controlling MOAP-1 turnover remains to be determined.

In addition to tight regulation of its basal levels, MOAP-1 is also rapidly upregulated in response to multiple apoptotic stimuli including serum withdrawal, etoposide, TRAIL and the endoplasmic reticulum stress inducer thapsigargin [192]. The increase in MOAP-1 protein arises through inhibition of its polyubiquitination and subsequent proteasomal degradation. Research findings demonstrate that elevation of MOAP-1 levels occurs prior to cell commitment to apoptosis and that the stabilization of MOAP-1 helps to sensitize cells to apoptosis by increasing the levels of activated Bax.

Intriguingly, stabilization of MOAP-1 in response to apoptosis employs the RING domain protein tripartite motif containing 39 (TRIM39) [193]. TRIM 39 has not yet been functionally characterized but belongs to the tripartite motif (TRIM) family of proteins that are commonly involved in innate immunity [194] and contains three zinc-binding domains including a RING, B box and coiled-coil region. Although a large number of proteins that contain RING domains also

function as E3 ligases [195], TRIM39 associates with MOAP-1 in a manner that promotes its stabilization rather than its polyubiquitination [193]. In particular, TRIM39 sensitizes cells to apoptosis by inhibiting MOAP-1 ubiquitination (through an unknown mechanism) and thus allows for the accumulation of this protein that can activate Bax.

1.4.4. Paraneoplastic antigens

In addition to its role as a pro-apoptotic molecule, MOAP-1 is also (unexpectedly) the fourth member of the paraneoplastic Ma antigen (PNMA) family and is consequently also known as PNMA4. Paraneoplastic antigens (also termed "onconeural antigens") are proteins that are restricted in expression to immune-privileged sites within the body (such as the brain) and are therefore recognized as foreign molecules by the immune system when aberrantly expressed at other sites [196, 197]. Remarkably, these foreign proteins are expressed by systemic tumors in a subset of cancer patients which subsequently triggers an immune-mediated anti-tumor response. Although this immune response is effective in suppressing tumor growth in certain individuals, activation of the immune system can also prove to have deleterious consequences. In some patients, this immune response is not only directed against the tumor itself but also towards the sites within the body that ordinarily express the protein. In the case of the brain, this immune response results in neuronal degeneration and the development of an autoimmune neurologic disease known as a paraneoplastic neurological disorder (PND). PNDs are rare and affect less than 1% of cancer

patients, yet are also the best known example of naturally occurring tumor immunity [198].

1.4.4.1. PNMA protein family

The PNMA family consists of six members (PNMAs 1-6) that were originally identified through screening of complementary DNA libraries using antibody-containing sera from patients with PNDs [199]. The first two members of this family, PNMA1 and PNMA2, were both cloned in 1999 and are expressed solely in the brain and/or testis [200, 201]. In contrast, PNMA3 is expressed in the brain, testis and several other systemic tissues [202] while MOAP-1 is ubiquitously expressed with higher levels in the heart and brain [190]. PNMA5 was recently identified as a brain-specific gene that is preferentially expressed in the association areas of the neocortex but has yet to be functionally characterized [203]. On the other hand, PNMA6 exists in two forms designated PNMA6A and PNMA6B which are genetically positioned in reverse orientations and share 99.2% amino acid identity [199].

The detection of antibodies to PNMAs 1-3 in PND patients is associated with disorders affecting the limbic system, brain stem and cerebellum but is not indicative of any particular cancer type [200-202, 204]. PNMAs 2-3 have been shown to localize to structures resembling nuclear bodies and evidence suggests that these proteins may be involved in mRNA biogenesis [202]. In support of this, PNMA3 possesses a zinc-finger motif that is common among transcription factors while PNMA2 contains a polypyrimidine tract that is often found in ribosomal proteins.

In contrast, MOAP-1 has a well-established role in apoptosis and- similar to PNMA5 and PNMA6- is not associated with the development of PNDs. Rather, MOAP-1 was identified as a PNMA family member as a result of extensive sequence similarity with the other PNMA proteins [187]. In particular, MOAP-1 displays the greatest amino acid sequence homology with PNMA1 (58%) which functions as a neuronal-specific pro-apoptotic molecule [205]. Interestingly, PNMA1 contains both a BH3-like domain and a conserved RASSF1A binding site similar to MOAP-1. However, PNMA1 does not associate with either Bax or RASSF1A [205] and therefore, although unknown, the mechanism by which it induces cell death presumably differs from that of MOAP-1.

1.5. RECEPTOR FOR ACTIVATED C-KINASE 1 (RACK1)

1.5.1. RACK proteins as PKC adaptors

Adaptor proteins carry out an important role in signaling biology by facilitating protein-protein interactions and the formation of molecular complexes, as well as regulating the localization of signaling proteins [206]. One well established family of adaptor proteins are the receptors for activated C-kinases (RACKs) that serve as shuttling molecules that regulate the subcellular localization of several signaling enzymes in addition to different activated protein kinase C (PKC) isozymes [207]. Upon activation, PKC is well-known for its translocation to the membrane and different subcellular compartments [208]. Currently, there are at least 10 different PKC isoforms that differ in their

sensitivity to phopshatidylserine, calcium and diacylglycerol (DAG) as cofactors [209]. Due to the extensive involvement of different PKC isozymes in multiple cellular processes [210], RACK proteins are also implicated in a wide range of biological phenomena. This section will focus on the first member of the RACK protein family, RACK1.

1.5.2. RACK1 scaffold protein

Receptor for activate C-kinase 1 (RACK1) is a 36 kDa protein that was first cloned in 1994 and serves as an adaptor for multiple PKC isozymes including its preferred binding partner PKC-βII [211]. The first demonstration for RACK1 as a shuttling protein was observed in dopamine receptor overexpressing Chinese hamster ovary (CHO) cells stimulated with either phorbol 12-myristate 13-acetate (PMA) or dopamine receptor agonist [212]. In this study, the authors found that stimulation of cells caused RACK1 and PKC-βII to move as a complex first to the cell periphery then to the perinuclear compartment where PKC-βII is finally active. Today, it is now well-recognized that RACK1 is important for targeting activated PKCs to their appropriate intracellular location. Although not a substrate of PKC itself, RACK1 binds within its C2 and V5 regions [213, 214] and the presence of RACK1 increases by several-fold PKC-mediated substrate phosphorylation [211].

As a homolog of the β subunit of G proteins, RACK1 assumes a β-propeller structure that contains seven Trp-Asp 40 (WD40) protein interaction domains [211]. Through its multiple WD40 repeats, RACK1 can simultaneously bind different signaling molecules. In addition to PKC, RACK1 can associate

with more than 80 different proteins including phospholipase Cγ [215]; a selection of pleckstrin homology domain-containing proteins [216]; protein tyrosine phosphatase μ [217]; cyclic AMP-specific phosphodiesterase PDE4D5 [218]; and Src family kinases [219, 220]. Consequently, the wide range of protein interactions involving RACK1 has implicated this scaffolding protein in multiple biological processes. Some of the biological functions of RACK1 include: regulation of cell proliferation via calcium signaling [221]; inhibition of Src kinase activity and cell growth [219]; modulation of integrin signaling [222, 223]; protein translation [224]; apoptosis [225, 226]; and multiple neuronal functions [227]. Two of these RACK1-dependent functions, eukaryotic translation and apoptosis, will be discussed in greater detail in the following sections.

1.5.3. Regulation of protein translation by RACK1

A number of different signaling pathways are involved in regulating the translation of proteins, several of which involve PKC [228]. Recently, RACK1 was also implicated as a protein involved in translational regulation. The first piece of evidence suggesting a role for RACK1 in protein synthesis came from results of a mass spectrometry study identifying RACK1 as a novel component of the yeast and human 40S ribosomal subunit [105]. Additionally, the authors also discovered that RACK1 was present at an equimolar concentration as other ribosomal molecules. This was followed by a cryo-electron microscopy study carried out a few years later demonstrating that RACK1 localizes to the head region near the mRNA exit channel and that its localization is conserved among various

eukaryotic ribosomes [229]. Recently, it has become evident that RACK1's ribosomal localization is important for at least some of its functions [230]. However, what is currently lacking is direct evidence relating its functions in signaling to its activities at the ribosome. Given RACK1's well-characterized function as an adaptor protein, the current hypothesis of RACK1's role at the ribosome is that it may be involved in the assembly or recruitment of signaling complexes that regulate translation.

In agreement with its originally identified adaptor protein function, one signaling molecule that RACK1 interacts with on mammalian ribosomes is activated PKC. During translation initiation, PKC phosphorylates eukaryotic initiation factor 6 (eIF6) which in turn enables the joining of 40S and 60S ribosomal subunits to form a functional 80S unit [231]. Although RACK1 and eIF6 are both present on ribosomes and interact with each other in the cytoplasm, there is currently no solid evidence connecting RACK1 function to eIF6. Nonetheless, the available data suggest that the release of eIF6 from 60S ribosomal subunits may involve a RACK1 and PKC-dependent pathway whereby RACK-1 mediated recruitment of PKC to the ribosome enables phosphorylation of eIF6.

Another proposed function for RACK1 at the ribosome includes regulating the translation of specific mRNAs. Depletion of RACK1 in cells has been shown to correlate with a selective decrease in the amount of ribosomal protein rpL25 due to a decrease in the recruitment of its mRNA to ribosomes [232]. On the other hand, RACK1 deletion in a separate study resulted in an increase in the levels of specific proteins [233]. *In S. cerevisiae*, RACK1-mediated translational regulation of

specific mRNAs is thought to be achieved by the recruitment of the mRNA binding protein Scp160 to the ribosome [234]. Therefore, RACK1 may be involved in either promoting or repressing translation depending on which proteins are involved and this likely occurs through an indirect mechanism.

1.5.4. Role of RACK1 in apoptosis

Src tyrosine kinase is a proto-oncogene that is implicated in several biological processes including regulation of the cell cycle, cell survival and cell motility [235]. Interestingly, RACK1 associates with - and is phosphorylated by - Src in a manner that is enhanced by PKC activation [219, 220, 236]. Although RACK1 regulates G1/S cell cycle progression by suppressing Src kinase activity [226], information concerning the pro-apoptotic functions of RACK1 indicates that its regulation of Src extends beyond the control of cell proliferation. RACK1 induces mitochondrial apoptosis by inhibiting Src activity and thus blocks the ability of Src to activate the Akt cell survival pathway [226]. Consequently, this leads to the activation of the transcription factor FOXO3, which functions as a strong inducer of both apoptosis and G_1 arrest. Additionally, RACK1 also promotes apoptosis by inhibiting the expression of anti-apoptotic proteins Bcl-2 and Bcl-X_L while inducing the expression of pro-apoptotic Bim through a Src-dependent mechanism [226]. Interestingly, RACK1 is also involved in mediating the degradation of BimEL through the ElonginB/C-Cullin2-CIS ubiquitin-protein ligase complex [505]. Inhibition of Src and the presence of Bim are both required for the oligomerization of Bax. Recent evidence shows that RACK1 promotes Bax

oligomerization by dissociating complexes of Bax and Bcl-X_L [225]. Moreover, RACK1 is involved in the depolarization of mitochondria membranes, release of cytochrome c and the activation of caspases 9 and 3 through the intrinsic pathway [226]. Therefore, RACK1 regulates cell growth through suppression of Src activity during both mitosis and apoptosis.

In contrast to RACK1's proapoptotic functions discussed above, it appears that this protein may also be involved in the negative regulation of T cell apoptosis [238]. Overexpression of RACK1 conferred resistance to dexamethasone-induced cell death and this correlated with translocation of PKCβ. This suggests that the activation of RACK1's preferred binding partner, PKCβ, might be involved in RACK1's anti-apoptotic effect in T cells.

1.5.5. Involvement of PKC and RACK1 in cancer

In 1982, Castagna et al. established a potential link between PKC activation and cancer development when it was found that the tumor promoting phorbol ester PMA could activate this family of enzymes [239]. Only a few years later, supporting evidence for PKC signaling in cancer became available. PKC expression was found to be elevated in malignant breast tumors relative to normal tissue [240]. Additionally, an inverse relationship was revealed between the level and activity of PKC and the level of estrogen receptor expression, which is also directly correlated with cancer prognosis [241, 242]. Not exclusive to breast cancer alone, a correlation between PKC levels and malignancy was also discovered in gastric cancer [243], colon carcinoma [244], pituitary tumors [245] and hepatocellular

carcinoma [246]. Given the close relationship between PKC and RACK1, it is not surprising that levels of RACK1 were also found to be altered in some human cancers. In particular, RACK1 expression is approximately fourfold greater in non-small cell lung cancer and eighteen times higher in colon carcinoma, relative to normal tissue [247]. Moreover, RACK1 was also shown to be upregulated during angiogenesis [247] and has also been identified as a predictor for poor clinical outcome in oral squamous carcinoma with an importance comparable to ki67 [248]. Additionally, in colon cancer, RACK1 is involved in downregulating the levels of the proapoptotic protein Fem1b which may contribute to apoptosis resistance during cancer progression [249].

In recent years, the role of RACK1 in breast cancer has been confounded by conflicting evidence. Research carried out by Al-Reefy et al. demonstrates that RACK1 levels are higher in normal breast tissue relative to their cancerous counterparts and that elevated RACK1 expression correlates with a good prognosis [250]. Thus, this is supportive of RACK1's pro-apoptotic function [226]. However, the opposite has also been shown to be true. In the same year, RACK1 was also identified as a biomarker for poor clinical outcome in breast cancer [251] and was shown to promote cancer cell proliferation, migration and metastasis both *in vitro* and *in vivo* [252], the latter of which occurs through activation of the RhoA/Rho kinase pathway [253]. One major difference between these two studies is that the results of Al-Reefy [250] and colleagues are based on actual patient samples whereas the research of Cao et al. [251, 252] has focused on the use of breast

carcinoma cell lines. Therefore, further investigation will be required in order to resolve these disparate findings.

1.6. UBIQUITINATION

1.6.1. Ubiquitin

Ubiquitin is a highly conserved 8.7 kDa molecule that is present in all eukaryotes and was originally purified by Goldstein and colleagues in 1975 [254]. Two years later, histone 2A (H2A) was identified as the first substrate to be modified by ubiquitin [255]. Although its significance and widespread function was unknown at the time, it is now clear that the addition of ubiquitin molecules to substrate proteins is involved in regulating virtually all aspects of cell biology [256]. This process, termed "ubiquitination", involves a conjugation reaction whereby an isopeptide bond is formed between the C-terminal glycine of ubiquitin and the epsilon amino group of a substrate protein's lysine residue [257]. Ubiquitin can be conjugated to proteins either as a single molecule (called "monoubiquitination") or as a chain of ubiquitins (known as "polyubiquitination"). As a highly globular and compact protein, ubiquitin is extremely resistant to temperature and pH alterations in addition to tryptic digestion [258]. Moreover, the process of ubiquitination is highly dynamic and allows for both the addition and removal of ubiquitin molecules from a protein [259]. As suggested by its name, ubiquitin can be found in virtually all tissue types and its ligation to substrate proteins is

recognized today as a major form of posttranslational modification with profound effects on the stability, function, localization or activity of its targets.

1.6.2. Ubiquitination process and enzymes

The ubiquitination reaction occurs in a three step process involving a key set of enzymes: 1) An activating enzyme (E1) that forms a thiol ester with the carboxyl group on the lysine C-terminal glycine in order to prepare it for nucleophilic attack, 2) a conjugating enzyme (E2) that carries the activated ubiquitin as a thiol ester, and 3) a ligase (E3) that transfers the activated ubiquitin from the E2 to the target lysine residue on a protein substrate [260]. Regardless of the biological outcome, all ubiquitin reactions known to date involve the same three steps and classes of enzymes.

In humans, only a few different E1 activating enzymes are responsible for activating all ubiquitination reactions [261]. In contrast, there are approximately forty E2 conjugating enzymes and over six hundred E3 ubiquitin ligases that help to ensure the specificity of ubiquitination. Each E2 conjugating enzyme can serve several E3 ligases; however, each E3 ligase can cooperate with only one or two different E2 enzymes in order to carry out its function [262, 263]. It is generally believed that E3 ligases are the most involved in substrate recognition and, hence, they are mainly responsible for the specificity and selectivity of ubiquitination. E3 ligases belong to one of three known protein families containing a HECT (Homologous to the E6-AP Carboxyl Terminus) [264], RING (Really Interesting New Gene) [265] or the more recently identified U-box [266] domains. HECT domains

differ from RING domains in that they contain a conserved cysteine residue that can form a thiol ester intermediate during a catalytic reaction whereas RING domains are characterized by the presence of a set of cysteine and histidine residues that serve as ligands for two zinc ions involved in stabilizing its globular conformation [264, 267]. On the other hand, U-boxes are similar in structure to RING domains but lack the metal chelating residues that are present in the latter [266].

The majority of ubiquitination reactions known to date typically only involve the three classes of enzymes described above. However, in some cases, an additional (E4) enzyme class known as ubiquitin chain elongation or assembly factors is also required [268]. This novel class of enzymes cooperate with E1, E2 and E3 enzymes during the polyubiquitination of certain proteins by binding to substrates that are already ligated with one or a few ubiquitin molecules and further catalyzing the ligation of additional ubiquitins in order to form a polyubiquitin chain [269]. Although some E4 enzymes also contain U-box domains similar to certain E3 enzymes, they compose a functionally distinct class of enzymes that cannot replace E3 function *in vivo* and moreover, have not been demonstrated to directly interact with E2 enzymes.

1.6.3. Ubiquitination signals

Whether or not a protein is ubiquitinated and the efficiency at which it occurs is determined by a number of different factors that contribute to substrate targeting including degradation signals or "degrons" [270]. One of the best characterized and possibly the simplest degradation signal to have been identified

is known as the N-degron and is applied as the N-end rule which governs the approximate half-life of a protein based on the properties of its N-terminal amino acid residue [271]. Although this rule can be used to provide a rough estimation of a protein's stability, it does not take into account the effect of amino acid modifications. According to this degradation rule, proteins contain either a stabilising or destabilising N-terminal amino acid residue and, in close spatial proximity, a specific internal lysine residue for ubiquitination [271, 272].

In general, most other ubiquitination signals are contained in short amino acid sequences. A second type of degron found in many short-lived proteins are PEST sequences which are stretches of amino acid residues rich in proline (P), glutamic acid (E), serine (S) and threonine (T) [273]. PEST sequences are typically flanked by several positively charged amino acids and often contain phosphorylation sites that are necessary for degradation [274-276]. In other proteins including the transcription factor STAT1 [277], CDK inhibitor Sic1p [278] and β-Catenin [279], phosphorylation alone can serve as a degradation signal by allowing for recognition by specific E3 ligases and is a commonly employed mechanism in biology. In contrast, phosphorylation can also serve as an inhibitory signal for degradation as seen in proteins such as c-Fos [280] and c-Jun [281].

Lastly, some degradation signals are conditionally active and include the so-called destruction boxes that are found in mitotic cyclins. During mitosis, the ubiquitination and subsequent degradation of cyclins is important for cell cycle progression and exit and is thus tightly controlled [282]. This is primarily achieved by regulating the activity of a key ubiquitin ligase known as the anaphase-

promoting complex (APC) that recognizes the conserved nine amino acid destruction box sequence [283]. As described earlier, the MOAP-1 binding partner and tumor suppressor RASSF1A plays a key role in regulating the APC through its interactions with C19ORF5/MAP1S and cdc20 [73, 75]. By restricting APC activity to late mitosis and G1 phase, the timing of cyclin degradation is coordinated in order to allow for controlled advancement in the cell cycle.

1.6.4. Ubiquitin chain assembly

Polyubiquitination and monoubiquitination of proteins both have important roles in regulating cell biology. Although polyubiquitination is historically the canonical form of ubiquitin modification, a relatively large number of monoubiquitinated proteins have been identified implicating the addition of single ubiquitin molecules in non-proteolytic biological processes [284]. In particular, monoubiquitination generally serves to regulate the location or activity of a protein. Although the biological significance of adding either one or multiple ubiquitin molecules to a substrate protein is not yet well understood, it is becoming clear that these two forms of modification are associated with different functional outcomes. One particularly interesting example is seen in the DNA polymerase processivity factor proliferating cell nuclear antigen (PCNA) which can be modified at the same site by monoubiquitination, polyubiquitination, or by the ubiquitin-like protein SUMO (small ubiquitin-like modifier) [285, 286] ultimately resulting in different consequences. However, the most commonly encountered

form of ubiquitin modification occurs in the form of polyubiquitination and, therefore, will constitute the major focus of this section.

The ability of ubiquitination to serve a wide range of functions is largely attributed to the fact that ubiquitin itself contains seven lysine residues to which additional ubiquitin molecules can be further conjugated in order to create distinct linkages [287]. During polyubiquitin chain assembly, the C-terminal glycine of one ubiquitin is joined to an internal lysine residue of the next ubiquitin molecule in order to form a chain. The internal lysine onto which subsequent ubiquitin molecules are attached serves as an important signal for downstream events. While polyubiquitin chains linked to K48 of ubiquitin are usually involved in signaling for protein degradation [262, 288], polyubiquitin chains linked through other lysines (for example, most commonly K63) are implicated in processes independent of proteolytic signaling [256, 261].

In general, it is thought that the polyubiquitination of proteins is the result of multiple conjugation reactions whereby ubiquitin molecules are added one at a time, and this is likely to be true since the concentration of free ubiquitin is much greater than that of preassembled ubiquitin chains [289]. However, it should also be noted that the transfer of preassembled polyubiquitin chains onto a substrate protein has previously been demonstrated *in vitro* and such chains have been shown to exist *in vivo* [290]. Thus, there remains a possibility that both ubiquitin pools - consisting of free molecules and preassembled chains - are utilized in the polyubiquitination process.

1.6.5. The ubiquitin-proteasome system

In 2004, Aaron Ciechanover, Avram Hershko and Irwin Rose were awarded the Nobel Prize in Chemistry for their research contributions leading to the discovery of a protein-regulating system known as the ubiquitin-proteasome system (UPS). Elucidation of the UPS first began in 1978 when a heat stable protein (later identified as ubiquitin) was found to be required for ATP-dependent proteolysis [291]. Two years later, work carried out by these Nobel laureates led to the hypothesis that ubiquitin ligation targets proteins for degradation [292]. Shortly thereafter, it was realized that the UPS serves as the principle mechanism by which short-lived proteins are degraded [293].

Selective protein degradation through the UPS regulates a diverse set of cellular processes by modulating levels of specific proteins influencing cell cycle progression [294], induction of inflammatory responses [295], transcriptional regulation [296] and multiple signal transduction pathways. Degradation through the UPS allows for rapid adjustments in the intracellular concentrations of a protein that may need to change with alterations in cell states. Additionally, ubiquitin-mediated proteolysis plays an important function by eliminating damaged or abnormal proteins. Typically, substrates that are targeted for proteolysis undergo K48-linked polyubiquitination resulting in the ligation of a chain of at least four sequentially attached ubiquitin molecules [288, 297]. On rarer occasions, the addition of single ubiquitin moieties may also be sufficient for targeting proteins for proteasomal degradation [298, 299]. Nevertheless, it appears that for certain proteins, multiubiquitination may help to accelerate the rate of proteolysis [288]. Another

possibility that has been suggested is that some proteins may be more susceptible to deubiquitination and therefore the addition of multiple ubiquitin molecules may be necessary in order for proper targeting to the proteasome [262].

During this process, the 26S proteasome plays a pivotal role by carrying out the proteolytic process. The 26S proteasome was the first threonine protease to be described [300] and is a 2.5 MDa complex that consists of a 20S core particle containing the protease catalytic sites and 19S regulatory cap complexes with multiple ATPase subunits [301]. During proteolysis, the 26S proteasome unfolds substrate proteins and translocates them into its interior using the energy acquired from ATP hydrolysis [302]. This is followed by hydrolysis of substrates into smaller peptides which can then be further degraded by peptidases into free amino acids. Ubiquitin molecules are also released from substrates and recycled for reuse after targeting to the proteasome [262] although it is not yet clear when ubiquitin molecules are released from a substrate during the degradation process.

Although ubiquitin is of central importance to proteasomal degradation, there are also exceptions whereby protein degradation by the 26S proteasome does not involve prior ubiquitination. One well-known example is for mammalian ornithine decarboxylase (ODC) [303]. In order to undergo ATP-dependent proteolysis by the 26S proteasome, ODC is not ubiquitinated but rather associates with the protein antizyme which - similar to ubiquitin - is not degraded by the process.

Interestingly, it also appears that certain proteins can be targeted for UPS-mediated degradation in a lysine-independent manner [304-306]. Although the sites of

ubiquitin addition remain unknown for the majority of these unconventional proteins, research has also demonstrated a noncanonical site of ubiquitination occurring at the N-termini of the transcription factor MyoD [307] and the latent membrane protein-1 of Epstein-Barr virus [308].

1.6.6. Ubiquitin-mediated non-proteolytic signaling

Although ubiquitination is best known for its role in signaling for proteolysis, this modification is also involved in regulating many non-proteolytic signaling events. One recently identified novel function of ubiquitin occurs during the DNA damage response where a number of key proteins are recruited in order to initiate repair. One of these proteins, PCNA, is involved in recruiting DNA polymerases during both DNA replication and post-replicative repair [285]. In response to chemical or UV-induced DNA damage, PCNA is monoubiquitinated in a Rad6 and Rad18-dependent manner [309] leading to the activation of translesion DNA synthesis [286]. Therefore, monoubiquitination of PCNA results in error-prone DNA repair. In contrast, polyubiquitination of PCNA of the same residue in a K63-linked manner by Ubc13/MmS2 and Rad5 signals for error-free repair of DNA damage. Not exclusive to PCNA-mediated DNA repair alone, ubiquitination is also involved in regulating both the Fanconi anemia and DNA double strand break repair pathways [310].

A second novel function of ubiquitin is its involvement in various aspects of transcriptional regulation including chromatin remodelling. One particular example is seen in the ubiquitination of yeast histone H2B which promotes the

site-specific methylation of histone H3 resulting in transcriptional silencing [311]. Interestingly, ubiquitin is also involved in regulating the function [312] and intracellular localization of several different transcription factors. The monoubiquitination of tumor suppressor p53 leads to its nuclear export [313] while polyubiquitination of transcription factors Smad3 [314] and hypoxia-inducible factor (HIF-α) [315] is required for their export from the nucleus.

Recent studies have revealed multiple additional non-proteolytic functions of ubiquitination including receptor endocytosis [316, 317], protein kinase activation [318], modulation of ribosomal function [319] and the lysosomal and vacuolar targeting of proteins [320, 321]. Most often, non-proteolytic signaling functions of ubiquitin involve atypical (non-K48-linked) ubiquitin chains including monoubiquitination or polyubiquitin linkages through K63 of ubiquitin. While K63-linked chains are the most well-studied atypical ubiquitin chain, all other lysine residues of ubiquitin have also been shown to be involved in chain formation *in vivo* [322]. Additionally, it is now also known that atypical ubiquitin chains can be of three different kinds: homotypic in which the lysine linkage is the same for all sequentially conjugated ubiquitin molecules; mixed-lineage when different lysine residues of consecutive ubiquitins are utilized in the formation of a chain; or heterologous whereby ubiquitin is connected with ubiquitin-like modifiers such as SUMO or NEDD8 (neural precursor cell expressed, developmentally downregulated 8) [322, 323]. Although there are still many unanswered questions surrounding the function and synthesis of atypical ubiquitin chains, it is becoming clear that there is an enormous versatility with which ubiquitin modifications can

occur. Thus, ubiquitin's involvement in regulating a diverse set of cellular events is largely due to differences in its location, number and type of lysine linkages.

1.7. RESEARCH OBJECTIVES

Apoptosis is a critical mechanism of tumor suppression. In response to death receptor signaling, MOAP-1 cooperates with the tumor suppressor protein RASSF1A in order to promote Bax activation and subsequently cell death [84,85]. Although epigenetic silencing of RASSF1A is a frequent event during early carcinogenesis [58, 60, 65], it is currently unknown if MOAP-1 expression may also be affected during cancer development or if it may possess anti-proliferative properties similar to RASSF1A. Therefore, in this research project we will examine the role of MOAP-1 as a candidate tumor suppressor protein. Additionally, we will also investigate the importance of non-degradative ubiquitination as a novel and previously undocumented mechanism of MOAP-1 regulation. Lastly, we will attempt to identify new interacting proteins for MOAP-1 in order to gain further information about this protein and its potential range of biological functions.

CHAPTER 2

Materials and Methods

2.1. Cell lines

The cell lines that were used in this study are listed in Table 2.1. The majority of these cell lines were obtained specifically for the generation of cell lysates to use for MOAP-1 expression analysis and were not maintained in cell culture. HCT 116 and HT29 cells were grown at 37°C and 5% CO2 in McCoy's 5A growth medium (Hyclone). U2OS and H1299 cells were grown under the same conditions in DMEM (High glucose, Hyclone) and RPMI 1640 media (Hyclone), respectively. All media types were supplemented with 10% bovine growth serum (Hyclone), 50 U/ml of penicillin and 50 ug/ml streptomycin solution (Hyclone). HEMa-Lp cells and melanoma cell lines A2058, WM35 and WM793 were obtained from Dr. Sujata Persad (Department of Pediatrics, University of Alberta). Breast cancer cell lines T47D, MDA-MB-468, MDA-MB-231, MCF-7 and SKBR3 were acquired from the lab of Dr. Ing Swie Goping (Department of Biochemistry, University of Alberta). Pediatric leukemia cell lines Molt-3, CEM, B1, KOPN8, TIB-202, W1, C1 and SEM were donated by Dr. Aru Narendran (Department of Pediatrics/Oncology, University of Calgary). Colon cancer cell lines SW480, Caco-2 and T84 were provided by Dr. Eytan Wine (Department of Pediatrics, University of Alberta). H1299 cell lines stably expressing pcDNA3-HA, pcDNA3-HA-RASSF1A, pXJ40-Myc-MOAP-1, pXJ40-Myc-MOAP-1 and pcDNA3-HA-RASSF1A, pXJ40-Myc-K264R-MOAP-1 and pcDNA3-HA-RASSF1A, or pXJ40-Myc-K278R-MOAP-1 and pcDNA3-HA-RASSF1A were generated by Dr. Shairaz Baksh.

Table 2.1. Cell lines used	
Cell line	**Tissue**
HEMa-Lp	Human epidermal melanocytes
A2058	Human melanoma lymph node metastasis
WM35	Human primary superficial spreading melanoma
WM793	Human primary superficial spreading melanoma
U2OS	Human osteosarcoma
Saos-2	Human osteosarcoma
MCF-10A	Human mammary epithelial cells
T47D	Human mammary adenocarcinoma
MDA-MB-468	Human mammary adenocarcinoma
MDA-MB-231	Human mammary adenocarcinoma
MCF-7	Human mammary adenocarcinoma
SKBR3	Human mammary adenocarcinoma
J77	Human T cell leukemia
THP1/TIB-202	Human acute monocytic leukemia
U937	Human histiocytic lymphoma
MOLT-3	Human acute lymphoblastic leukemia
CEM	Human acute lymphoblastic leukemia
B1	Human acute myeloid leukemia (relapsed as acute lymphoblastic leukemia)
KOPN8	Human acute lymphoblastic leukemia
W1	Human juvenile myelomonocytic leukemia
C1	Human pre-B acute lymphoblastic leukemia
SEM	Human acute lymphoblastic leukemia
DAOY	Human medulloblastoma
SKNAS	Human neuroblastoma
SHSY5Y	Human neuroblastoma
HT29	Human colon adenocarcinoma
HCT 116	Human colorectal carcinoma
SW480	Human colorectal adenocarcinoma
Caco-2	Human colorectal adenocarcinoma
T84	Human colorectal carcinoma
H1299	Human non-small cell lung carcinoma
A549	Human lung carcinoma

2.2. DNA transfection

2.2.1. DNA plasmids

All plasmids used in this study are listed in Table 2.2. pcDNA3-GFP-RACK1 and pEGFP-Bcl-X_L were provided by Dr. Chris Cartwright (Department of Medicine, Stanford University) and Dr. Michele Barry (Department of Microbiology & Immunology, University of Alberta), respectively.

2.2.2. DNA transfection protocol

Cells were cultured as described in section 2.1 and seeded in 6-well dishes one day prior to transfection. Cell transfection was performed by mixing plasmid DNA with polyethyleneimine (PEI; Polysciences, USA) at a ratio of 1 μg DNA: 4 ul PEI in 400 μl of serum-free growth media. The DNA/PEI mixture was allowed to incubate at room temperature for 15 minutes. During this incubation period, the cells to be transfected were washed 3 times with serum-free media. The PEI/DNA mixture was added drop-wise to each cell well containing 2 ml of complete growth media and mixed gently by hand. Cells were allowed to grow in culture overnight. The following day, transfection media was aspirated and replaced with 1.5 ml of complete media.

2.3. Protein methodology

2.3.1. Antibodies

All antibodies used in this study are listed in Table 2.3 along with their optimal usage concentrations and source.

Table 2.2. List of DNA plasmids used

Plasmid	Description
pcDNA3-HA	Expresses HA tag; used as empty vector control
pcDNA3-HA-RASSF1A	Expresses wild type RASSF1A
pXJ40-Myc-MOAP-1 (WT)	Expresses wild type MOAP-1
pXJ40-Myc-MOAP-1 (K264R)	Expresses MOAP-1 with lysine to arginine mutation at amino acid 264
pXJ40-Myc-MOAP-1 (K278R)	Expresses MOAP-1 with lysine to arginine mutation at amino acid 278
pEGFP-N1-MOAP-1	Expresses wild type MOAP-1
pEBG-GST	Expresses GST tag; used as empty vector control
pEBG-GST-MOAP-1	Expresses wild type MOAP-1
pcDNA3-GFP-RACK1	Expresses wild type RACK1
pEGFP-Bcl-X_L	Expresses wild type Bcl-X_L

Table 2.3. Antibodies used		
Antibody	**Dilution/Amount**	**Source**
Immunoprecipitation		
Mouse anti-myc	25 µl/1 ml cell lysate	Baksh Lab
Western blotting		
Mouse anti-GFP (B-2)	1:150	Santa Cruz Biotechnology
Mouse anti-HA	1:100	Baksh Lab
Mouse anti-c-Myc (9E10)	1:200	Santa Cruz Biotechnology
Mouse anti-RACK1 (B-3)	1:200	Santa Cruz Biotechnology
Mouse anti-Ubiquitin (P4D1)	1:500	Santa Cruz Biotechnology
Rabbit anti-ERK1 (C-16)	1:2000	Santa Cruz Biotechnology
Rabbit anti-ERK2 (C-14)	1:2000	Santa Cruz Biotechnology
Rabbit anti-MOAP-1 (11009)	1:500	QED Bioscience
Rabbit anti-PARP (9542)	1:1000	Cell Signaling
Anti-mouse IgG, HRP-linked	1:7000	GE Healthcare
Anti-rabbit IgG, HRP-linked	1:7000	GE Healthcare
Confocal microscopy		
Mouse anti-myc	1:50	Baksh Lab
Anti-mouse Alexa Fluor 555 (A21424)	1:250	Invitrogen
Flow cytometry		
Annexin V, Alexa Fluor 647 conjugate (A23204)	1:50	Invitrogen

2.3.2. Cell lysis and immunoprecipitation

The appropriate cell stimulants were added to cells prior to harvest (Tumor necrosis factor alpha [TNFα, Peprotech]; Phorbol 12-myristate 13-acetate [PMA, Sigma Aldrich]. Cells were collected into a 1.5 ml eppendorf using a cell scraper and washed twice with 1 x phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, 1.4 mM KH2PO4, pH 7.4) in order to remove growth media and potential bovine growth serum contaminants. Following washing, cells were lysed in 1 ml of RIPA lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 2 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride [PMSF] and 5 μg/ml aprotinin) and rotated at 4°C for 15 minutes in order to ensure complete cell lysis. Immunoprecipitation (IP) was subsequently performed using the collected cell lysates by the addition of the correct antibody and incubated overnight with rotation at 4°C. The next day, 15 μl of packed volume protein A or protein G sepharose beads (GE Healthcare) were added to each IP sample and incubated for one hour while rotating at 4°C. IP samples were then washed twice with 1 x PBS, resuspended in 100 μl 1 x PBS with protein loading dye, and protein complexes resolved by SDS-PAGE.

2.3.3. SDS-polyacrylamide gel electrophoresis

All protein samples were resolved on 10% polyacrylamide gels using pre-stained protein molecular weight markers (Bio-Rad, 161-0374EDU) as size ladders. IP and whole cell lysate samples were boiled at 100°C for 7 minutes

using a heating block prior to loading onto the gel. Large gels were run overnight at approximately 80 V in 1 x running buffer (0.0125 M Tris pH 8.3, 0.096 M glycine, 0.05% SDS) until resolved the following morning. Mini gels were run for approximately 1.5 hours at 150 V in 1 x running buffer until resolved.

2.3.4. Gel transfer techniques

2.3.4.1. Semi-dry transfer

Proteins resolved using a mini acrylamide gel were transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore) using a semi-dry transfer apparatus and buffer containing 50 mM Tris, 380 mM glycine, 0.1% SDS and 20% methanol. Prior to transfer, PVDF membranes were activated by soaking in pure methanol for approximately 20 seconds. Semi-dry transfer was performed for two hours at 450 mA and followed by membrane blocking for 30 minutes in 10% milk powder diluted in 1 x Tris buffered saline plus Tween-20 (TBS-T; 500 mM Tris pH 7.4, 100 mM NaCl, 0.05% Tween-20).

2.3.4.2. Wet transfer

Proteins resolved using a large acrylamide gel were transferred onto PVDF membranes (Millipore) using a wet transfer apparatus and buffer containing 25 mM Tris, 192 mM glycine and 20% methanol. Prior to transfer, PVDF membranes were activated by soaking in pure methanol for approximately 20 seconds. Wet transfer was performed at 4°C for three hours at 250 mA and

followed by membrane blocking for 30 minutes in 10% milk powder diluted in 1 x TBS-T.

2.3.5. Western blotting

Following membrane blocking, PVDF membranes were immunoblotted with the antibodies and concentrations listed in Table 2.3. Antibodies were diluted in 5% milk powder in TBS-T and incubated with membranes overnight on a shaker at 4°C. Membranes were subsequently washed three times for five minutes with 1 x TBS-T to remove any unbound primary antibody and incubated on a shaker at room temperature with the appropriate horseradish peroxidase (HRP)-linked secondary antibody diluted in 5% milk powder in 1 x TBS-T for one hour. Membranes were washed again three times for five minutes with 1 x TBS-T prior to visualization of Western blots using enhanced chemiluminescence (ECL; GE Healthcare) and x-ray film (Fuji RX).

2.4. Fixed-cell immunofluorescence confocal microscopy

HCT 116 cells were seeded onto 1.5 mm square coverslips (Fisher Scientific) in a 6-well dish and transfected the following day with the appropriate DNA plasmids using PEI as described in section 2.2.2. Two days after transfection, cells were gently washed twice using 1 x PBS before adding 3.7% paraformaldehyde dissolved in 1 x PBS in order to attach cells to coverslips. After a 15 minute incubation period at room temperature, cells were permeabilized by adding 0.1% Triton X-100 in 1 x PBS for 5 minutes at room temperature.

Blocking was performed for 30 minutes at room temperature in 5% bovine growth serum and 0.1% Tween-20 dissolved in 1 x PBS. Blocking solution was removed from cells prior to adding primary antibody solution and incubated overnight at 4°C. Thereafter, cells were incubated with Alexa Fluor 555 secondary antibody for 30 minutes in the dark at room temperature. In between each step following fixation, cells were washed twice with 1 x PBS. Cells on coverslips were mounted onto glass slides (Fisher Scientific) using approximately 10 µl of Vectashield with DAPI (Vector Labs) as mounting medium. Coverslips were allowed to dry overnight in the dark at room temperature and sealed to glass slides the next day by applying nail varnish to the coverslip periphery. Slides were visualized using a Leica SP5 confocal microscope at 458 nm to detect cell nuclei stained with DAPI, 488 nm to detect GFP fluorescence and 543 nm to detect MOAP-1 when using Alexa Fluor 555 as a secondary antibody. Confocal images were obtained using the Leica Application Suite LAS AF imaging software. Slides were subsequently stored at -20°C in order to preserve fluorescent signal.

2.5. Calf intestinal phosphatase treatment

To evaluate the potential phosphorylation of MOAP-1 in C1 and SEM pediatric leukemia cell lines, treatment of cell lysates was performed using calf intestinal alkaline phosphatase (CIP; New England BioLabs). Approximately 50 µg of C1 or SEM cell protein lysate was combined with 100 units of CIP diluted in NEBuffer 4 (New England BioLabs) and incubated at 37°C for 60 minutes. Protein dephosphorylation was then assessed by Western blotting.

2.6. MTT cell proliferation assay

H1299 cells were seeded at 8000 cells per well in a 96 well plate and allowed to grow overnight. The following day, cell media was aspirated and replaced with 100 µl of complete growth media and 3.6 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma Aldrich). After 3 hours, media containing MTT was aspirated and 50 µl of DMSO was added to each well with resuspension of purple formazan product. Absorbance measurements were read at 560 nm using a Perkin Elmer 2030 multilabel plate reader (VICTOR X4). Significance of data was evaluated by performing a Student's t-test (two-tailed).

2.7. Apoptosis detection assay by fluorescence-activated cell sorting

H1299 cells were stimulated to undergo apoptosis by adding 50 ng/ml TNFα and 10 µg/ml CHX for 10 hours. Cells were harvested by trypsinization and collected into polystyrene round-bottom fluorescence-activated cell sorting (FACS) tubes (BD Falcon). Two micrograms of Annexin V solution (Alexa Fluor 647 conjugate, Invitrogen) was diluted in 100 µl of annexin V binding buffer (BD Pharmingen) and added to cells for 15 minutes at room temperature in the absence of light. After incubation, 400 µl of annexin V binding buffer was added to dilute each sample along with 0.1 µg of DNA intercalating agent propidium iodide (PI; BD Pharmingen). FACS was carried out using a FACSCalibur flow cytometer (Becton-Dickinson) by sequential gating on the FL3 (PI) and FL4 (Annexin V-Alexa Fluor 647 conjugate) channels. Data were acquired for 8000 cells per

sample and analysis was performed using CellQuest software. Significance of data was evaluated by performing a Student's t-test (two-tailed).

2.8. Animal Experiments

2.8.1. Mouse splenocyte isolation

All mice used for splenocyte isolation were male and an average of 13 weeks of age with the strain background C57BL/6 with the exception of Rassf5a knockout mice that were from the C57BL/6.129 background. Six genotypes were used: Wild type (Charles River Laboratories, USA), *Moap-1*$^{-/-}$ (Dr. Victor Yu, National University of Singapore), *Rassf5a*$^{-/-}$ (Dr. Sean Lee, National Institutes of Health, USA), *Rassf1a*$^{-/-}$ (Dr. Gerd Pfeifer, Beckman Research Institute, USA) and *Rassf1a*$^{-/-}$/*Moap-1*$^{-/-}$ (generated at the University of Alberta). Mice were sacrificed by CO_2 narcosis and spleens removed by surgical dissection. Using two frosted glass slides (Fisher Scientific), spleens were crushed by applying a rubbing motion to either side of the organ. The cell suspensions generated were pelleted and supernatants resuspended in 5 ml of red blood cell lysing buffer (Sigma Aldrich). 1 ml of sterile bovine growth serum was carefully added to the bottom of each cell suspension and allowed to sit at room temperature for 5 minutes prior to centrifugation. Following cell pelleting, supernatants were decanted and isolated splenocytes were resuspended in complete growth media. Cell counting was performed under a microscope using a haemocytometer and

significance of the data was evaluated by performing a Student's t-test (two-tailed).

2.8.2. Xenograft tumor assays

The establishment of human tumor xenografts was performed in male athymic nude mice that were 8 to 10 weeks of age (Taconic Laboratories #NCRNU-M, CrTac:NCr-FoxN1Nu). HCT 116 cells were transfected with the appropriate DNA constructs using the method outlined in section 2.2.2 and harvested by trypsinization two days later. Transfected cells were resuspended in a 3:1 ratio of complete growth media to matrigel basement membrane matrix (BD pharmingen). One hundred fifty microlitres of cell suspension (~1 x 10^6 cells) was injected subcutaneously into the left and right flanks of athymic nude mice using a 21G needle in order to determine the tumor promoting potential of wild type MOAP-1 or its lysine mutants. Mice were monitored over the course of 35 days for tumor formation and size and euthanized prior to the end of experiment if tumors exceeded 20 mm in diameter. Tumor sizes were calculated using the formula for spherical volume and data evaluated by performing a Student's t-test (two-tailed).

2.9. Microarray Analyses

2.9.1. Oncomine meta-analysis of human cancer microarrays

Differential expression analysis was performed for MOAP-1 in the normal versus cancer category using the publicly available Oncomine cancer microarray

database, version 4.4 Research Edition (Compendia Bioscience, Ann Arbor MI). Microarray datasets available through Oncomine are downloaded from public websites or provided by the authors upon request [324, 325]. Oncomine independently processes and normalizes each dataset used in their analyses and employs t-statistics with false discovery rates as a corrected measure of significance. All data are log-transformed, median centered per array and standard deviation normalized to one per array. We assigned a cut threshold of fold change ≥2 and $p \leq 0.05$ 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.

2.9.2. MOAP-1 and RASSF1A expression analysis in neuroblastoma patients

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.

2.9.3. Analysis of MOAP-1 expression in breast cancer patients

Breast cancer microarray data were made available to us by Dr. John Mackey (Department of Oncology, University of Alberta) and have previously

been used for a publication on DEAD box 1 [326]. Briefly, 176 primary, treatment-naive breast cancer samples and 10 normal breast tissue samples were obtained from the Canadian Breast Cancer Foundation Tumor Bank and used for gene expression microarray analysis. Patient information was also collected under Research Ethic Board Protocol ETH-02-86-17. Total RNA was isolated from frozen samples using Trizol and Qiagen RNeasy columns and were subjected to linear amplification and Cy3 labeling prior to hybridization to Agilent Whole Human Genome Arrays using Agilent Technologies Kits (One color Low RNA Input Linear Amplification Kit Plus, One Color RNA Spike-In Kit and Gene Expression Hybridization Kit). Microarray gene expression data were extracted and evaluated for quality using Feature Extraction Software 9.5 (Agilent Technologies) and subsequently normalized and analyzed using GeneSpring GX 7.3 (Agilent Technologies). Using the breast cancer microarray data provided to us, data calculations and sorting for MOAP-1 expression were performed using Microsoft Excel 2010. Data for MOAP-1 expression from 176 patients and 10 normal individuals were $1og_2$ transformed and grouped into populations of normal and breast cancer patients. Breast cancer patients were further sub-divided based on subtypes: luminal A (ER+ve, PR+ve, HER2-ve), luminal B (ER+ve, PR+ve, HER2+ve), Her2-amplified (ER-ve, PR-ve, HER2+ve) or triple-negative (ER-ve, PR-ve, HER2-ve) using the available patient clinical information. Changes in average MOAP-1 expression during breast cancer and for each cancer subtype were assessed relative to normal breast tissue and significance of data were evaluated by Student's t-tests (two-tailed). MOAP-1 expression levels were also

examined with respect to several clinical and pathological features of interest by first calculating a low/high MOAP-1 expression cut-off through receiver operating characteristic (ROC) curve analysis. For each category of interest, patients were then sorted into groups based on whether they displayed either high or low levels of MOAP-1 expression. Correlations between levels of MOAP-1 expression and each feature of interest were evaluated by Student's t-test (two-tailed).

2.9.4. Gene expression profiling of human tumor xenografts

HCT 116 human tumor xenografts were established in athymic nude mice as described in section 2.8.2 and allowed to grow for 35 days prior to surgical excision. Tumors were stored in RNAlater solution (Qiagen) and used for RNA isolation according to manufacturer's protocol with Qiagen RNeasy kits. RNA samples with RNA integrity numbers (RIN) greater than 7.0 were used in this study and were subjected to linear amplification and Cy3 labeling prior to hybridization to Agilent Whole Human Genome Arrays using Agilent Technologies Kits (One color Low RNA Input Linear Amplification Kit Plus, One Color RNA Spike-In Kit and Gene Expression Hybridization Kit). Gene expression profiling was carried out for three individual RNA samples for each construct that was analysed. The arrays were scanned using an Agilent scanner and data extracted and evaluated for quality using Feature Extraction Software 10.5.1 (Agilent Technologies). Data normalization and analysis was performed using GeneSpring GX 11.5.1 (Agilent Technologies). Microarray expression data were subjected to one-way analysis of variance (ANOVA), post-hoc analysis by

Turkey's HSD test and Benjamini and Hochberg false discovery rate multiple testing correction. Gene probes that lacked GenBank accession numbers were filtered out from the dataset as were genes that displayed very low expression in most samples. Normalized data were $\log_2$ transformed and averaged over three replicates per construct.

2.9.4.1. Canonical pathway and biological function analysis of xenograft gene expression changes

Post-analysis of gene expression changes in HCT 116 xenografts was performed using Ingenuity Pathway Analysis software (IPA; Ingenuity systems, www.ingenuity.com) in order to determine the biological functions and canonical pathways most strongly associated with our data. 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. Significance of the association between the dataset and canonical pathway was measured by: 1) providing a ratio of the number of molecules from the dataset that map to the pathway divided by the total number of molecules involved in the canonical pathway, and 2) Fisher's exact test to calculate a p-value to determine the probability that the association between the genes and the canonical pathway is explained by chance alone. Graphical representations of selected canonical

pathways were also generated using IPA with up- or down-regulated molecules from our dataset represented in red and green, respectively.

2.9.4.2. Venn analysis of differentially expressed genes

Venn analysis was performed using GeneSpring GX 11.5.1 (Agilent Technologies) in order to generate lists of shared and exclusive genes between two dataset comparisons. Microsoft Excel 2010 was used to generate a selection of genes from each group and Venn diagrams created with Microsoft Word 2010.

2.10. GST-MOAP-1 pull-down assay

HT29 cells were transiently transfected with pEBG-GST or pEBG-GST-MOAP-1 as described in section 2.2.2 and stimulated with TNFα (20 ng/ml) for the indicated times. Cell lysates were generated and subsequently incubated with glutathione sepharose beads in order to bind GST control or GST-MOAP1 and any associated protein complexes prior to resolution by SDS-PAGE on a 10% acrylamide mini gel. The subsequent steps were performed as described under the "Blue-Silver" Coomassie staining protocol obtained from the lab of Dr. Richard Fahlman (Department of Biochemistry, University of Alberta). Briefly, the acrylamide gel was fixed in 50% ethanol and 2% phosphoric acid for 2 hours on a shaker at room temperature and washed twice for 20 minutes in double distilled water. Blue-Silver Coomassie staining solution (0.67 M ammonium sulphate, 1.4 mM Coomassie Blue G-250, 10% phosphoric acid and 20% methanol) was

incubated overnight with the gel on a shaker and followed by rinsing with double distilled water until clear. Protein bands of interest were excised from SDS-PAGE gel using a sterile scalpel over a light box and sent for in-gel digestion and protein identification by liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis at the Institute for Biomolecular Design (University of Alberta).

CHAPTER 3

MOAP-1 functions as a tumor suppressor protein and demonstrates potential prognostic value in human cancer

3.1. INTRODUCTION

MOAP-1 is a proapoptotic protein that is required for caspase-dependent apoptosis in response to activators of both the intrinsic and extrinsic pathways of cell death [187, 189]. During death receptor signaling, the ability of MOAP-1 to induce cell death is dependent on its ability to associate with the bona fide tumor suppressor protein RASSF1A [84, 85]. As a result of this interaction, MOAP-1 undergoes a necessary conformational change that exposes its BH3-like domain through which it can bind and activate Bax. Therefore, the interaction between MOAP-1 and RASSF1A downstream of death receptors is required for the induction of apoptosis.

Although RASSF1A serves as a critical MOAP-1 binding partner, its frequent inactivation and loss of expression is observed in a wide range of human cancers [58, 60, 65]. While we know that MOAP-1 is an important modulator of Bax activity and mitochondrial-linked cell death, it is currently unknown if MOAP-1 also possesses anti-proliferative properties analogous to RASSF1A or if its expression is also altered during cancer. Indeed, the ability of cells to evade apoptosis is considered a hallmark of nearly all cancers and contributes to the expansion of tumor cell populations [327]. Given that MOAP-1 cooperates with RASSF1A to modulate apoptosis, it is plausible that MOAP-1 may also be involved in tumor suppression in a similar manner as RASSF1A and that it, too, may become inactivated during neoplastic progression. Therefore, we were interested in investigating a potential tumor suppressor function for MOAP-1 that may be important in the prevention of cancer. Based on this study, our results indicate that MOAP-1 functions as a tumor

suppressor protein both *in vitro* and *in vivo* and also frequently displays decreased expression in human cancers. Equally important, reduced MOAP-1 expression appears to have significant prognostic value for at least some cancer types and correlates with poor patient outcome for both neuroblastoma and breast cancer.

3.2. RESULTS

3.2.1. MOAP-1 is a cytoplasmic protein and accumulates in intracellular puncta

A previous study investigating the intracellular localization of MOAP-1 demonstrated through cell fractionation, confocal microscopy and a series of detergent treatment experiments that MOAP-1 is a mitochondria-associated protein localized to the outer mitochondrial membrane [189]. This raised the question about the role of MOAP-1 at the mitochondria under non-stimulated conditions and the significance of this localization given that MOAP-1 possesses cytoplasmic functions away from the mitochondria during apoptosis. Previously in our lab, confocal microscopic analysis of MOAP-1 had demonstrated a general pan-cytoplasmic staining pattern for this protein that was not reminiscent of a mitochondrial association (Baksh et al., unpublished observations). Thus, these results were in contrast to what was originally reported for the localization of MOAP-1 [189]. Given the important connection between a protein's intracellular localization and its normal biological function, we decided to examine once again the subcellular localization of MOAP-1 in non-apoptotic cells in order to gain a

better understanding about this protein and how it may function during apoptosis. HCT 116 colon carcinoma cells were transiently transfected with pXJ40-Myc-MOAP-1 or pEGFP-N1-MOAP-1 and were subsequently analysed by confocal microscopy.

Consistent with our previous lab findings, both Myc-MOAP-1 and GFP-MOAP-1 exhibited pan-cytoplasmic staining patterns (Fig. 3.1.). Noticeably, we did not observe the subcytoplasmic granular staining pattern that is typically seen for mitochondrial-associated proteins [328]. However, in some of these cells, MOAP-1 regularly accumulated in cytoplasmic puncta that are larger in the presence of Myc-MOAP-1 and less pronounced with GFP-MOAP-1 overexpression (Fig. 3.1A, B, Top panels). Although we have not yet investigated the identity of these punctate structures, one possibility is that they colocalize with certain ribosomal proteins that we have identified as having a novel connection to MOAP-1 [329, 330]. One important consideration to take into account when comparing our consistent pan-cytoplasmic staining patterns for MOAP-1 to the mitochondrial localization reported by Tan et al. [189] is the difference in cell lines used for analysis. For example, Tan and colleagues previously demonstrated a mitochondrial localization for MOAP-1 in 293T and MCF-7 cells whereas the experiment shown here was performed using HCT 116 cells. It is possible that MOAP-1 may be both cytoplasmic and localized at the mitochondria and that the ratio of the quantities present at each location may differ depending on cell type. However, confirmation of the true localization of MOAP-1 still requires the

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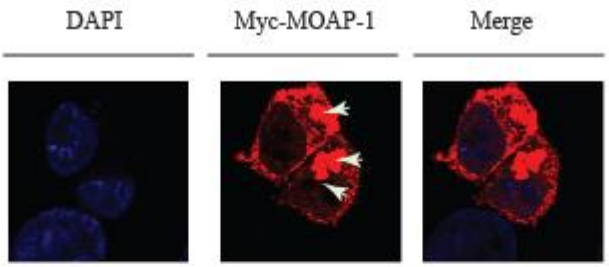

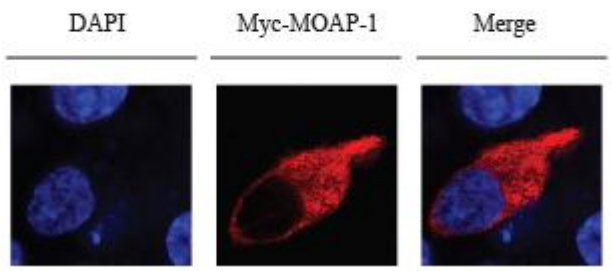

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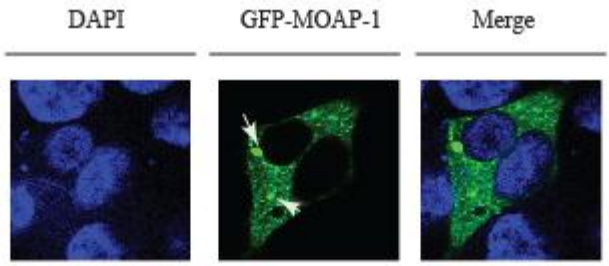

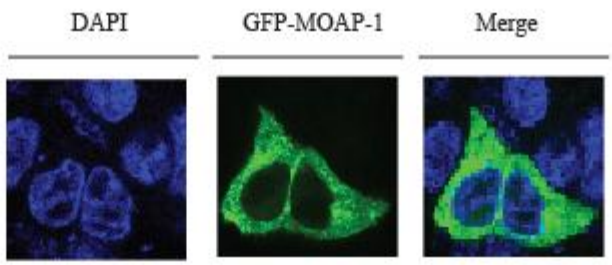

Figure 3.1. MOAP-1 exhibits a pan-cytoplasmic staining pattern and accumulates in cytoplasmic puncta. HCT116 cells were transfected onto square coverslips with (A) Myc-tagged MOAP-1 construct or (B) GFP-tagged MOAP-1. Forty-eight hours post-transfection, cells in (A) were immunostained with mouse anti-Myc antibody and goat anti-mouse Alexa 555 secondary antibody. Cellular localization of MOAP-1 was visualized by confocal microscopy using a Leica SP5 microscope. Arrows in the top images of (A) and (B) point to MOAP-1 accumulation in puncta.

analysis of a broader range of cell lines and may require possibly more intensive techniques including cellular fractionation. Nonetheless our current understanding of MOAP-1's intracellular localization is that it is not a strictly mitochondrial-associated protein as it has been previously reported.

3.2.2. MOAP-1 expression is reduced in multiple human cancers

A loss or decrease in the expression of tumor suppressor genes commonly occurs during cancer development and subsequently promotes the growth and proliferation of neoplastic cells [327]. To gain insight as to whether MOAP-1 may normally function as a tumor suppressor protein, we decided to begin our investigation by assessing for any changes in its expression in human cancer. A meta-analysis of microarray data was performed using the online cancer microarray database Oncomine (Compendia Bioscience, Ann Arbor, MI) [324]. In this program, each dataset is processed and normalized independently with the differential expression analysis module employing t-statistics and false discovery rates as a corrected measure of significance. With this in mind, differential expression analysis of MOAP-1 was carried out in samples from normal versus malignant tissues with data subsequently compiled from all available microarray studies meeting the threshold fold-changes ≥ 2 and $p \leq 0.05$. The results that were obtained from this meta-analysis indicate that MOAP-1 expression is markedly reduced in multiple types of human cancer (Fig 3.2). Results from several microarray studies support on average a significant downregulation of MOAP-1 in brain cancers and sarcomas and the currently available datasets suggest that this

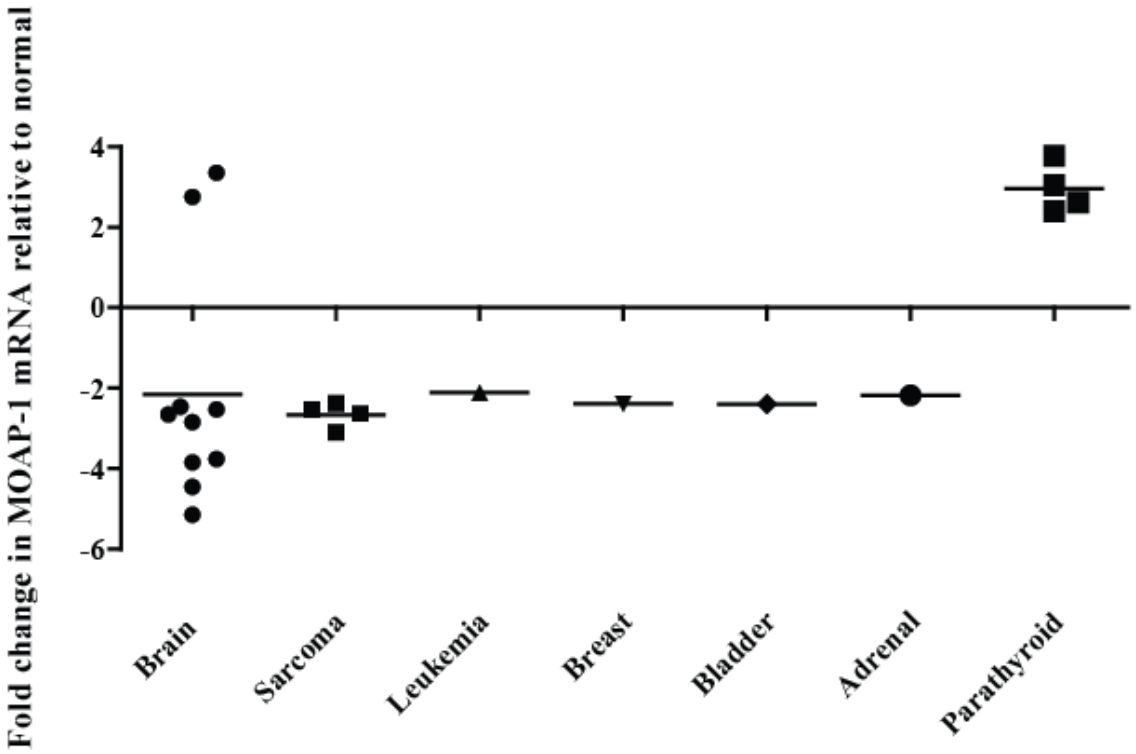

Figure 3.2. Meta-analysis of microarray data reveals downregulation of MOAP-1 in human cancers. Differential expression analysis of MOAP-1 in normal versus cancer tissues was performed using the cancer microarray database Oncomine (Compendia Bioscience, Ann Arbor, MI). Results from individual microarray studies are each represented by a single point on this plot. 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. Threshold values for all analyses: fold- change ≥ 2, $p \leq 0.05$.

may also be common among leukemias, breast, bladder and adrenal cancers. Evidently, MOAP-1 expression is significantly elevated in parathyroid cancer which is in contrast to the rest of the data that we retrieved. Given the rarity and poorly understood etiology of parathyroid malignancy [331], it is difficult to ascertain why MOAP-1 would be upregulated in this form of endocrine cancer. However, loss of expression of the parathyroid cancer-associated gene *CDC73* is thought in some cases to contribute to the pathogenesis of this cancer by promoting apoptosis [331, 332]. Additionally, several oncogenic mutations have been shown to promote - rather than suppress - apoptosis [333] and, therefore, it is possible that alterations in *MOAP-1* during cancer development may result in its elevated expression and aberrant contribution to carcinogenesis. Nonetheless, the overall average reduction in MOAP-1 expression in human cancer suggests that loss of this protein is important for tumorigenesis to occur and thus supports a potential tumor suppressor function for this protein.

To examine if MOAP-1 expression is also altered in human cancer cell lines, we decided to test for any expression changes in MOAP-1 that may support the findings from our microarray meta-analysis. Cell lysates derived from a variety of different human cancer cell lines were collected and resolved by SDS-PAGE prior to Western blotting against MOAP-1. In support of a general reduction in *MOAP-1* expression during cancer, a loss or decrease in MOAP-1 protein levels was detected in multiple cell lines derived from melanoma, bone and breast cancers (Fig. 3.3). Additionally, *MOAP-1* expression was found to be retained in both non-transformed cell lines HEMa-LP and MCF-10A albeit at

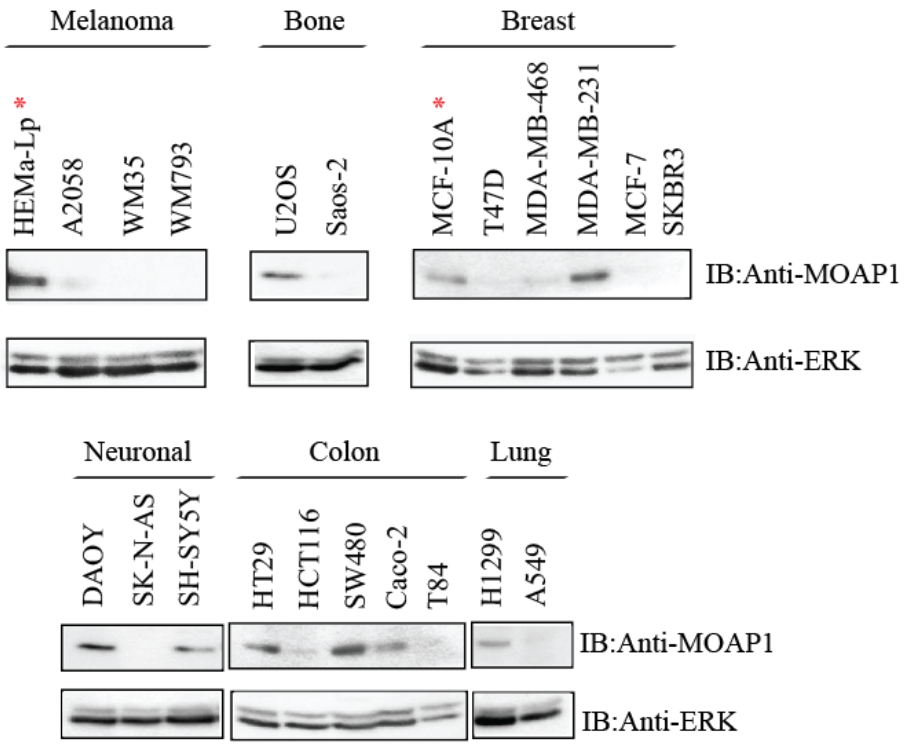

Figure 3.3. MOAP-1 expression is reduced in multiple transformed human cancer cell lines. Western blotting (IB) was performed to analyse levels of MOAP-1 expression in the indicated cell lines. Red asterisks signify a non-transformed positive control cell line.

different levels (Fig. 3.3). Noticeably, MOAP-1 expression is detectable in the p53-positive U2OS cell line but is absent from the p53-null cell line Saos-2. Meanwhile, MOAP-1 is also absent from mutant p53-containing cell lines MDA-MB-468 and T47D. However, we note that the expression of MOAP-1 does not always correlate with the status of p53 for each of the cell lines tested.

In brain, colon and lung cancer cell lines, expression of MOAP-1 is slightly more variable (Fig. 3.3) but this may reflect the genetic heterogeneity among these cancers as well as differences in their tissues of origin. In our brain cancer cell line panel, MOAP-1 is present at higher levels in DAOY medulloblastoma cells than in two metastatically derived neuroblastoma cell lines, SK-N-AS and SH-SY5Y. Similarly, T84 cells generated from a lung metastasis display the lowest levels of MOAP-1 among our selection of colon cancer cell lines tested. These results suggest a possible association between levels of MOAP-1 expression within a cell and its metastatic potential. In comparison to MOAP-1, RASSF1A expression is frequently downregulated in several of the same cancer types tested including breast [334, 335], skin [336], brain [337] and colorectal [338] malignancies. *RASSF1A* is also highly epigenetically silenced in small cell lung cancer but occurs less commonly in non-small cell lung cancer [339-341]. Therefore, these results suggest that a combined loss of MOAP-1 and RASSF1A expression may be present in some cancer types and may possibly contribute to malignant cell growth.

Recurrent chromosomal abnormalities involving the genetic locus of *MOAP-1*, 14q32, are frequently observed in malignant B- and T-lymphoid

diseases including chronic lymphocytic, acute lymphoblastic and adult T-cell leukemias [342]. As it is currently unknown if *MOAP-1* is directly involved in these cancer-associated chromosomal alterations, we decided to evaluate MOAP-1 expression in a number of leukemic cell lines. Our Western blotting results indicate that MOAP-1 protein levels are decreased in many of the leukemia cell lines tested (Fig. 3.4A). However, MOAP-1 expression does not appear to correlate with the cancer subtype and whether it is acute lymphocytic (ALL), acute myelocytic (AML) or T-cell (TCL) leukemia. Intriguingly, repeated immunoblotting of these leukemia cell lines consistently displayed a higher migrating band for MOAP-1 in both C1 and SEM pediatric ALL cell lines. This suggested to us that perhaps MOAP-1 was present in a post-translationally modified form in both C1 and SEM cells. As an initial hypothesis, we decided to test the possibility that the higher migrating bands in C1 and SEM represented a phosphorylated form of MOAP-1. To assess this hypothesis, a calf intestinal alkaline phosphatase (CIP) treatment was performed on C1 and SEM cell lysates prior to SDS-PAGE and Western blotting. Surprisingly, CIP treatment resulted in a collapse of the higher migrating MOAP-1 bands to their expected sizes based on molecular weight thereby supporting our hypothesis that MOAP-1 may be phosphorylated in C1 and SEM (Fig. 3.4B). While the results presented in Fig. 3.4B are an exciting and potentially novel finding, confirmation of this apparent MOAP-1 modification still awaits. If indeed our preliminary results are correct, phosphorylation of MOAP-1 may have important biological and functional consequences not only in relation to its role in apoptosis but possibly in cancer

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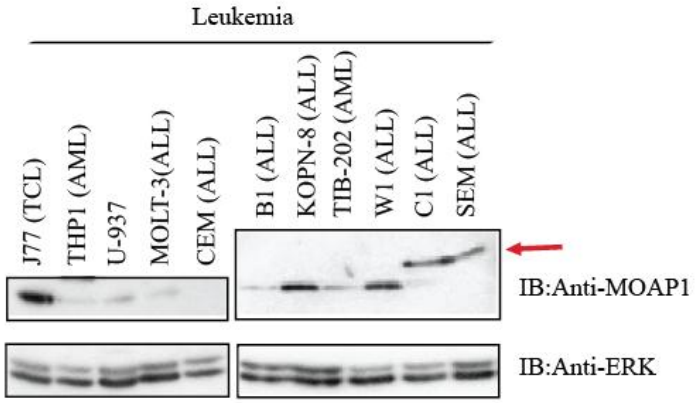

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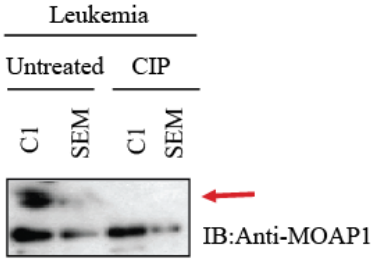


Figure 3.4. MOAP-1 expression is reduced in several human leukemia cell lines and is present in a modified form in C1 and SEM cells. (A) Western blotting (IB) was performed to evaluate MOAP-1 expression levels in the indicated cell lines. Note: 1) the presence of a higher migrating band for MOAP-1 in C1 and SEM pediatric ALL cell lines (red arrow); 2) THP1 is also known as TIB-202; 3) B1 cells display biphenotypic features of early B and myeloid lineages [508]. (B) The slower migrating bands for MOAP-1 (red arrow) are sensitive to treatment with calf intestinal phosphatase (CIP) thereby suggesting that they represent a phosphorylated form of MOAP-1 protein.

development as well. It is interesting to note that although MOAP-1 levels are reduced in many of the leukemia cell lines tested, *RASSF1A* is infrequently methylated in this cancer type [343, 344]. Nonetheless, given that MOAP-1 functions downstream of RASSF1A in death receptor-dependent cell death, we speculate that this pathway may be inhibited in some leukemias.

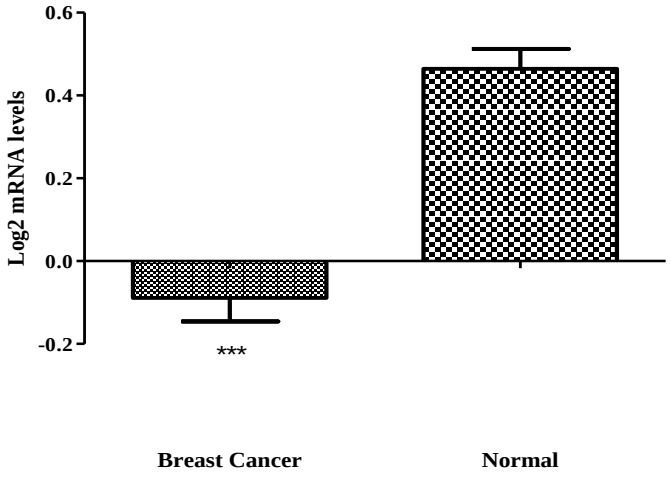
Overall, the results from our microarray meta-analysis and immunoblotting demonstrate a loss or reduction in *MOAP-1* expression throughout a wide range of human cancers. Together, our results point towards a potential tumor suppressor function for MOAP-1 whose expression may be reduced during carcinogenesis.

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

Breast cancer is the most frequently diagnosed malignancy and also the leading cause of cancer-associated deaths among women worldwide [345]. It is estimated that approximately 30% of patients originally diagnosed with early stage breast cancer will later relapse with advanced or metastatic disease [346]. In developed countries, it has also been predicted that roughly 6 to 10% of breast cancer patients present with metastatic disease at the time of diagnosis [347]. Therefore, one of the current goals in breast cancer research is to develop more effective treatment options as well as to identify new prognostic and predictive markers that will facilitate both diagnosis and disease management.

Breast cancer is a heterogeneous disease that consists of multiple biological subtypes that differ based on their clinical, pathological and molecular features [348]. The presence of hormone receptors and the levels of expression of human epidermal growth factor receptor 2 (Her2) both have a tremendous influence on the clinical management and outcomes of breast cancers. Currently, four major breast cancer subtypes can be distinguished based on the expressions of estrogen receptor (ER), progesterone receptor (PR) and Her2 and are classified as the following in 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-) [348]. Luminal A breast cancers are the most common subtype that respond well to adjuvant hormone therapy and have the best overall patient prognosis. In contrast, there are currently no targeted-therapies available for the treatment of triple-negative breast cancers. These cancers account for approximately 15% of all breast cancer cases and are associated with the worst overall and disease-free survival rates [348, 349]. Due to the prevalence of breast cancer in the human population and the finding that MOAP-1 expression is reduced in this form of cancer (Fig. 3.2, 3.3), we decided to further investigate MOAP-1 expression changes through a more detailed analysis of patient microarray data. In collaboration with Dr. John Mackey from the University of Alberta (Department of Oncology), we were able to access a set of breast cancer microarray data generated from 176 primary, treatment-naive breast cancer samples and 10 normal breast tissue samples [326]. Our assessment of *MOAP-1* expression in this dataset supports our initial finding of decreased levels

A)



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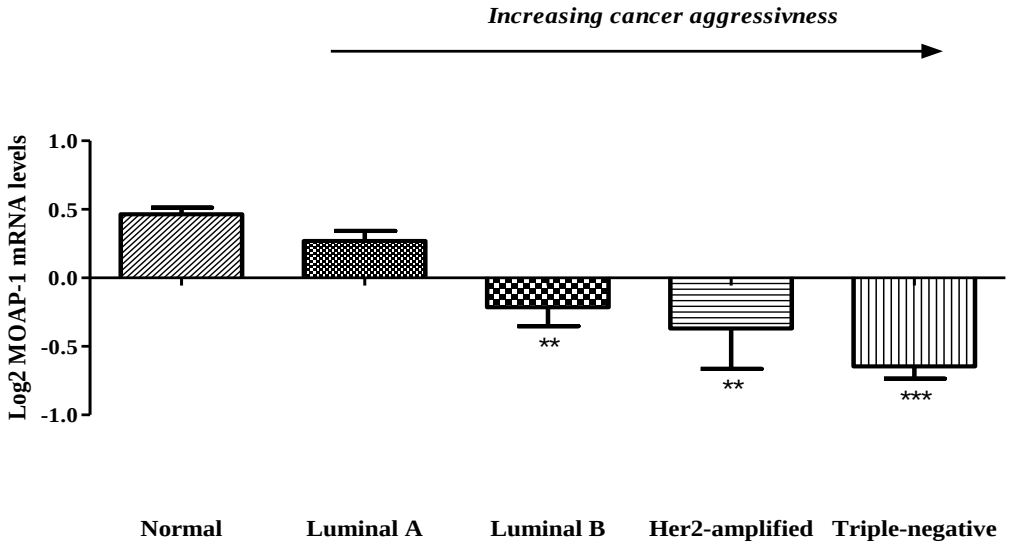

Figure 3.5. MOAP-1 expression is reduced in breast cancer patients and correlates with increasing cancer aggressiveness. (A) A comparison of MOAP-1 mRNA levels in cancerous versus normal breast tissue. n breast cancer= 176, n normal= 10; p=2.15E-09. (B) Average MOAP-1 expression is significantly reduced in 3 out of 4 major breast cancer subtypes relative to normal breast tissue. (Luminal A: p=0.34, n=75; Luminal B: p=0.0018, n=19: HER2-amplified: p=0.0067, n=8; Triple negative: p<0.0001, n=56; Normal: n=10.
Data in collaboration with Dr. John Mackey, University of Alberta.

of MOAP-1 in breast cancer and indicates an approximate fivefold difference in expression between breast tumor samples and normal breast tissue (Fig. 3.5A). We next sought to determine MOAP-1 expression levels among the four major breast cancer subtypes. Using Microsoft Excel, breast cancer patients were sorted based on luminal A, luminal B, Her2-amplified or triple-negative classifications prior to calculating the average MOAP-1 expression levels for each subgroup. Note that eighteen patients were excluded from this assessment since they could not be classified.

The results from this analysis revealed a steady and significant decrease in MOAP-1 expression that correlated with increasing breast cancer aggressiveness based on subtype (Fig. 3.5B). Although there was no significant difference between the levels of MOAP-1 in normal breast tissue and luminal A breast tumors ($p=0.3395$), there was a highly significant and noticeable difference in MOAP-1 expression when comparing normal breast tissue to luminal B, Her2-amplified and triple-negative breast cancers. These results suggest that MOAP-1 expression is downregulated during breast cancer progression and may subsequently contribute to the poor patient outcomes associated with more aggressive breast cancer subtypes. To determine if MOAP-1 expression is associated with particular clinical or pathological features of breast cancer, we next analysed patient clinical records and sorted the data with respect to different variables of interest. A threshold cut-off value for high and low MOAP-1 expression was calculated through receiver operating characteristic (ROC) curve analysis using MedCalc statistical software with the aid of Ryan Heit (Research

technician, Dr. Luc Berthiaume Lab). Based on the cut-off value that we obtained (threshold=0.1124), we first sorted patients with respect to our parameter of interest and then secondly grouped them based on whether they expressed high (log2 mRNA>0.1124) or low (log2 mRNA≤0.1124) levels of MOAP-1. Although we failed to find 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 (Fig. 3.6). Negative receptor status in breast cancer is associated with a less favourable prognosis compared to tumors that do express hormone receptors and also do not benefit as greatly from endocrine therapies [350]. Given that ER-negative and PR-negative tumors correlate with a number of different characteristics that are typically unfavourable to a patient, it is interesting that MOAP-1 is significantly lower among these tumor types and is consistent with the hypothesis that reduction of MOAP-1 may correlate with increased cancer aggressiveness. Overall, the results from our collaborative microarray analysis confirm that MOAP-1 expression is reduced in breast cancer patients and reveals a correlation between levels of MOAP-1 and patient prognosis predicted based on cancer subtype or hormone receptor status.

3.2.4. 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

	No. patients with low MOAP-1 (log2 mRNA≤0.1124)	No. patients with high MOAP-1 (log2 mRNA>0.1124)	p
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

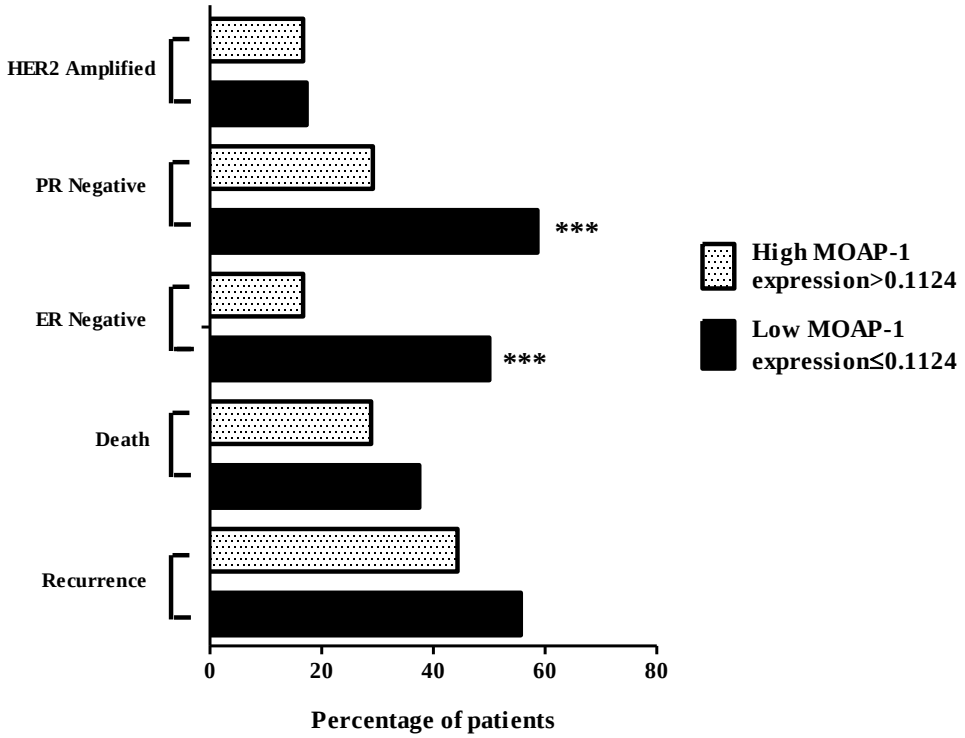

Figure 3.6. MOAP-1 expression is significantly reduced in ER-negative and PR-negative breast cancers. Breast cancer patient data was sorted based on levels of MOAP-1 expression with relation to various clinical and pathological features of interest as shown. ROC curve analysis calculated MOAP-1 expression cut-off at 0.1124 (low MOAP-1 expression ≤0.1124, highMOAP-1 expression >0.1124). ER-negative breast cancers: n=64, p=7.54E-05; PR-negative breast cancers: n=94, p=1.19E-06. Data in collaboration with Dr. John Mackey, University of Alberta.

solid tumor in childhood [351]. These tumors are often characterized by varying degrees of cellular differentiation and tend to develop spontaneously with familial forms of neuroblastoma accounting for only 1% of all cases [352]. Several genetic features of neuroblastoma have been identified that serve quite accurately as predictive markers of clinical outcome. Favourable tumors that are associated with a good patient prognosis often express high levels of the TrkA neurotrophin receptor [353]. In contrast, poor clinical outcome normally correlates with tumors that display amplification of the *MYCN* oncogene [354] and which express high levels of the neurotrophin receptor TrkB and its ligand brain-derived neurotrophic factor (BDNF) [355].

Remarkably, neuroblastoma tumors display the highest rate of spontaneous regression among all human cancers and it has been suggested that this may be attributed to delayed activation of normal apoptotic signaling pathways [356]. The anti-apoptotic Bcl2 protein is present at high levels in most neuroblastomas with expression levels inversely correlated to the degree of cellular differentiation and apoptosis [357, 358]. Additionally, elevated caspase expression in neuroblastoma is often accompanied by the expression of other favourable prognostic markers and correlates with improved disease outcome [359]. Given the alterations in apoptosis that are observed in neuroblastoma [360], we wanted to determine whether or not expression levels of MOAP-1 might also influence patient outcomes in this cancer.

In collaboration with Dr. Rogier Versteeg (University of Amsterdam, Netherlands), we were able to obtain MOAP-1 and RASSF1A expression data

from 88 neuroblastoma patients based on mRNA quantification. Data and statistical analysis was carried out by Dr. Rogier Versteeg who categorized neuroblastoma patients as either high or low expressers for MOAP-1 and - as a control - RASSF1A and subsequently correlated these data with patient survival. The Kaplan-Meier data shown here display a similar trend for both our candidate and bona fide tumor suppressor proteins, MOAP-1 and RASSF1A, respectively (Fig. 3.7). Neuroblastoma patients expressing high levels of MOAP-1 have a significantly increased overall survival probability than those who express low levels of this protein (Fig. 3.7A). Likewise, we also see a significantly improved survival rate among neuroblastoma patients expressing high RASSF1A, which reinforces previous findings demonstrating frequent inactivation of this gene corresponding to poor disease outcome [361, 362] (Fig. 3.7.B). Considering that both MOAP-1 and RASSF1A are essential components of death receptor-mediated apoptosis [84, 85], the findings here support previous reports of deregulated apoptosis in neuroblastoma and identifies the reduction of MOAP-1 and RASSF1A expression levels as a potential contributor to poor patient prognosis. The results from this dataset demonstrate a direct and significant correlation between expression levels of MOAP-1 and RASSF1A and survival probability in neuroblastoma.

3.2.5. MOAP-1 inhibits cell proliferation in culture

A fundamental property that is shared by many tumor suppressor proteins is the ability to negatively regulate cell growth and proliferation. So far, our data

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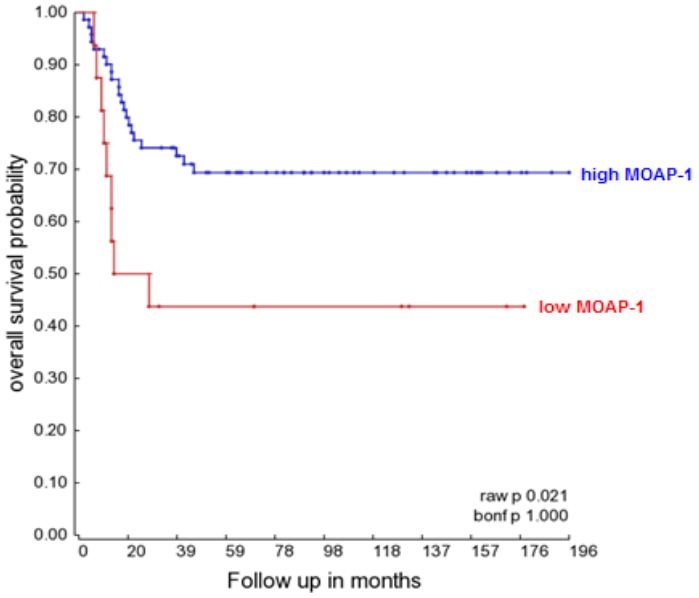

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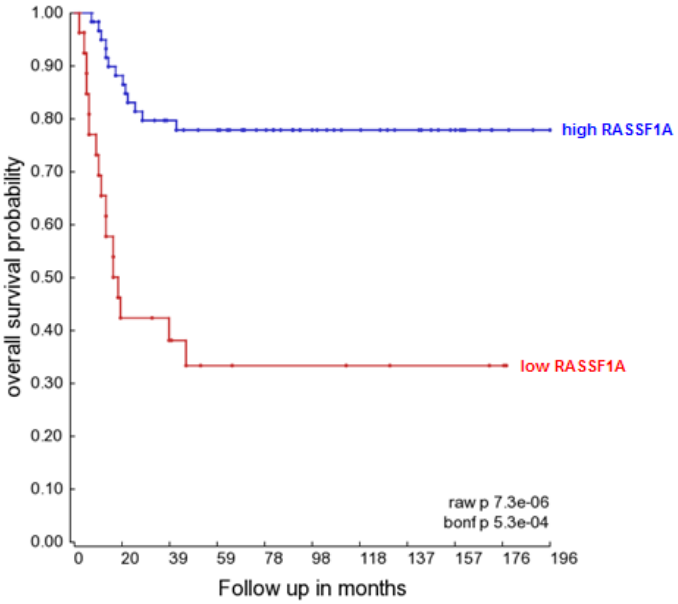


Figure 3.7. MOAP-1 expression levels directly correlate with survival probability in neuroblastoma. Kaplan-Meier survival curves demonstrating a correlation between expression levels of (A) MOAP-1 (n high expression=72, n low expression=16; expression cut-off: 700.1) and (B) RASSF1A (n high expression =61, n low expression =27; expression cut-off: 101.7) with overall patient survival probability from neuroblastoma. Data generated by Dr. Rogier Versteeg, University of Amsterdam (Netherlands).

suggest that MOAP-1 may function as a tumor suppressor protein as its expression is downregulated in multiple human cancers and reduced levels of MOAP-1 correlate with increased breast cancer aggressiveness and decreased patient survival in neuroblastoma. To test if MOAP-1 possesses the ability to inhibit cell proliferation, H1299 non-small cell lung carcinoma cells stably expressing MOAP-1 were generated. Given that H1299 cells express low to undetectable levels of endogenous MOAP-1 [85, 192], we decided that cells from this line would possess an ideal genetic background to create our stable transfectants. Additionally, RASSF1A serves as a key MOAP-1 binding partner that is required for inducing the pro-apoptotic function of MOAP-1 and is not endogenously expressed in H1299 cells [63]. Therefore, we decided to create two MOAP-1 stably expressing cell lines either with or without the addition of RASSF1A overexpression to assess the role of RASSF1A in MOAP-1's potential growth suppressive functions.

To verify the functionality and the pro-apoptotic activities of our ectopically expressed MOAP-1 and RASSF1A constructs, we performed a cell death assay followed by fluorescence-activated cell sorting (FACS) analysis. H1299 cells stably expressing vector control, RASSF1A alone, or MOAP-1 with and without RASSF1A overexpression were stimulated to undergo cell death with the addition of 50 ng/ml TNFα and 10 μg/ml cycloheximide (CHX) for ten hours. The addition of CHX was imperative in order to promote TNFR1-mediated cell signaling towards apoptosis as opposed to inflammation [174]. Our FACS results demonstrated increased levels of apoptosis in H1299 cells stably expressing

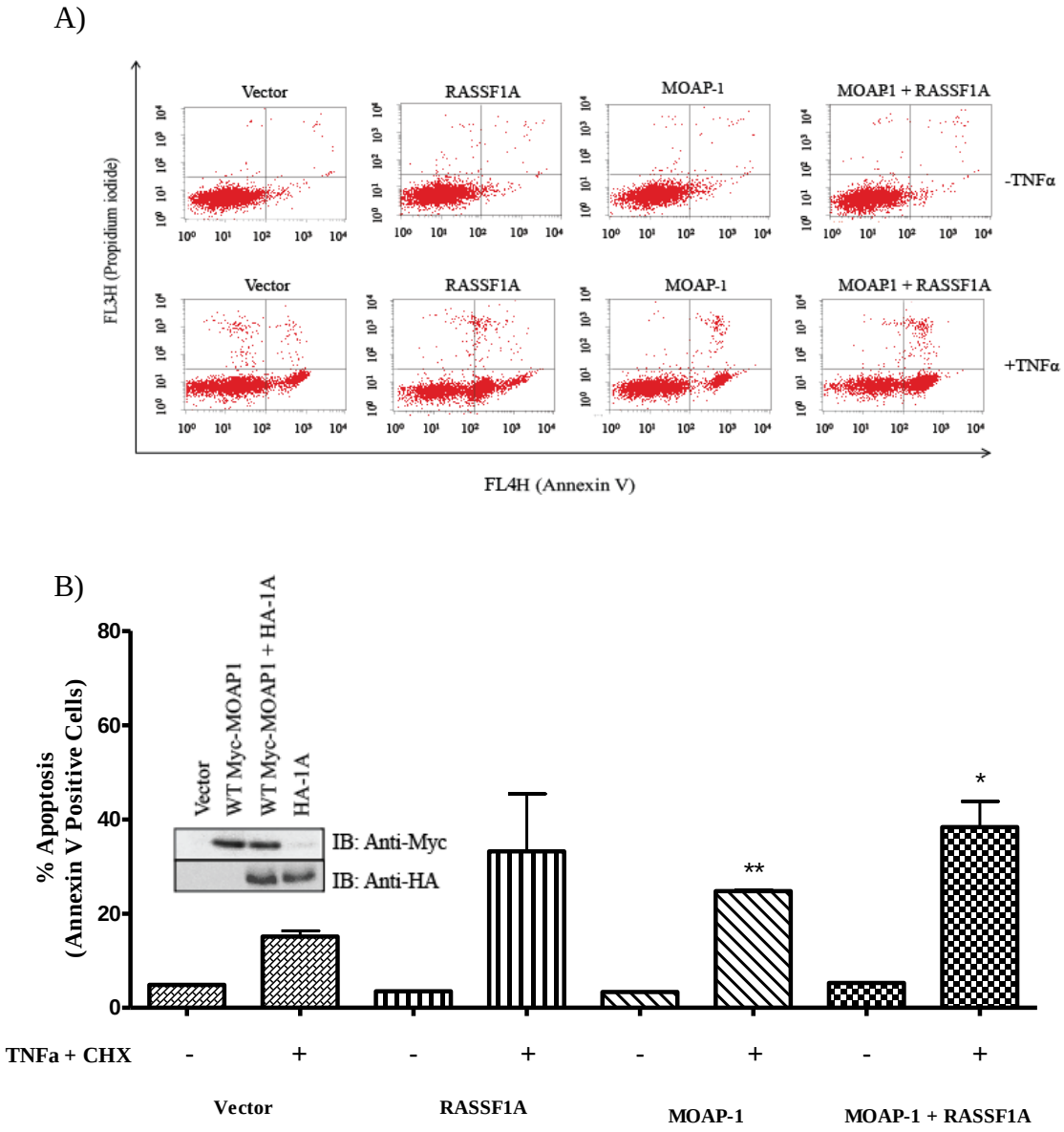
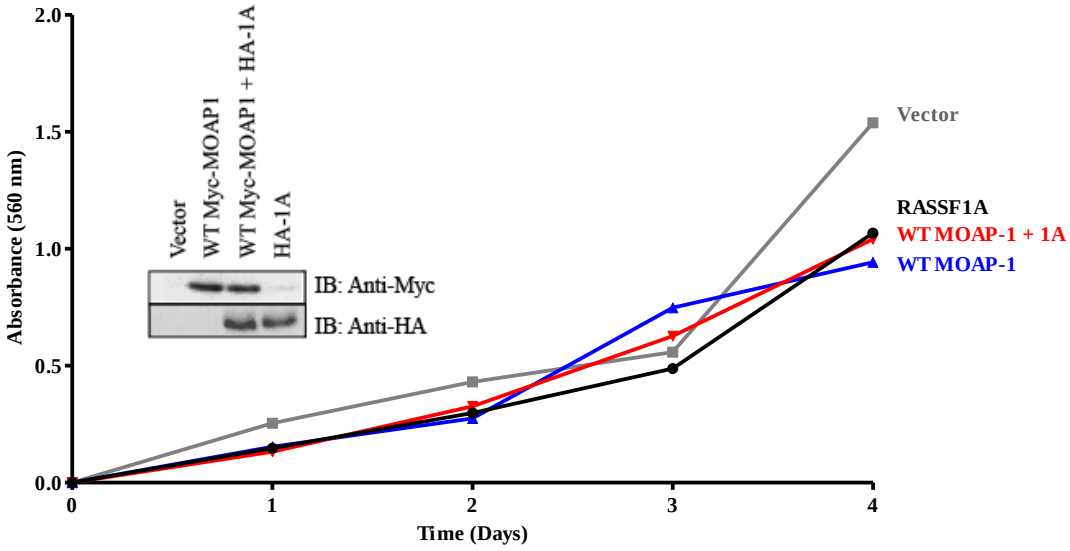


Figure 3.8. Stable overexpression of RASSF1A and MOAP-1 in H1299 cells results in increased apoptosis upon TNFα stimulation relative to vector. H1299 cells stably expressing vector, HA-RASSF1A, Myc-MOAP-1 or Myc-MOAP-1 with HA-RASSF1A were grown to ~90% confluency prior to stimulation with TNFα (50 ng/ml) and cycloheximide (CHX, 10 ug/ml). After 10 hours, cells were harvested and stained with propidium iodide and annexin V to detect apoptotic cells by FACS analysis. (A) Scatterplots of propidium iodide (y-axis) versus annexin V (x-axis) staining. (B) Average percentage of cell death for H1299 cells stably expressing the indicated DNA constructs. Results are from three individual trials. Inset: Western blot to confirm the expression of HA-RASSF1A (1A) and Myc-MOAP-1. Vector: n=4; RASSF1A: n=3, p=0.2147; WT MOAP-1: n=3, p=0.0082; WT MOAP-1+1A: n=4, p=0.0143.

RASSF1A and MOAP-1 when stimulated to undergo cell death (Fig. 3.8), which is in accordance with previous findings [84, 85]. Since our H1299 stable cells were behaving as expected, we decided to test the anti-proliferative propertiesof these cells as a next step.

Using the MTT colorimetric assay for cell proliferation first described by Mosmann [363], we analyzed the growth of our H1299 stable transfectants in culture over the course of four days. In this assay, the underlying principle is based on the reduction of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) into a coloured formazan product by metabolically active cells. When dissolved, the intensity of the formazan solution is directly proportional to the number of viable cells within a population. Stable expression of RASSF1A resulted in a significantly lower rate of cell proliferation relative to vector control cells. These results are expected since RASSF1A it is an established tumor suppressor protein [364] (Fig. 3.9). Similarly, MOAP-1 was also capable of inhibiting cell proliferation to a comparable and significant extent as RASSF1A (Fig. 3.9). However, the additional expression of RASSF1A in H1299 cells had no substantial effect on the ability of MOAP-1 to suppress cell growth ($p=0.0224$) (Fig. 3.9) and thus presumptively suggests that MOAP-1 may possess the ability to inhibit cell proliferation independently of RASSF1A. Nevertheless, this remains a preliminary hypothesis that remains to be further substantiated. Together, these results demonstrate a growth suppressive property possessed by MOAP-1 that can effectively inhibit cell proliferation in culture.

A)



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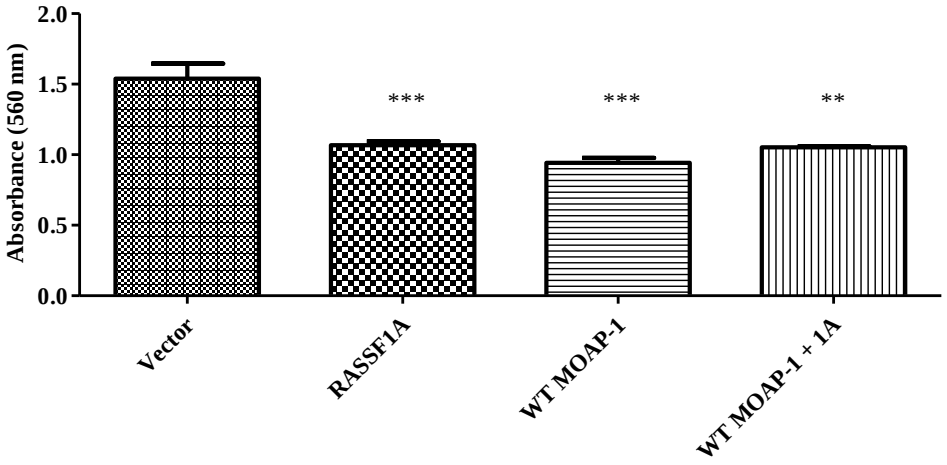


Figure 3.9. MOAP-1 can inhibit H1299 cell proliferation in culture. H1299 cells stably expressing vector, HA-RASSF1A, Myc-MOAP-1 or Myc-MOAP-1 with HA-RASSF1A were seeded at 8000 cells per well in a 96 well plate. Cells were incubated with 3.6 mM 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 3 hours each day followed by dissolution of formazan product and absorbance reading at 560 nm. (A) A plot of absorbance readings over the course of four days for each of the four H1299 cell lines stably expressing the indicated constructs at right. Inset: Western blot to confirm the expression of HA-RASSF1A (1A) and Myc-MOAP-1. (B) Average absorbance readings at Day 4 for H1299 cells stably expressing the indicated DNA constructs. Vector: n=8; RASSF1A: n=8, p=0.0008; WT MOAP-1: n=6, p=0.0006; WT MOAP-1+1A: n=5, p=0.0047.

3.2.6. Loss of Moap-1 results in increased spleen cellularity in mice

The data presented so far including the ability of MOAP-1 to inhibit cell proliferation in culture highly suggests that this protein possesses an inherent tumor suppressor function. To determine if we could observe the growth inhibitory properties of MOAP-1 during normal *in vivo* biology, we decided to investigate whether there were any particular features of Moap-1 knockout mice that would support its role as a tumor suppressor protein. Over the course of studies involving *Moap-1*$^{-/-}$ mice in our lab, we regularly observed the presence of enlarged spleens (splenomegaly) in the absence of Moap-1 when compared to wild type mice. The spleen is the largest secondary lymphoid organ and is involved in blood filtration as well as in the initiation of both humoral and cell-mediated immune responses [365]. However, the presence of splenomegaly is associated with a broad range of different diseases including neoplasia [366]. To analyze the significance and the extent of the effects of Moap-1 loss on splenic growth, splenocyte counts were performed for wild type, *Moap-1*$^{-/-}$, *Rassf1a*$^{-/-}$, and *Moap-1*$^{-/-}$*/Rassf1a*$^{-/-}$ double knockout mice. As an additional experimental control, splenocyte isolation and counts were also performed for mice lacking Rassf5a, a second member of the RASSF protein family that is also believed to function as a tumor suppressor [51].

Our results indicate that *Moap-1*$^{-/-}$ mice have significantly increased spleen cellularity relative to wild type animals (Fig. 3.10). Similar results were also observed with the loss of either Rassf1a alone or in combination with Moap-1 (Fig. 3.10). The presence or absence of Moap-1 had no significant effect

(p=0.2126) on the results seen in Rassf1a knockout mice (Fig. 3.10) and is consistent with our *in vitro* MTT results (Fig. 3.9) suggesting that the tumor suppressor functions of Rassf1a and Moap-1 may not be dependent on each other. Although we were expecting that *Rassf5a*$^{-/-}$ mice would also display increased spleen cellularity based on its reported tumor suppressor function, cell counts from these animals were comparable to wild type mice (Fig. 3.10). This suggests that perhaps the tumor suppressor function of Rassf5a may not be active in all tissue types.

That *Moap-1*$^{-/-}$ mice have increased spleen cellularity and size supports an anti-proliferative function for this protein. Interestingly, splenomegaly is also observed in other genetically modified mice with knockout of tumor suppressor genes. Mice deficient for the tumor suppressor phosphatase and tensin homolg gene *Pten* develop splenomegaly that is thought to be caused by an impairment in Fas-mediated apoptosis [367]. Similarly, the deletion of the gene encoding runt-related transcription factor 1/acute myeloid leukemia 1 (Runx1/AML1) causes splenomegaly and lymphomas in adult mice [368]. Although the role of MOAP-1 in Fas-induced cell death has not been studied in detail, unpublished results from our lab indicate that MOAP-1 may be involved in this process since knockdown cells are resistant to Fas-mediated apoptosis. In humans, an association also exists between the loss of tumor suppressor genes, neoplasia and the development of splenomegaly. Patients with lymphocytic leukemia often acquire deletions in the genetic locus of the ATM kinase tumor suppressor gene and this has been shown to correlate with the development of splenomegaly [369].

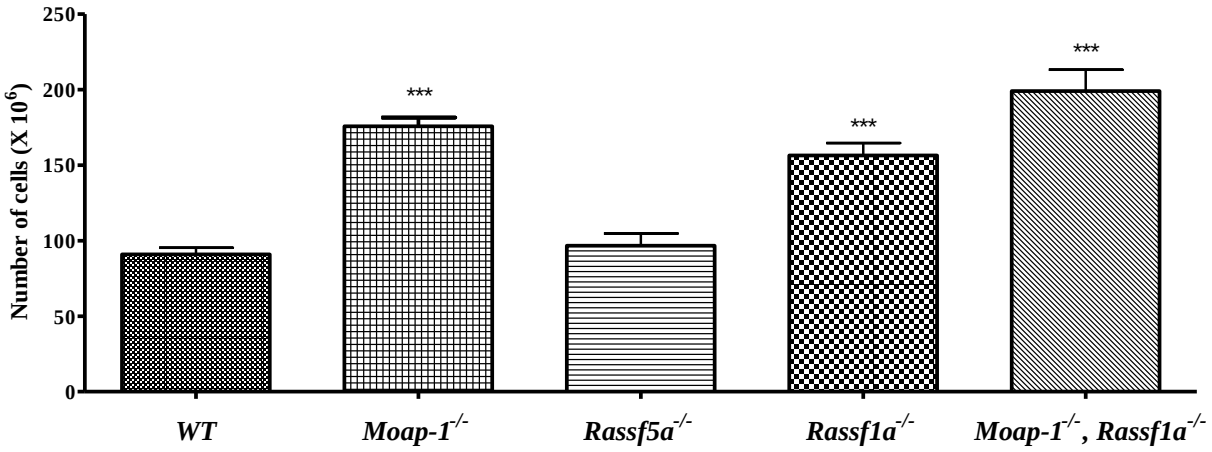


Figure 3.10. *Moap-1*$^{-/-}$ mice display increased spleen cellularity. Mice of the above indicated genotypes were sacrificed by CO_2 narcosis prior to spleen isolation, cell suspension and red blood cell lysis. Splenocytes were stained with trypan blue prior to cell counting. Average age of mice at time of sacrifice was 13 weeks. Wild type (WT): n=9; Moap-1$^{-/-}$: n=5, p=<0.0001; Rassf5$^{-/-}$: n=5, p=0.5034; Rassf1a$^{-/-}$: n=10, p=<0.0001; Moap-1$^{-/-}$, Rassf1a$^{-/-}$: n=7, p=<0.0001.

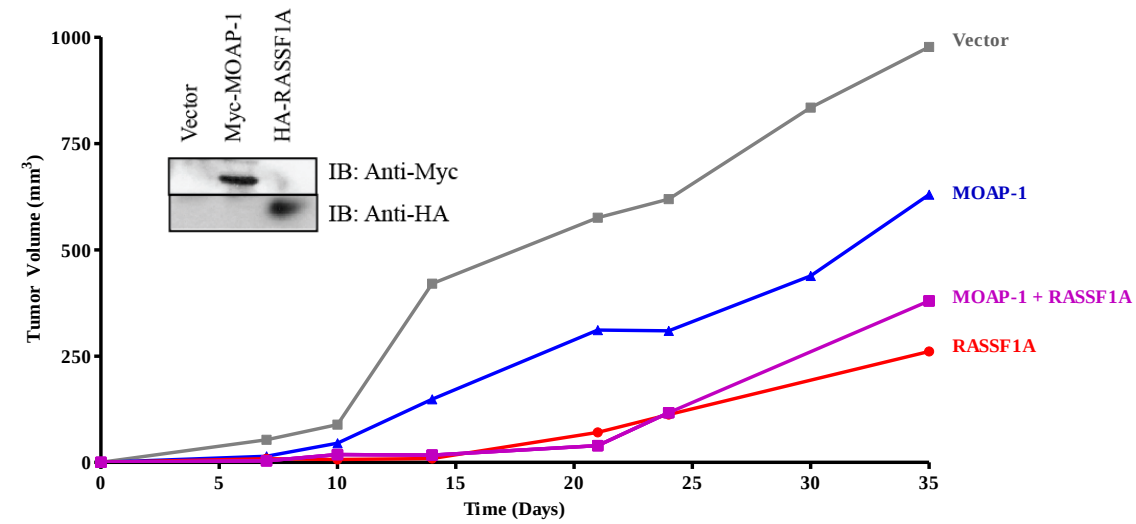
Still, we cannot rule out the possibility that the presence of splenomegaly in *Moap-1*$^{-/-}$ mice may also signify the presence of an underlying disease [366]. Considering that splenomegaly is associated with a multitude of disease ranging from infection to inflammation and also haematological disorders (including leukemia and lymphoma) [366], future additional investigation is required to fully understand the significance of our findings. Nonetheless, these results suggest a role for Moap-1 that is either directly or indirectly involved in negatively regulating splenocyte proliferation and also proposes other biological functions for Moap-1 that extend beyond apoptosis and tumor suppression.

3.2.7. MOAP-1 inhibits tumor formation *in vivo*

To directly examine and validate the tumor suppressor function of MOAP-1 *in vivo*, xenograft tumor assays were performed in athymic nude mice which lack a functioning immune system [370]. Although MOAP-1 possesses the ability to effectively inhibit cell proliferation in culture (Fig. 3.9), *in vivo* models for tumor growth still remain a gold standard for the assessment of candidate tumor suppressor genes by recapitulating an environment that more accurately reflects true biology. To verify the growth inhibitory function of MOAP-1, HCT 116 colorectal carcinoma cells overexpressing vector control or MOAP-1 with or without RASSF1A were injected subcutaneously into the left and right flanks of athymic mice. This was followed by a regular, periodic examination of tumor growth and size over the course of the next 35 days. Despite the slow and similar rates of tumor development during the initial ten days post-injection, a highly

apparent and significant difference in the rates of tumor formation emerged post-day 15 of our experiments (Fig. 3.11A). The results from our xenograft tumor assays demonstrate that MOAP-1 can significantly inhibit tumor formation in HCT 116 cells relative to vector control (Fig. 3.11A, B). Interestingly, the average rate of tumor growth due to the combined expression of MOAP-1 and RASSF1A was reduced from that of MOAP-1 alone but remained at borderline significance (Fig. 4.8; $p=0.045$). While these data are in contrast to our MTT and spleen cellularity results that demonstrate no appreciable effects of RASSF1A on MOAP-1-mediated growth inhibition (Fig. 3.9, 3.10), we intend to resolve the exact contributions of RASSF1A to the tumor suppressor function of MOAP-1 in future studies. Nonetheless, preliminary xenograft tumor assays involving DAOY medulloblastoma and H1299 non-small cell lung cancer cells indicates that MOAP-1 can inhibit tumor formation involving other cell types and thus its ability to negatively regulate cell proliferation is not exclusive to HCT116 cells (data not presented). In summary, we have validated a bona fide tumor suppressor function for the proapoptotic protein MOAP-1.

A)



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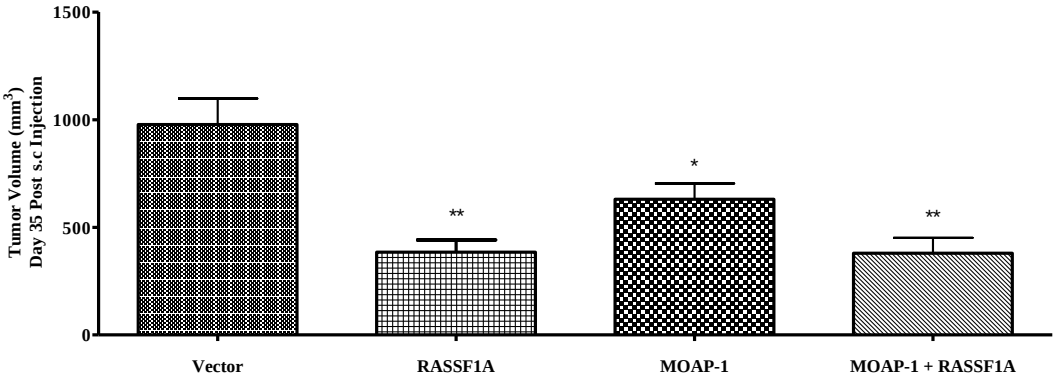


Figure 3.11. MOAP-1 can inhibit tumor formation *in vivo*. HCT 116 colorectal carcinoma cells were transiently transfected with empty vector, HA-RASSF1A, or Myc-MOAP-1 with or without HA-RASSF1A. Two days post-transfection, cells were harvested and resuspended in matrigel solution prior to subcutaneous injection into the left and right flanks of athymic mice. Tumor sizes were measured at regular intervals over the course of 35 days and calculated based on the formula for spherical volume ($4/3\pi r^3$, r=radius). (A) Tumor growth curve of mice injected with HCT 116 cells overexpressing the indicated DNA constructs. Inset: Western blotting (IB) to verify the expression of HA-RASSF1A and Myc-MOAP-1. (B) Static plot of average tumor volumes in mice 35 days post-subcutaneous injection. Vector: n=8; RASSF1A: n=7, p=0.0025; MOAP-1: n=16, p=0.0299, MOAP1+RASSF1A: n=8, p=0.0045.

3.3. DISCUSSION AND FUTURE DIRECTIONS

3.3.1. Biological roles of the MOAP-1 tumor suppressor protein

Early studies on MOAP-1 had previously alluded to a potential tumor suppressor function for this protein given its significant role in Bax-mediated apoptosis [84, 189]. Indeed, the ability of cells to undergo apoptosis is a critical mechanism of tumor suppression [327, 371]. However, our data suggest that MOAP-1 may also possess other biological functions - in addition to its role in cell death - that may be important for its ability to mediate growth suppression. For instance, MOAP-1 is able to significantly inhibit cell proliferation in the absence of endogenous RASSF1A (Fig. 3.9, 3.10) even though RASSF1A is required for MOAP-1-mediated Bax activation during death receptor signaling [84, 85]. Therefore, this implies that the ability of MOAP-1 to inhibit cell growth is not solely dependent on its role in TNFα- or TRAIL-induced cell death. Additionally, this may also suggest that MOAP-1 possesses RASSF1A-independent tumor suppressor functions. Previous reports indicate that MOAP-1 is also involved in the induction of apoptosis through the intrinsic pathway of cell death [189] while RASSF1A is not [84] so this remains a possible mechanism of growth suppression for MOAP-1 that does not involve RASSF1A and which may help to explain our results. However, we would like to pursue a more thorough investigation in order to determine the true interdependence between MOAP-1- and RASSF1A-mediated tumor suppressor functions.

Preliminary findings from a GST-MOAP-1 pull-down assay followed by mass spectrometry analysis also suggests additional non-apoptotic functions for

MOAP-1 that may contribute to its growth inhibitory function including roles in both protein translation and the regulation of actin dynamics (discussed in Chapter 6, Fig. 6.1). Several translational regulators possess tumor suppressor functions including von Hippel-Lindau (VHL) [372], DEAD-box protein 3 (DDX3) [373] and programmed cell death protein 4 (PDCD4) [374], and the aberrant translation of mRNAs resulting in altered gene expression is known to be involved in carcinogenesis [375]. Similarly, the deregulation of actin dynamics is an important contributor to the epithelial-mesenchymal transition resulting in cancer metastasis [376]. Therefore, it will be interesting to further investigate the involvement of MOAP-1 in other biological processes that may be important for its tumor suppressor function.

3.3.2. Regulation of MOAP-1 expression during cancer development

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 [192]. However, this process is inhibited during early apoptosis and results in the rapid upregulation of MOAP-1 protein that sensitizes cells to cell death through the activation of Bax. Consequently, levels of MOAP-1 are post-translationally regulated during normal cell biology. The results that we obtained from our analysis of the Oncomine cancer microarray database (Fig. 3.2) and immunoblotting for *MOAP-1* expression in cancer cell lines (Fig. 3.3, 3.4) indicate a downregulation or absence of MOAP-1 during carcinogenesis. Furthermore, the reduction of MOAP-1 levels during breast cancer proceeds in a

progressive manner that correlates with increasing cancer aggressiveness. The inactivation of its interacting partner, RASSF1A, is well-known to be caused by promoter methylation in nearly all cases of its expression loss during cancer development and quantitative analysis indicates an association between high levels of *RASSF1A* methylation and poor patient outcomes for breast cancer [65, 377]. Intriguingly, a computer-based program analysis of the MOAP-1 promoter region carried out in collaboration with Dr. Gerd Pfeifer (Beckman Research Institute, Duarte, CA) indicates the presence of 19 CpG islands and thus raises the possibility that MOAP-1 expression may also be epigenetically silenced during cancer development (Baksh et al., unpublished observations). To test the biological susceptibility of the MOAP-1 promoter region to methylation, future work may involve the methylation-specific polymerase chain reaction (PCR) of DNA isolated from patient tumor biopsies and transformed cell lines. An alternative yet equally interesting hypothesis for the decrease in MOAP-1 expression during cancer may also result from elevated proteasomal degradation rather than downregulated expression (Dr. Victor Yu, National University of Singapore). Thus, it is possible that the deregulation of MOAP-1 ubiquitination enzymes during carcinogenesis may contribute to decreased intracellular levels of MOAP-1 protein. However, given that microarray results indicate the presence of decreased MOAP-1 mRNA levels in a wide range of human cancers (Fig. 3.2, 3.5, 3.7), it is most likely that increased MOAP-1 ubiquitination and degradation may only be a contributing factor (if at all) and not solely responsible for its downregulation.

A number of potential transcription factor binding sites have also been located within the MOAP-1 promoter region and includes consensus sequences for the CREB [378], Pax5, Myc, STAT1 and NF-κB transcription factors (unpublished results). Deregulation of the activities of each of these transcription factors are implicated in cancer development and may result in either the loss or overexpression of gene transcriptions [379-383]. Amplification of *MYC* is observed in multiple cancer types and is also overexpressed in parathyroid tumors [384]. Considering that *MOAP-1* may be overexpressed in parathyroid cancers based on our Oncomine results and contains a potential binding site for the MYC transcription factor, increased Myc activity may be responsible for the elevation of MOAP-1 protein levels. However, the biological significance of increased MOAP-1 levels during parathyroid cancer warrants further investigation. We propose that alterations in two potential transcriptional regulatory mechanisms - promoter methylation or altered transcription factor activity - may account for the changes in MOAP-1 expression in human cancer.

3.3.3. The prognostic value of MOAP-1 in human cancer

The identification of biological markers in cancer is often for the purpose of facilitating the diagnosis and treatment of patients and has proven to be extremely beneficial and useful in many clinical settings. In addition to the reduced expression of MOAP-1 in human cancer, we have also discovered a direct correlation between levels of MOAP-1 protein in patients and their outcomes for breast and neuroblastoma malignancies. Our results indicate that

reduced *MOAP-1* expression correlates with breast cancer progression towards the high-risk triple-negative subtype and also significantly correlates with negative ER and PR receptor statuses (Fig. 3.5, 3.6). With regards to neuroblastoma, low levels of *MOAP-1* expression significantly correlate with decreased patient survival and, on average, reflects an approximate thirty percent decrease in survival probability (Fig. 3.7). Considering that we have identified MOAP-1 as a tumor suppressor protein, it is not unforeseeable that these associations exist. Correlations between the expression levels of various tumor suppressors and their abilities to predict cancer prognosis has been demonstrated for multiple proteins including RASSF1A [385, 386], p53 [387], p16 [388] and pRb [389]. We predict that further studies on MOAP-1 expression in other patient populations and cancer types will reveal a more accurate reflection of the suitability of MOAP-1 as a potential prognostic marker in cancer. However, the preliminary results that we have obtained so far with regards to breast cancer and neuroblastoma are promising in that MOAP-1 may very well have significant prognostic value in other human cancers.

3.3.4. Potential phosphorylation of MOAP-1 protein in leukemia

Protein phosphorylation is a reversible post-translational modification that is highly involved in nearly all biological processes and plays a key role in signal transduction events [390]. The observation that MOAP-1 resolves at a higher migration after SDS-PAGE of C1 and SEM protein lysates in addition to their sensitivity to phosphatase treatment strongly suggests that MOAP-1 is present in a

phosphorylated state in both of these leukemia cell lines (Fig. 3.4). What is particularly interesting is the finding that the entire pool of detectable MOAP-1 protein in C1 and SEM are putatively modified and, furthermore, that this altered form of MOAP-1 is not present in any of the other cell lines or cancer types that were tested.

C1 and SEM are both derived from pediatric ALL patients but do not seem to share any similarities based on what is currently known about these two cell lines [391, 392]. The higher migrating bands for MOAP-1 that are present in C1 and SEM are noticeably absent from the other ALL cell lines tested (Fig. 3.4) and therefore suggests that the phosphorylation of MOAP-1 is unique to certain cases or subtypes of leukemia. While the novel discovery of what appears to be a modified form of MOAP-1 in two ALL cell lines may have important implications in normal cell and leukemia biology, further investigation is still required. One challenge that we have encountered over the course of validation Western blotting for MOAP-1 in C1 and SEM is the relative instability of its modified form. Although we are able to consistently detect the putative phosphorylated form of MOAP-1 during the initial stages of our experiments, we observe a loss or detachment of the modification from MOAP-1 over time such that this protein begins to resolve at its expected migration following SDS-PAGE. It is important to note that the prolonged storage of C1 and SEM cell lysates in sample buffer may have partially contributed to this observation. Nonetheless, we are currently unable to explain this phenomenon but expect that future studies will help to resolve this peculiarity.

In continuation of this investigation, it would be interesting to carry out a more detailed analysis of MOAP-1 in C1 and SEM that may lead to the identification of its phosphorylation site(s) as well as the kinase that is responsible for its phosphorylation. Results obtained at medium stringency from the online software Scansite [393] suggests that MOAP-1 may possess two potential kinase binding sites for PKCμ and Erk based on consensus sequence motifs. These kinases are involved in the regulation of cell growth and apoptosis, and are additionally implicated in human cancer [394-396].

At this time, there are many unanswered questions concerning the putative phosphorylation of MOAP-1 that we would like to better understand. For instance, the biological function of MOAP-1 phosphorylation, the difficulty in detecting this modification in other cell lines, and the significance of this finding in relation to ALL are all areas of intense interest. We anticipate that the answers to these questions will reveal a wealth of information about MOAP-1 biology in the future.

3.4. CONCLUSION

In this chapter, we have demonstrated that MOAP-1 has the capacity to inhibit tumor formation and therefore functions as a true tumor suppressor protein. Similar to other established tumor suppressor genes, *MOAP-1* expression is reduced in multiple human cancers and decreases in its expression correlate with poor prognosis in relation to breast cancer and neuroblastoma. Surprisingly, our results indicate that MOAP-1 may possess additional growth regulatory

functions aside from apoptosis and therefore provide an exciting starting point for future investigations.

CHAPTER 4

Non-degradative ubiquitination of MOAP-1 is essential for its tumor suppressor function

4.1. INTRODUCTION

Protein ubiquitination has multiple regulatory functions during cell signaling that are independent of proteolysis [256]. In addition to modulating protein interactions [397], substrate ubiquitin conjugation is also involved in altering both the functions and intracellular locations of signaling molecules [256]. Previous studies have demonstrated that MOAP-1 is subject to ubiquitin-mediated proteasomal degradation under normal cellular conditions [192, 193]. We report here that MOAP-1 is also regulated by non-degradative ubiquitination at lysine (K)-278 through a process that may also involve K264. Surprisingly, neither K264 nor K278 are involved in regulating the intracellular localization of MOAP-1 under normal cellular conditions and, additionally, are not required for its ability to induce apoptosis through the extrinsic pathway of cell death. The ubiquitination of MOAP-1 at K278 proceeds in a PKC-dependent manner and is important for its ability to mediate growth inhibition. Although K264 is dispensable for MOAP-1 ubiquitination, mutation at this site results in ubiquitination with altered kinetics and impairs its ability to suppress tumor formation. However, the pro-proliferative effects of K264 mutation in MOAP-1 are far less detrimental than genetic change at K278. Our results indicate that PKC-dependent MOAP-1 ubiquitination may be required for its tumor suppressor function and we hypothesize that K264 may serve a regulatory role by possibly facilitating the modification at K278.

4.2. RESULTS

4.2.1. Identification of a PKC-dependent ubiquitination of MOAP-1

The MOAP-1 interacting protein RASSF1A is a substrate of protein kinase C (PKC) and contains a conserved region 1 (C1) domain that is typically involved in phorbol ester and diacylglycerol (DAG) binding [398]. Phosphorylation of RASSF1A by PKC is required for the ability of RASSF1A to modulate the microtubule network and can be induced with the compound phorbol 12-myristate 13-acetetate (PMA) [398]. PMA shares close similarity with the natural glycerol ester DAG and is frequently employed in biochemical research as an activator of both conventional and novel PKC isoforms [399].

Over the course of studies investigating the effects of PMA stimulation on RASSF1A in cells, a discovery was made by Dr. Shairaz Baksh that MOAP-1 ubiquitination could be induced by PKC activation. Given that MOAP-1 ubiquitination could be detected in the absence of proteasomal inhibition, this represented a non-degradation associated form of the modification. Prior to the commencement of my graduate studies, PKC stimulation via the use of PMA was performed to induce the ubiquitination of MOAP-1. Analysis of MOAP-1 ubiquitination sites was carried out by liquid chromatography with tandem mass spectrometry (LC-MS/MS) in order to identify the potential site(s) of modification. The principle behind this procedure relies on a 114 Da mass shift due to the C-terminal diglycine of ubiquitin that remains attached on the substrate lysine residue following tryptic digestion [400]. The results that were obtained from this analysis with peptide coverage of 41% revealed a putative ubiquitination site

at K516 of GST-MOAP-1 that corresponds to K278 in untagged MOAP-1 protein (Fig. 4.1). Additionally, a second MOAP-1 ubiquitination site was also suggested to reside at K264. Therefore, K264 and K278 were two potential lysine residues within MOAP-1 that may be responsible for its PKC-dependent ubiquitination. The identification of these two MOAP-1 lysine residues as potential sites of modification serves as the basis for the remainder of this chapter where we investigate the involvement of K264 and K278 in the ubiquitination of this protein and their importance in MOAP-1 biology.

4.2.2. Lysine residues K264 and K278 are not required for regulating the intracellular localization of MOAP-1

The appropriate subcellular localization of proteins is important for the execution of normal biological function and is often regulated by reversible post-translational modifications including non-degradative ubiquitination [256]. To begin our investigation of K264 and K278 as potential sites for MOAP-1 ubiquitination, we first assessed the importance of these two sites for the intracellular localization of this protein. HCT 116 colon carcinoma cells were transiently transfected with Myc-tagged wild type, K264R or K278R MOAP-1 constructs that had previously been cloned into pXJ40 vectors. This was followed by immunofluorescence staining of cells and analysis by confocal microscopy. Cells transfected with either K264R or K278R MOAP-1 displayed similar pan-cytoplasmic staining patterns that were comparable to wild type MOAP-1 (Fig. 4.2). Additionally, K264R MOAP-1 accumulated in distinct cytoplasmic puncta analogous to wild

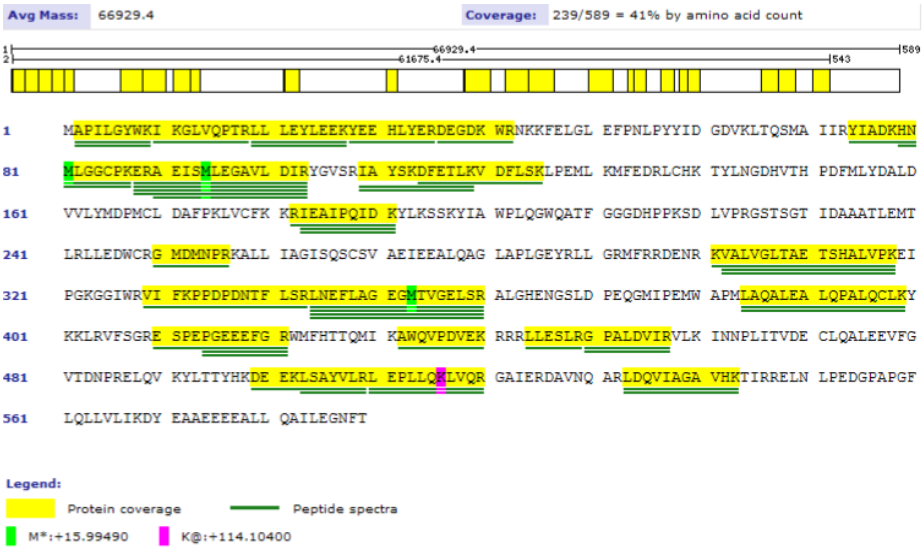


Figure 4.1. MOAP-1 is ubiquitinated in response to stimulation of cells with PKC activator phorbol 12-myristate 13-acetate (PMA). COS-7 cells were transiently transfected with pEBG-GST-MOAP-1 and stimulated two days later for 1 hour with PMA (150 ng/ml). Cells were harvested and lysed prior to GST pull-down, SDS-PAGE and coomassie blue staining. Shown here is the sequence of GST-MOAP-1 from PMA-treated cells. Trypsin digestion and LC-MS/MS analysis was performed to identify a putative MOAP-1 ubiquitination site at K516 in GST-MOAP-1 corresponding to K278 in untagged MOAP-1protein. Peptide coverage (yellow regions): 41% by amino acid count. (Data for this figure was generated by Dr. Shairaz Baksh).

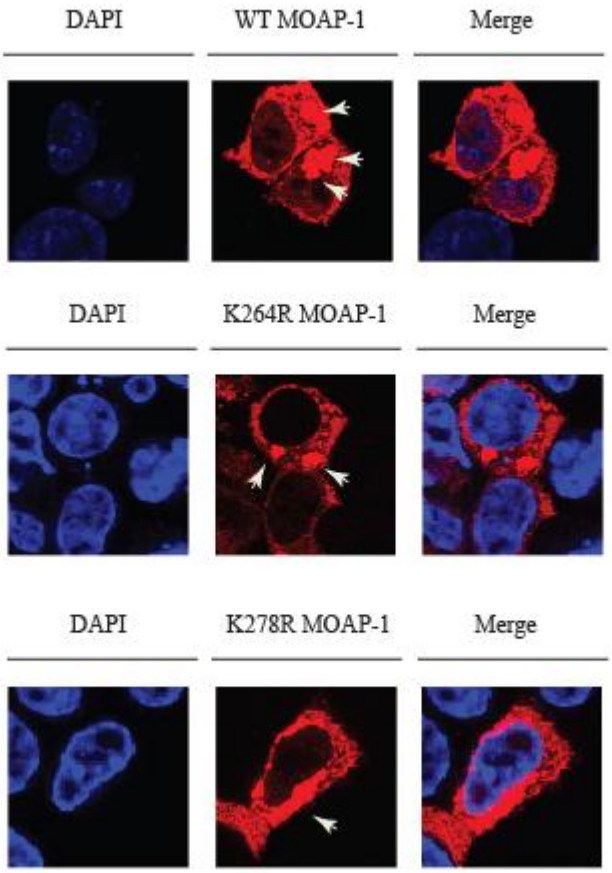


Figure 4.2. WT, K264R and K278R MOAP-1 exhibit similar pan-cytoplasmic staining patterns. HCT 116 cells cultured on square coverslips were transfected with Myc-tagged constructs for wild type (WT), K264R or K278R MOAP-1. Forty-eight hours post-transfection, cells were immunostained with mouse anti-myc antibody and goat anti-mouse Alexa 555 secondary antibody. Cellular localization of MOAP-1 was visualized by confocal microscopy using a Leica SP5 microscope. Arrows in the above images point to areas of MOAP-1 accumulation in puncta.

type protein (Fig. 4.2). While the presence of K278R MOAP-1 in punctate structures is not as clearly visible when compared to K264R or wild type MOAP-1, it is apparent that K278R MOAP-1 is present at selectively higher concentrations in certain cytoplasmic regions of the cell (Fig 4.2). However, the significance of MOAP-1 intracellular accumulation remains to be further investigated. Our results indicate that wild type, K264R and K278R MOAP-1 possess similar localizations within the cell. Therefore, ubiquitination at either K264 or K278 in MOAP-1 is not required for regulation of its intracellular location under normal growth conditions.

4.2.3. MOAP-1 is ubiquitinated on residue K278 in response to PKC activation

Protein ubiquitination can frequently induce a functional change without noticeably altering substrate localization [256, 261]. To directly test the requirement of K264 and K278 in PKC-dependent MOAP-1 ubiquitination, U2OS osteosarcoma cells were transfected with either wild type or lysine mutants K264R and K278R of MOAP-1. Two days later, transiently transfected cells were stimulated with PMA in order to induce MOAP-1 ubiquitination prior to lysis, immunoprecipitation, SDS-PAGE and Western blotting. Our results indicate that ubiquitination of wild type MOAP-1 can be detected within 30 minutes following stimulation but occurs with delay with mutation of K264 (Fig. 4.3). On the other hand, mutation of K278 in MOAP-1 consistently results in a complete abrogation of MOAP-1 ubiquitination in response to PKC activation (Fig. 4.3). Therefore,

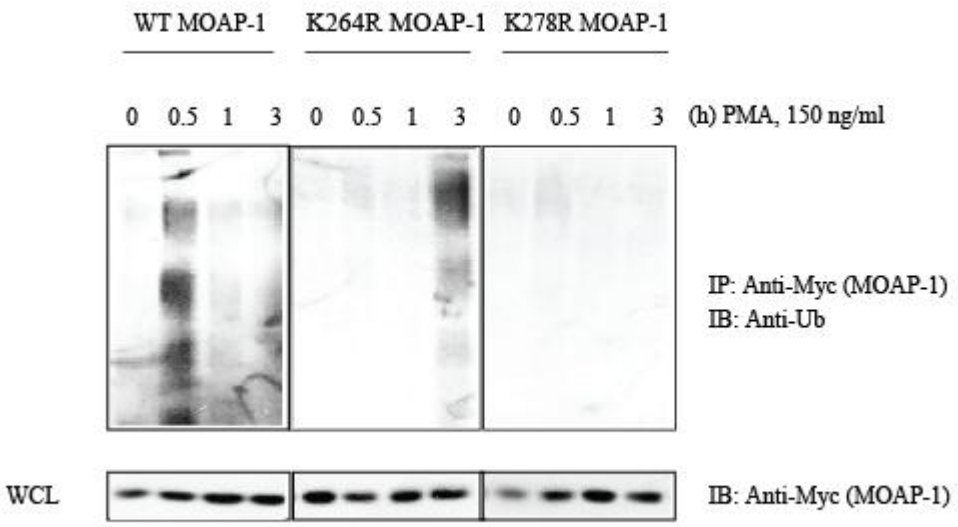


Figure 4.3. MOAP-1 ubiquitination is abrogated with mutation at K278. U2OS cells were transiently transfected with Myc-tagged constructs of WT, K264R or K278R MOAP-1. After 48 hours, cells were stimulated with 150 ng/ml phorbol 12-myristate 13-acetate (PMA) prior to harvest and immunoprecipitation (IP) with mouse anti-Myc antibody. Immunoblotting (IB) was performed with the indicated antibodies. WCL: whole cell lysate.

this strongly suggests that K278 in MOAP-1 is the site of PMA-stimulated modification. However, the significance of K264 is more ambiguous considering that MOAP-1 ubiquitination is retained with its mutation yet does not proceed with the same kinetics as with wild type MOAP-1. Consequently, K264 may not be absolutely required for PKC-dependent MOAP-1 ubiquitination but may still be biologically significant for other currently unknown reasons. Delayed ubiquitination of MOAP-1 may have important biological significance if the timely and rapid modification is required for its normal cellular function. It is possible that mutation of K264 may impede the recruitment of the E3 ligase responsible for MOAP-1 ubiquitination thereby resulting in delayed modification. This theory, along with other possibilities, warrants further investigation. Together, these results demonstrate that PKC-dependent MOAP-1 ubiquitination occurs at K278 and also suggest a role for K264 in MOAP-1 biology.

4.2.4. Ubiquitination of MOAP-1 involving K264 or K278 is not required for association with cell death proteins Bcl-x_L or RASSF1A

Cell signaling during apoptosis involves a defined sequence of events including multiple changes in protein associations that ultimately promote cell death [146]. As a proapoptotic protein, MOAP-1 has been shown to interact with several molecules involved in cell death including death receptors, Bcl2 family members and other proapoptotic proteins [84, 85, 189, 190]. To investigate the importance of ubiquitination and the residues K264 and K278 in the ability of MOAP-1 to induce apoptosis, we began by investigating whether or not mutations

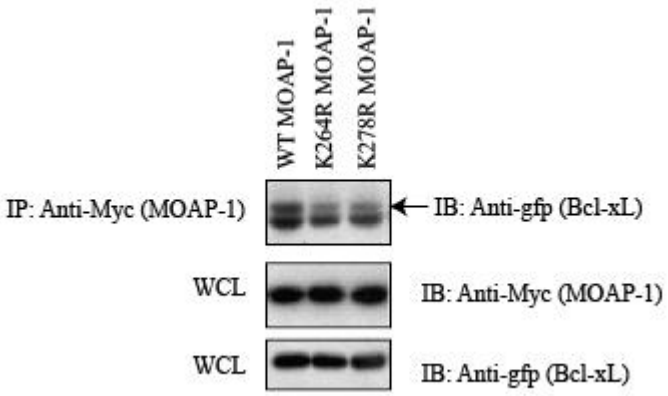
at these lysine residues would affect its associations with known interacting proteins.

In addition to Bax, one of the first molecules that MOAP-1 was shown to interact with is the anti-apoptotic Bcl-X_L protein [190]. Although Bcl-X_L association does not require the BH3-like domain present within MOAP-1, it is believed that this interaction involves the N- and C-terminal regions of the latter molecule. However, the amino acid sequences involved have not yet been determined. Given that MOAP-1-mediated cell death is blocked by Bcl-X_L overexpression, we were interested in investigating whether its ubiquitination involving K264 or K278 was critical for this interaction. Accordingly, HCT 116 cells were transfected with Myc-tagged wild type, K264R or K278R MOAP-1 along with GFP-Bcl-X_L. Protein complexes were immunoprecipitated with an anti-Myc antibody followed by Western blotting using an anti-GFP antibody against Bcl-X_L. In agreement with previous results [190], we were able to detect an association between wild type MOAP-1 and Bcl-X_L (Fig. 4.4A). Similarly, an interaction between K264R and K278R MOAP-1 with Bcl-X_L was also observed albeit at slightly reduced levels (Fig. 4.4A). Nonetheless, their ability to associate with Bcl-X_L to a nearly comparable extent as wild type MOAP-1 indicates that neither a K264R or K278R change perturbs this association.

Using a similar approach and methodology as with Bcl-X_L, we also decided to evaluate the ability of K264R and K278R MOAP-1 to interact with the tumor suppressor protein RASSF1A. During the extrinsic pathway of apoptosis, MOAP-1 interacts with RASSF1A following its recruitment to death receptors [84,

[85]. The association of MOAP-1 with RASSF1A is critical for its ability to induce apoptosis since it requires a RASSF1A-mediated conformational change that facilitates its subsequent interaction and activation of proapoptotic Bax. Previously, Foley and colleagues had established that the basic region [202]KRRR in MOAP-1 is required for its association with RASSF1A [85]. However, it is also possible that ubiquitination of MOAP-1 involving K264 and K278 may also be important for this interaction. Thus, if modification at K278 is necessary for association with RASSF1A or if ubiquitination requires K264 during the modification process, we would expect to observe a loss of interaction in the presence of lysine mutations at these residues. HCT 116 cells were transiently transfected with Myc-tagged wild type, K264R or K278R MOAP-1 along with HA-tagged RASSF1A. Two days post-transfection, cells were stimulated with TNFα plus cycloheximide (CHX) in order to induce cell death and to promote signaling towards apoptosis as opposed to inflammation [174]. This was followed by immunoprecipitation using an anti-Myc antibody and immunoblotting with an anti-HA antibody in order to detect RASSF1A association. Our results from Western blotting demonstrate that wild type, K264R and K278R MOAP-1 are each capable of associating with RASSF1A (Fig. 4.4B). Mutation at K278 appears to result in a slight decrease in its ability to interact with RASSF1A but still permits an association that does not dramatically differ from wild type or K264R MOAP-1 (Fig. 4.4B). Therefore, the ability of MOAP-1 to associate with RASSF1A does not require K264 or K278. Altogether, our findings suggest that

A)



B)

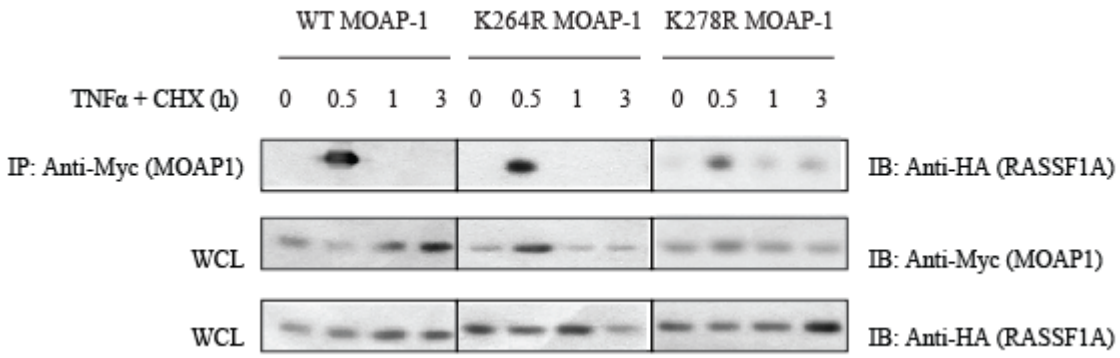


Figure 4.4. K264 and K278 are not critical residues for MOAP-1 association with Bcl-XL or RASSF1A. HCT 116 cells were transfected with Myc-tagged WT, K264R or K278R MOAP-1 along with (A) GFP-Bcl-X_L or (B) HA-RASSF1A. Two days after transfection, cells were (A) left unstimulated or (B) treated with 50 ng/ml TNFα plus 10 μg/ml cycloheximide (CHX), prior to harvest and cell lysis. Immunoprecipitation (IP) was performed with the indicated antibodies and followed by SDS-PAGE and Western blotting (IB). WCL: whole cell lysate. Note: the band detected below GFP-Bcl-X_L in (A) represents the mouse IgG heavy chain of the antibody used for IP.

the ability of MOAP-1 to interact with anti or pro-apoptotic proteins may not require its PKC-mediated ubiquitination.

4.2.5. PKC-dependent ubiquitination of MOAP-1 is not required for its ability to induce apoptosis through the death receptor-dependent signaling pathway

To confirm whether or not ubiquitination and lysine residues K264 or K278 in MOAP-1 are required for its ability to induce apoptosis, we next sought to perform a quantitative cell death assay. Results from Western blotting indicate that neither K264 nor K278 are important for MOAP-1 to be either prevented from inducing apoptosis through association with Bcl-X_L (Fig. 4.4A) or primed to activate Bax by interaction with RASSF1A (Fig. 4.4B). This suggests that perhaps PKC-dependent ubiquitination of MOAP-1 is not required for its proapoptotic function. To directly assess this possibility, H1299 lung carcinoma cells stably expressing either RASSF1A or MOAP-1 alone, or RASSF1A with the addition of wild type, K264R or K278R MOAP-1, were stimulated to undergo cell death with a ten hour treatment of TNFα plus CHX. During apoptosis, caspase-mediated activation of DNAses results in the cleavage of DNA into nucleosomal (180 bp) and oligonucleosomal (multiples of 180 bp) fragments [401]. Additionally, multiple changes in the plasma membrane occur during apoptosis including a redistribution of phopshatidylserine from the inner to the outer leaflet [180]. These characteristic features of apoptosis can be quantitatively assessed through the use of specific dyes followed by flow cytometry analysis. Following TNFα treatment, H1299

apoptotic cells were subsequently stained with the DNA intercalating agent propidium iodide and the phopshatidylserine binding protein annexin V prior to fluorescence activated cell sorting (FACS). On average, cells expressing both RASSF1A and wild type MOAP-1 displayed close to 40% apoptosis after treatment with TNFα and CHX and induced cell death to a significantly greater extent than vector control cells (Fig. 4.5A,B). Similar results were also obtained in the presence of either K264R or K278R MOAP-1 (Fig. 4.5A,B), thereby demonstrating that mutation at K264 or K278 do not impair the ability of MOAP-1 to induce apoptosis. Indirectly, these results also indicate that ubiquitination involving K264 or K278 in MOAP-1 is not required for its proapoptotic function.

As a further validation that K264 and K278 in MOAP-1 are not important for its role in apoptosis, a second type of analysis was performed. Poly(ADP-ribose) polymerase-1 (PARP-1) is an enzyme that plays a key role in the detection and repair of DNA strand breaks by catalyzing the NAD^+-dependent transfer of ADP-ribose to nuclear substrates and signaling for the recruitment of DNA repair proteins [402]. However, during apoptosis, PARP-1 is proteolytically cleaved from a 116 kDa full-length peptide to a signature 89 kDa and 24 kDa set of fragments. PARP-1 cleavage is often considered to be a hallmark of apoptosis and is also frequently employed as an assay to monitor cell death [403]. Therefore, we chose to verify our results from FACS analysis by Western blotting for cleaved PARP-1 in apoptotic cells. H1299 cells stably expressing RASSF1A or wild type MOAP-1 alone, or RASSF1A with wild type, K264R or K278R MOAP-1, were

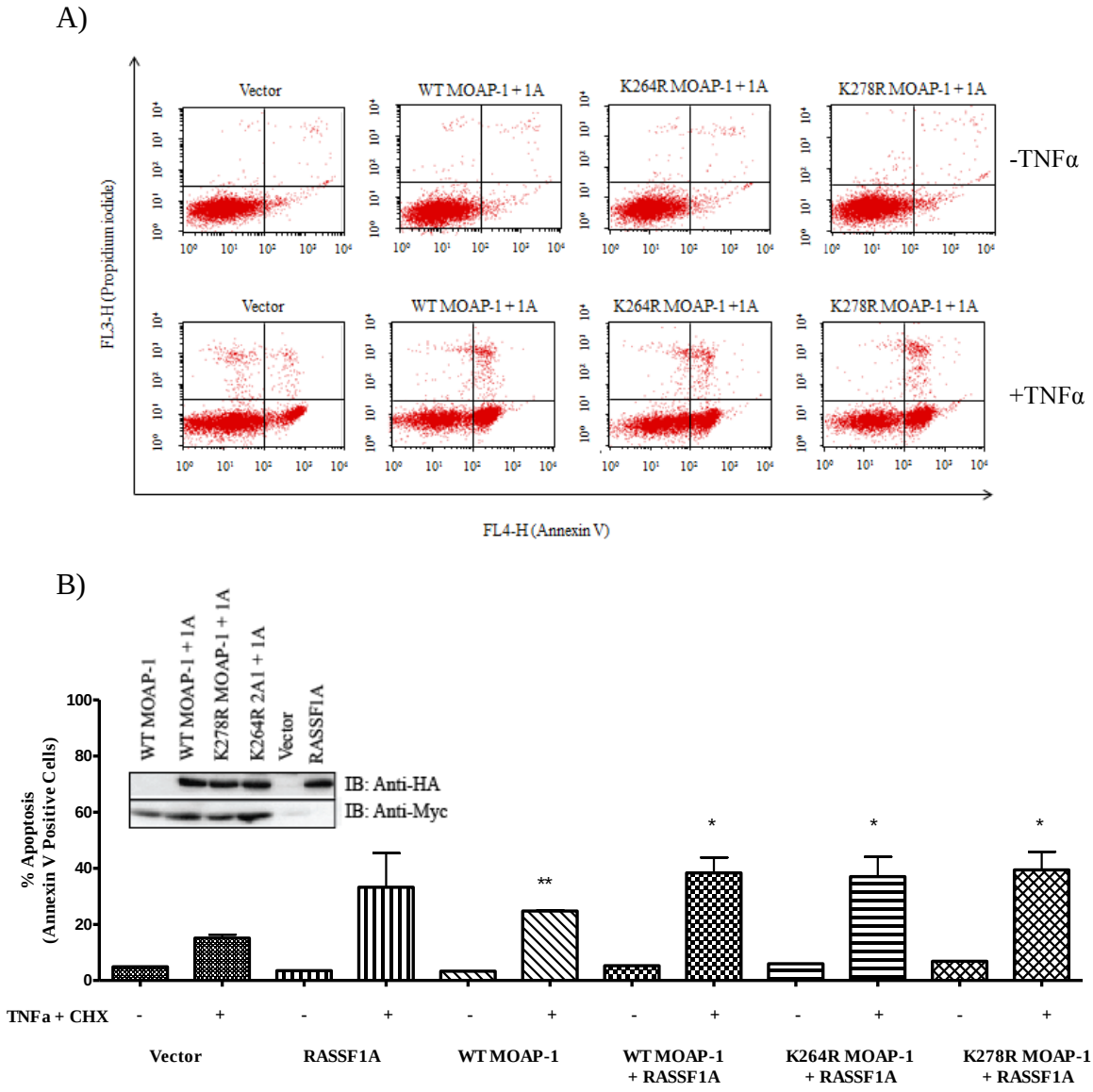


Figure 4.5. WT, K264R and K278R MOAP-1 share the same capacity to induce death receptor-mediated apoptosis. H1299 cells stably expressing the indicated vector, HA-RASSF1A or Myc-MOAP-1 constructs were grown to ~90% confluency prior to stimulation with TNFα (50 ng/ml) and cycloheximide (CHX, 10 μg/ml). After 10 hours, cells were harvested and stained with propidium iodide and annexin V to detect apoptotic cells by FACS analysis. (A) Scatterplots of propidium iodide (y-axis) versus annexin V (x-axis) staining. (B) Average percentage of cell death for H1299 cells stably expressing the indicated DNA constructs. Results are from three individual trials. Inset: Western blot to confirm the expression of HA-RASSF1A (1A) and Myc-MOAP-1. WT MOAP-1: p=0.0082; WT MOAP-1 + RASSF1A: p=0.0143; K264R MOAP-1 + RASSF1A: p= 0.0375; K278R MOAP-1 + RASSF1A: p=0.0203.

stimulated with TNFα and CHX for up to sixteen hours in order to induce DNA damage and cell death. Western blotting was subsequently performed in order to assess the amount of PARP-1 cleavage present. Accumulation of the 89 kDa cleaved PARP-1 fragment was detected after seven hours of treatment and was observed at similar levels in cells expressing RASSF1A with the addition of wild type, K264R or K278R MOAP-1 (Fig. 4.6). Comparable results were also observed after sixteen hours of cell stimulation where total cleavage of cellular PARP-1 was detected in the presence of either wild type or lysine mutants of MOAP-1 and was also present at higher levels than in cells expressing wild type MOAP-1 or RASSF1A alone (Fig. 4.6). Similar findings were also obtained in transiently transfected HCT 116 cells (results not shown). Overall, these results are in accordance with our findings from FACS analysis and support the conclusion that ubiquitination of MOAP-1 involving K264 or K278 is not required for its ability to induce death receptor activated apoptosis in cells.

4.2.6. Ubiquitination involving K264 and K278 is required for MOAP-1's tumor suppressor function

Thus far, we have demonstrated that MOAP-1 is ubiquitinated at K278 in response to PKC activation and that K264 may also be important for MOAP-1 biology given that its mutation results in ubiquitination with altered kinetics (Fig. 4.3). However, our results so far have failed to ascribe the importance of MOAP-1 ubiquitination to its proapoptotic function. Lysine mutants K264R and K278R MOAP-1 are both capable of interacting with cell death proteins Bcl-X_L and

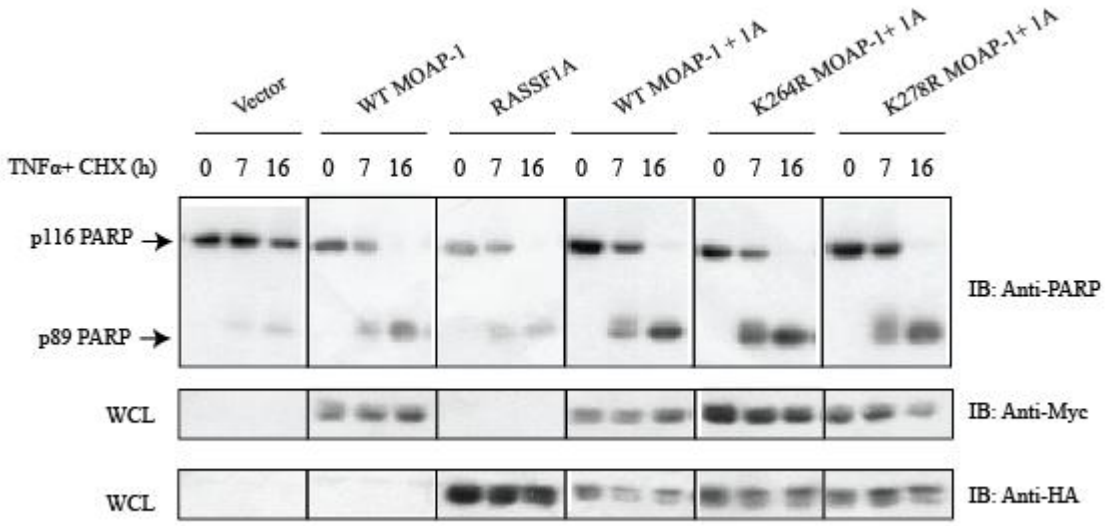
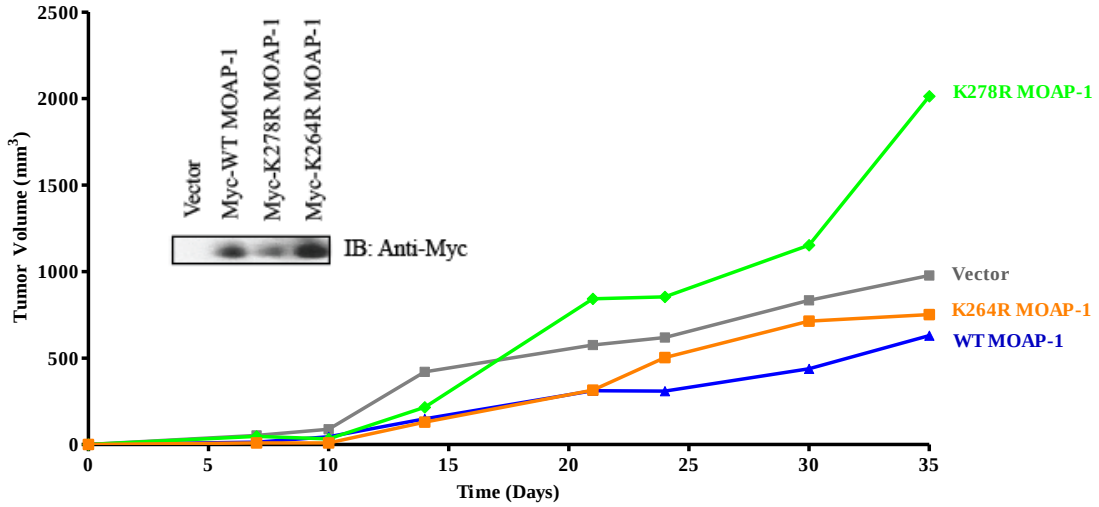


Figure 4.6. PARP cleavage is not affected by K264 or K278 MOAP-1 mutations. H1299 cells stably expressing vector control or the indicated combinations of Myc-tagged MOAP-1 and HA-RASSF1A (1A) constructs were stimulated with TNFα (50 ng/ml) + CHX (10 μg/ml) for the indicated times. Western blotting (IB) was performed to assess PARP cleavage. WCL: whole cell lysate.

RASSF1A and can also induce apoptosis with the same capacity as wild type MOAP-1 (Fig. 4.4, 4.5, 4.6). Meanwhile, our results concerning the tumor suppressor function of wild type MOAP-1 also strongly suggest that it extends beyond its role in cell death. For instance, wild type MOAP-1 can inhibit cell proliferation in the absence of it proapoptotic binding partner RASSF1A (Fig. 3.9, 3.10, 3.11). Logically, we therefore next proceeded to evaluate the importance of K264 and K278 in MOAP-1's tumor suppressor function.

Previously, we had employed a xenograft mouse tumor assay in order to confirm the growth inhibitory function of wild type MOAP-1 (Fig. 3.11). To directly examine the potential importance of MOAP-1 ubiquitination involving K264 and K278 in its tumor suppressor function, we decided once again to utilize the human tumor xenograft mouse model as a highly informative and biologically relevant *in vivo* test. HCT 116 cells transfected with wild type, K264R or K278R MOAP-1 were injected subcutaneously into the left and right flanks of athymic mice. Regular and periodic measurements of tumor growth and size were performed over the course of the next 35 days. Although wild type MOAP-1 was found to significantly inhibit tumor formation relative to vector control ($p=0.0213$), mutation at K264 resulted in a partial loss of tumor suppressor function as cells expressing this MOAP-1 lysine mutant behaved comparably to cells transfected with empty vector ($p=0.1966$) (Fig. 4.7A,B). Noticeably, mutation at K278 in MOAP-1 not only resulted in complete abolishment of its tumor suppressor function but also functioned as an oncogenic mutation ($p=0.0002$) (Fig. 4.7A,B).

A)



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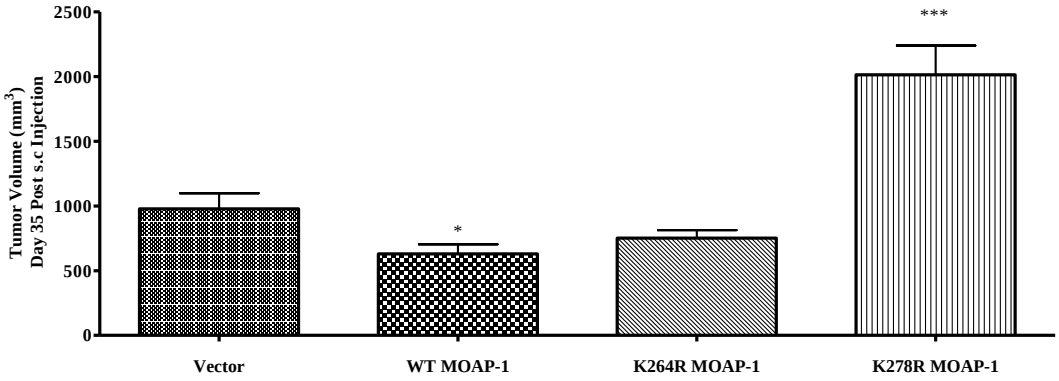


Figure 4.7. MOAP-1 ubiquitination at K278 is required for its tumor suppressor function *in vivo*. HCT 116 colorectal carcinoma cells were transiently transfected with empty vector or Myc-tagged WT, K264R or K278R MOAP-1. Two days post-transfection, cells were harvested and resuspended in matrigel solution prior to subcutaneous injection into the left and right flanks of athymic mice. Tumor sizes were measured at regular intervals over the course of 35 days and calculated based on the formula for spherical volume ($4/3\pi r^3$, r=radius). (A) Tumor growth curve of mice injected with HCT 116 cells overexpressing the indicated DNA constructs. Inset: Western blotting (IB) to verify the expression of HA-RASSF1A and Myc-MOAP-1. (B) Static plot of average tumor volumes in mice 35 days post-subcutaneous injection. Vector: n=18; WT MOAP-1: n=17, p=0.0213; K264R MOAP-1: n=10, p=0.1966; K278R MOAP-1: n=8, p= 0.0002.

Tumors that formed in the presence of overexpressed K278R MOAP-1 were, on average, approximately twofold larger than with vector control cells (Fig. 4.7A,B). These results clearly demonstrate that MOAP-1 residues K264 and K278 are important for its tumor suppressor function. However, the relative importance of these two lysine residues differs to a great extent and may possibly reflect how directly involved they are in the ubiquitination of MOAP-1. While K278 is absolutely required for PKC-dependent MOAP-1 ubiquitination and may serve as the actual site of modification, mutation of K264 only impairs - but still permits - MOAP-1 ubiquitination (Fig. 4.3). Given these results, further examination of the significance of K264 and K278 in MOAP-1 biology will be necessary.

4.2.7. Overexpression of RASSF1A cannot suppress the tumorigenicity of K264R or K278R MOAP-1 mutations

RASSF1A is a bona fide tumor suppressor protein [364] and cooperates with MOAP-1 during death receptor-mediated apoptosis [84, 85]. To determine if overexpression of RASSF1A can minimize the tumorigenicity observed with K264R or K278R MOAP-1 mutations, a xenograft mouse tumor assay was performed using HCT 116 cells cotransfected with WT, K264R or K278R MOAP-1 and RASSF1A. The results from this experiment were subsequently compared to our results from mice injected with cells solely transfected with either wild type or one of two lysine mutants of MOAP-1 (Fig. 4.7) in order to determine the effects of RASSF1A overexpression. Although RASSF1A

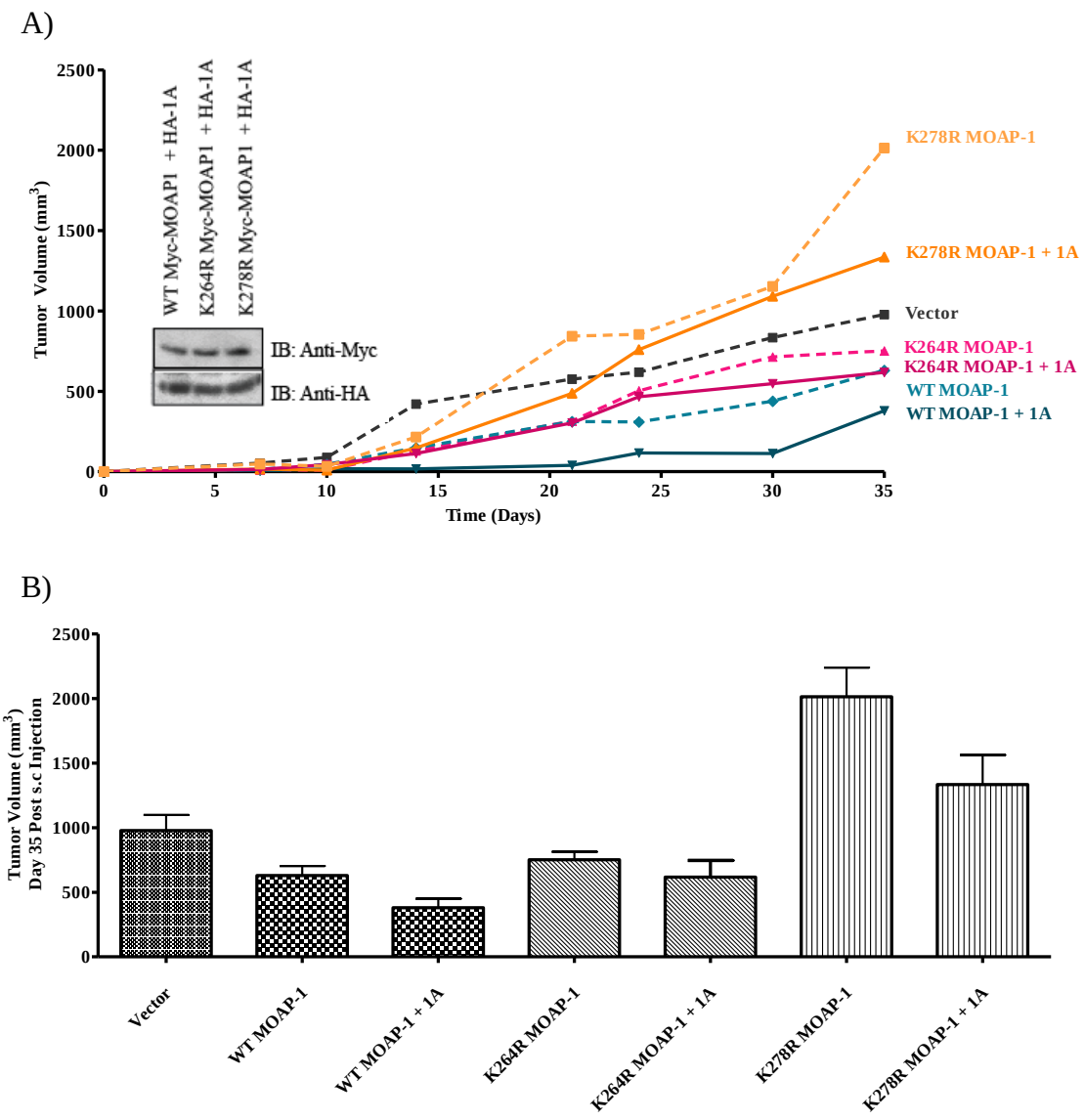


Figure 4.8. RASSF1A overexpression cannot suppress the tumorigenicity of K264R and K278R MOAP-1 mutations *in vivo*. HCT 116 colorectal carcinoma cells were transiently transfected with the indicated combinations of HA-RASSF1A (1A) and Myc-tagged WT, K264R or K278R MOAP-1. Xenograft tumor assay was performed as described in Fig. 4.7. (A) Tumor growth curve of mice injected with HCT 116 cells overexpressing the indicated DNA constructs. Solid lines display the progress of tumor growth for cells transfected with Myc-tagged WT or mutant MOAP-1 constructs with the addition of HA-1A overexpression. For comparison, dashed lines show an underlay of data presented in Fig. 4.7. Inset: Western blotting (IB) to verify the expression of HA-1A and Myc-MOAP-1 constructs. (B) Static plot of average tumor volumes in mice 35 days post-subcutaneous (s.c) injection. Vector: n=18; WT MOAP-1: n=17, with 1A: n=8, p=0.045; K264R MOAP-1: n=10, with 1A: n=10; p=0.3623; K278R MOAP-1: n=8, with 1A: n=12 p= 0.0581.

possesses a growth inhibitory function that impinges on several areas in cell biology, the additional overexpression of this protein had no significant influence on tumor growth due to exogenous K264R or K278R MOAP-1 expressions (Fig. 4.8; p=0.3623 and 0.0581, respectively). It is worth mentioning, however, that the overexpression of RASSF1A appears to have a greater tumor suppressive effect on the oncogenic growth induced by K278R than K264R MOAP-1 mutants. This may suggest that the loss of tumor suppressor function due to K278R MOAP-1 mutation involves a biological process that is also shared by RASSF1A and therefore may be partially complemented by the latter protein. Preliminary results from additional xenograft mouse tumor experiments involving transiently transfected DAOY medulloblastoma cells (data not shown) and stably transfected H1299 lung carcinoma cells (Fig 4.9A,B,C) also support the observation that mutation at K264 or K278 in MOAP-1 also results in a loss of growth suppression. Consequently, our results reveal that K278 (and to a lesser extent K264) are essential to the tumor suppressor function of MOAP-1. Given that MOAP-1 residues K264 and K278 are also involved in its PKC-dependent ubiquitination, we believe that this post-translational modification of MOAP-1 must also be important for its growth inhibitory function. Further investigation will certainly be required in order to fully understand the significance of these findings.

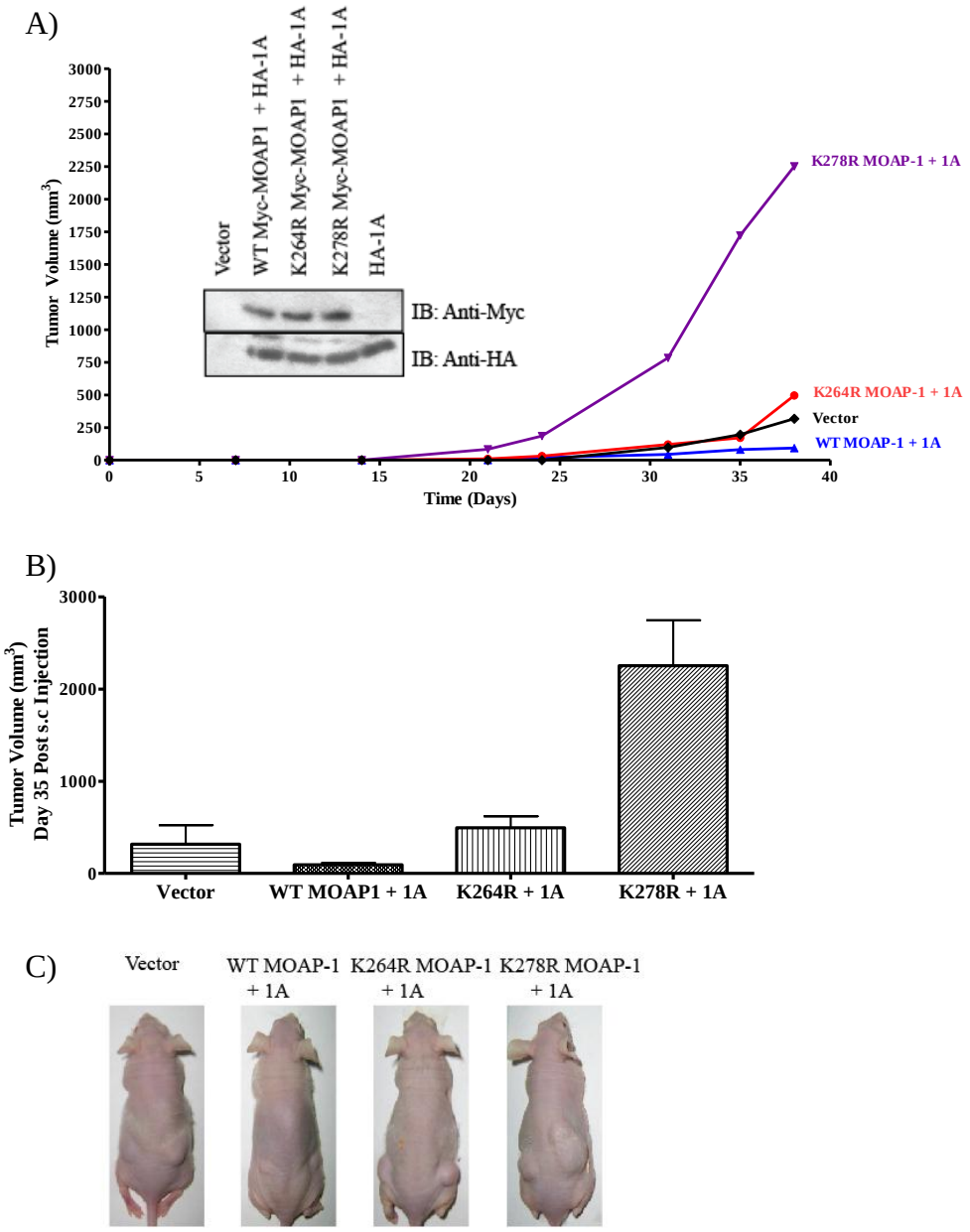


Figure 4.9. Tumorigenicity of K264R and K278R MOAP-1 mutations remain unaffected by RASSF1A overexpression *in vivo*. H1299 lung carcinoma cells stably co-expressing Myc-tagged WT, K264R or K278R MOAP-1 and HA-RASSF1A (1A) were utilized in a xenograft tumor assay that was performed as described in Fig. 4.7. (A) Tumor growth curve of mice injected with H1299 cells overexpressing the indicated DNA constructs. Inset: Western blotting (IB) to verify the expression of HA-1A and Myc-MOAP-1 constructs. (B) Static plot of average tumor volumes in mice 35 days post-subcutaneous (s.c) injection. Vector: n=2; WT MOAP-1 with 1A: n=4, p=0.1562; K264R MOAP-1 with 1A: n=3, p=0.4810; K278R MOAP-1 with 1A: n=6, p=0.0765. (C) Representative images of nude mice with s.c tumors in the left and right flanks 35 days post-injection.

4.3. DISCUSSION AND FUTURE DIRECTIONS

4.3.1. Differential roles of K264 and K278 in MOAP-1's tumor suppressor function

It is quite interesting yet also peculiar that two different lysine residues in MOAP-1- K264 and K278- are both important for its non-degradative ubiquitination and tumor suppressor function. By inference, these data also implicate MOAP-1 ubiquitination as an important post-translational modification required for the ability of this protein to inhibit malignant growth. Our results concerning the importance of K278 have been consistent over the duration of this study in that they indicate that this residue serves as the site of PKC-dependent ubiquitination (Fig. 4.3). Our data also clearly demonstrate that loss of MOAP-1 ubiquitination through genetic mutation at K278 not only results in a loss of tumor suppressor function but also promotes oncogenicity (Fig. 4.7). Therefore, it is possible that mutation at K278 may have a dominant negative effect in MOAP-1 cell biology. Interestingly, some of the findings that we have observed for K278R MOAP-1 may potentially be explained by what is currently known for one of the most widely mutated tumor suppressor proteins in human cancer, p53. Oncogenic mutations in the DNA binding domain of p53 account for the vast majority of its mutations and impairs its ability to transactivate the transcription of genes involved in processes such as DNA repair, apoptosis or growth arrest [28]. Given that p53 functions as a tetramer, mutations in its DNA binding domain display a dominant negative behaviour towards its wild type form [33]. However, in addition to interfering with its own normal functions, mutant p53 can also impair other

cellular processes that typically do not involve its activity including p53-independent apoptosis [404]. These findings may help to explain our observations with K278R MOAP-1 and suggests that mutation at this site not only impedes MOAP-1-mediated tumor suppression but may also affect other MOAP-1-independent cellular processes. Considering that carcinogenesis generally involves both oncogenic gain of function activity and loss of tumor suppressor function, it is conceivable that the loss of MOAP-1 ubiquitination at K278 may contribute to both these events.

In contrast to K278 in MOAP-1, K264 may not be essential for MOAP-1 ubiquitination. However, mutation at this residue results in altered kinetics of post-translational modification and causes a partial loss of MOAP-1-mediated tumor suppressor function (Fig. 4.3, 4.7). Together, these results suggest that K264 may fulfill a regulatory function in MOAP-1 ubiquitination by possibly facilitating modification at K278. Previously mentioned is the hypothesis that K264 may somehow participate in the recruitment of the putative MOAP-1 E3 ligase, or other proteins or enzymes involved in the ubiquitination process. This plausible explanation may account for the delayed modification observed with K264 mutation (Fig. 4.3). However, that MOAP-1 ubiquitination is still retained with K264 mutation also indicates that this residue is dispensable for this process and therefore other mechanisms must be in place in order to ensure MOAP-1 modification. Nonetheless, altered MOAP-1 ubiquitination appears to have significant impact on its ability to suppress tumor formation considering the loss of tumor suppressor function that is observed with K264R MOAP-1 mutants (Fig.

4.7). It is worth mentioning that loss of MOAP-1 ubiquitination has consistently been observed for K278R MOAP-1 mutants but that results with our K264R MOAP-1 constructs have been somewhat less consistent in that normal kinetics and loss of ubiquitination have also been observed albeit infrequently. This confounds our data with respect to the significance of K264 in MOAP-1 and makes it difficult to ascertain what potential functions it may serve. In spite of this, results from our xenograft tumor assays support the proposition that K264 is biologically important to the tumor suppressor function of MOAP-1 and by extension, also its ubiquitination. Additional studies will be necessary in order to better understand the exact significance of K264 in the MOAP-1 ubiquitination process.

4.3.2. Potential role of PKC in MOAP-1-mediated growth inhibition

Currently, MOAP-1 is only known to function as a proapoptotic molecule through its role in Bax activation [84, 189]. However, our results indicate that the ability of MOAP-1 to suppress tumor formation extends beyond its role in death receptor-mediated apoptosis and also requires its PKC-dependent ubiquitination. To date, there are ten different isozymes that belong to the PKC protein family and whose range of functions include regulation of cell growth and apoptosis, transcription, immune responses and receptor desensitizaton [209, 405]. These PKC isoforms differ in their sensitivity to phosphatidylserine, calcium and DAG as cofactors, and several of these enzymes are known to have opposing functions. Given that PKC functions as a key signaling molecule in a variety of different

cellular processes, there are a number of different ways in how it may contribute to MOAP-1-mediated tumor suppression. However, a logical and general hypothesis is that PKC may contribute to the activation of MOAP-1's growth inhibitory function through the induction of MOAP-1 ubiquitination. Indeed, PKC is known to regulate the ubiquitination of multiple proteins including the dopamine transporter [406], Akt [407], cationic amino transporter 1 (CAT-1) [408] as well as itself [409].

Given that protein phosphorylation commonly serves as a priming signal for ubiquitination [410], it is also possible that MOAP-1 may serve as a PKC substrate. In support of this, Scansite analysis [393] of MOAP-1 at medium stringency suggests that this protein contains a potential PKCμ binding site. Additionally, the accumulation of phosphorylated MOAP-1 protein in C1 and SEM pediatric leukemia cell lines (Fig 3.4) suggests a possible block in the non-degradative ubiquitination of this protein that would certainly impair its tumor suppressor function (Fig 4.7). Thus, the possible inability of MOAP-1 to undergo PKC-dependent ubiquitination in these cell lines may have been a contributing factor to these cancers. Of interest, the MOAP-1 binding protein RASSF1A is also regulated by PKC phosphorylation in a manner that is required for its tumor suppressor function although it is independent from ubiquitination [398]. Therefore, it will be interesting to further investigate the ability of PKC to phosphorylate MOAP-1 and also determine how it may be involved in the regulation of its ubiquitination.

Lastly, our results suggest that MOAP-1 may possess other biological functions in addition to its role in extrinsically activated apoptosis and that, collectively, they may contribute to MOAP-1's tumor suppressor function. As one possibility, it is still plausible that MOAP-1 may mediate its growth inhibitory effects through its function in the intrinsic pathway of apoptosis. Although the mechanism of its role in this pathway has not yet been elucidated, evidence suggests that MOAP-1 serves a similar function as its role in death receptor-mediated cell death through activation of Bax [189]. Although we attempted a preliminary investigation of the importance of K264 and K278 in MOAP-1's ability to induce intrinsically activated apoptosis, our results were inconclusive. Therefore, we are currently unable to comment on the importance of this second apoptotic pathway to MOAP-1's tumor suppressor function as it remains to be further investigated.

4.3.3. Future investigations in the MOAP-1 ubiquitination process

Thus far, we have identified two sites (K264 and K278) in MOAP-1 that are important for both its ubiquitination and tumor suppressor function. In continuation of this study, there are several additional areas of interest that we would also like to examine. Of greatest importance to this study would be to determine the signaling pathways involved in MOAP-1 ubiquitination and the mode of this protein's tumor suppressor function, with relation to the significance of K264 and K278. However, additional investigations will be required in order to help us realize this final goal.

Polyubiquitination of proteins occurs in a processive manner through the conjugation of one ubiquitin molecule to the next through specific lysine residues in ubiquitin [263]. Our current hypothesis is that PKC-dependent MOAP-1 ubiquitination likely proceeds through a K63-linked mechanism since it is the most common type of ubiquitin linkage typically associated with non-degradative functions [322]. However, protein ubiquitination involving other lysine linkages have also been observed in cell signaling processes and are equally probable possibilities. Given that certain ubiquitin chain formations are also associated with different E2 and E3 ubiquitin enzymes, it will be interesting to investigate the type of lysine linkage involved in MOAP-1 ubiquitination so as to gain insight into its potential regulators.

Preliminary studies that have been carried out by Dr. Shairaz Baksh suggest that TNF receptor-associated factor 2 (TRAF2) may actually function as the putative E3 ligase responsible for PKC-dependent MOAP-1 ubiquitination (unpublished results). MOAP-1 contains two potential binding sites for TRAF2 based on the consensus TRAF2 binding motif (S/A/T/PxE/Q E) where x is any amino acid. Within MOAP-1, these potential sites for TRAF2 are located at ^{175}EPGEEEFGRW (part of intra-electrostatic association within MOAP-1) and ^{331}DYEAAEEEEAL (a site involved in death domain interaction with TNFR1, underlined residues are the TRAF2 binding sites). Early studies reveal that PMA-stimulated MOAP-1 ubiquitination is absent from *Traf2*$^{-/-}$ mouse embryonic fibroblasts (Baksh et al., unpublished observations). In further support of TRAF2 as the potential MOAP-1 E3 ligase is its known association with PKC. During

TNFα signaling, PKC is involved in directing the membrane localization and K63-linked ubiquitination of TRAF2 and is important for NF-κB activation [411]. However, a previous study has also demonstrated that MOAP-1 interacts with the RING domain-containing E3 ligase TRIM39 [193]. Surprisingly, data indicate that TRIM39 is involved the stabilization of MOAP-1 during apoptosis through inhibition of its polyubiquitination and proteasomal degradation. Nonetheless, it is possible that TRIM39 may also be involved in PKC-dependent MOAP-1 ubiquitination and therefore remains to be tested. Thus, further validation studies will be required to potentially confirm the role of TRAF2 or TRIM39 in PKC-dependent MOAP-1 ubiquitination.

4.4. CONCLUSION

The results from this chapter clearly demonstrate that PKC-dependent ubiquitination of MOAP-1 at K278 is required for its tumor suppressor function. Our results also indicate that K264 is important for both MOAP-1 ubiquitination and tumor suppression; however, this residue likely serves a different function than K278. Given that mutation at K264 leads to a loss of growth inhibition while genetic change at K278 promotes oncogenic growth, we hypothesize that K264 may serve a regulatory function in MOAP-1 biology whereas K278 is the site actually required for mediating MOAP-1's tumor suppressor function.

CHAPTER 5

Microarray expression analysis of HCT 116 xenograft tumors reveals potential mechanisms of MOAP-1 mediated growth suppression

5.1. INTRODUCTION

Thus far, we have identified MOAP-1 as a novel tumor suppressor protein and have uncovered a mode of post-translational modification that is essential to its function. Data indicates that PKC-dependent MOAP-1 ubiquitination is important for its ability to suppress tumor formation yet is not required for its ability to induce extrinsically-activated cell death (Fig. 4.5, 4.6, 4.7). Together, these results suggest that death receptor-dependent apoptosis is not the sole source of MOAP-1's tumor suppressor function and we are, therefore, interested in investigating other possible mechanisms that may explain how MOAP-1 inhibits cell proliferation and tumor formation.

To gain insight into potential proteins and molecular pathways that may be involved in the tumor suppressor function of MOAP-1, gene expression profiling was performed on tumors formed by xenograft assays in nude mice. Tumors were excised from athymic nude mice injected with HCT 116 cells overexpressing vector control, RASSF1A or wild type, K264R or K278R MOAP-1 upon termination of the experiment 35 days post-injection. RNA extraction was performed using three representative xenograft tumors for each construct that was tested and were subsequently subjected to microarray expression analysis using the Agilent platform with the skilled help of research technologist Lillian Cook and Dr. Kathryn Graham (Department of Oncology, University of Alberta). As a measure of quality control, gene expression changes were analysed in replicates of three for each construct that was tested using the RNA isolated from three

different xenograft tumors. Data normalization and analysis was performed by Dr. Kathryn Graham using GeneSpring GX 11.5.1 (Agilent Technologies).

As anticipated, our microarray transcription analysis generated a large volume of data. Consequently, our preliminary assessment required a simplification of our dataset. With the microarray results from this assay, we decided to focus on gene expression changes with potential relevance to cancer development that would provide a possible explanation for the cell proliferation and tumor growth characteristically inhibited or induced by either wild type or lysine mutants of MOAP-1, respectively. In particular, we were interested in expression changes for genes encoding proteins involved in cell proliferation and death, gene expression or DNA repair as common and possible methods of tumor suppression. Using Ingenuity Pathway Analysis software (IPA, Ingenuity systems) [412] we were able to organize our differential gene expression results based on biological function and their involvement in canonical signaling pathways. This chapter will discuss and summarize some of the results from our microarray study.

5.2. RESULTS AND DISCUSSION

5.2.1. Analysis of gene expression changes induced by wild type MOAP-1 overexpression

One of the primary objectives of this microarray analysis was to identify potential biological processes and patterns of gene expression that would help us

to better understand MOAP-1 as a novel tumor suppressor protein and how it may exert its growth inhibitory function, with vector control as a basis for comparison. Microarray gene expression profiling of xenograft tumors due to wild type MOAP-1 versus vector control overexpressing cells revealed a total of 1434 differentially expressed results based on a twofold expression cut-off with probability (p-)values less than or equal to 0.05. Using the "core analysis" function in IPA, we were able to interpret gene expression changes in the context of biological functions and signaling pathways. The data presented in Table 5.1 represent a selection of differentially expressed genes generated using the functional analysis module in IPA, while Table 5.2 displays several signaling pathways that were identified as being most significant to this dataset based on Fisher's exact test using the IPA canonical pathway analysis function. Our results reveal significant changes for a large list of genes encoding proteins with potential growth regulatory or tumor suppressor functions (Table 5.1). As point of fact, the top three biological functions most strongly associated with the disregulated molecules in our wild type MOAP-1 dataset are involved in the cell cycle (155 molecules with p-values 1.30E-08 to 6.89E-03), cell death (290 molecules with p-values 3.21E-07 to 6.36E-03), and gene expression (191 molecules with p-values 6.23E-07 to 6.84E-03).

Table 5.1. Genes differentially expressed by WT MOAP-1 relative to Vector[a]

Biological association[b]	GenBank accession no.	Probe Name	Gene Symbol	Description	Avg.[c] 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	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

Table 5.1 *continued*. Genes differentially expressed by WT MOAP-1 relative to Vector[a]

Biological association[b]	GenBank accession no.	Probe Name	Gene Symbol	Description	Avg.[c] fold change	p-value
Gene expression	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

Table 5.1 *continued*. Genes differentially expressed by WT MOAP-1 relative to Vector[a]

Biological association[b]	GenBank accession no.	Probe Name	Gene Symbol	Description	Avg.[c] fold change	p-value
DNA replication, recombination, and repair	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-03
	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]Genes differentially expressed in WT MOAP-1 relative to vector control HCT 116 xenografts.
[b]Biological associations were assigned by functional analysis in IPA. Each molecule is represented by only one biological association in cases where more than one may be applicable.
[c]Average (Avg.) fold change. Negative values denote downregulation, positive values indicate upregulation.

Table 5.2. Potential canonical pathways disregulated by WT MOAP-1 relative to vector[a]

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.

5.2.1.1: A potential p53-dependent mechanism of wild type MOAP-1 tumor suppressor function

Of particular interest, *TP53* encoding the tumor suppressor protein p53, was found to be upregulated by over three-fold in the presence of wild type MOAP-1 relative to vector (Table 5.1). Several molecules involved in p53-dependent signaling pathways and which participate in a variety of growth regulatory processes were also present at elevated levels (Fig. 5.1, Table 5.1). As one of the most well-studied tumor suppressor proteins in cancer research, p53 is implicated in DNA repair, induction of cell cycle arrest, and apoptosis, and is mutated in over 50% of human tumors [413]. Loss of the MOAP-1 binding protein *Rassf1a* in mice has been shown to synergize with *p53* inactivation during tumorigenesis [69]. It is possible that MOAP-1 cooperates with p53 in tumor suppression either directly or indirectly due to overlapping biological functions. p53-mediated apoptosis can be activated by either intrinsic or extrinsic cell death signals and proceeds via the mitochondrial-dependent apoptotic pathway [414]. p53 also regulates death receptor-dependent apoptosis by modulating the cell surface expression of receptors including Fas [415] and TRAIL receptor-2 [416]. Given that MOAP-1 is involved in both the intrinsic and extrinsic pathways of apoptosis [84, 85, 189, 192], it is conceivable that MOAP-1 may function along with p53 in certain signal transduction pathways.

Interestingly, several p53 transcriptional targets were also found to be differentially expressed in the presence of overexpressed wild type MOAP-1 (Table 5.1). These include upregulation of the DNA replication and repair protein

proliferating cell nuclear antigen (PCNA, Fig. 5.1) [417] and cell death receptor Fas [415], as well as downregulation of cell surface protein epidermal growth factor receptor (EGFR) [418] (Table 5.1). Together, these gene expression changes are involved in the maintenance of genomic integrity and also protect against uncontrolled cell proliferation. Additionally, several p53 regulatory proteins were also present at elevated levels including cyclin-dependent kinase 8 (CDK8) [419], cell division cycle 14 homolog B (CDC14B) [420] and transforming growth factor beta regulator 1 (TBRG1/NIAM) [421]. The differential expression of these p53 targets and regulators strongly suggests that the elevated levels of p53 also correlate with its increased activity in the presence of wild type MOAP-1.

The decision as to whether p53 induces cell cycle arrest or apoptosis in response to cellular stresses is not fully understood but is thought to be influenced by the availability of its coactivators that ultimately determine which target genes are induced [414]. One particular molecule that functions as an important determinant in p53-regulated cell fate is the transcription factor Myc. Myc has been shown to inhibit growth arrest in response to DNA damage [422, 423] and promotes apoptosis by blocking p53-dependent transcription of the cyclin-dependent kinase inhibitor p21 [424]. As a result, Myc prevents the induction of cell cycle arrest and, instead, promotes apoptosis. Given that p53 is upregulated in the presence of wild type MOAP-1, it is interesting that certain components involved in Myc-mediated apoptosis are also upregulated (Fig. 5.2, Table 5.2). This suggests a connection between p53, MOAP-1 and apoptosis as a plausible mode of tumor suppression. Meanwhile, other molecules from additional p53-dependent

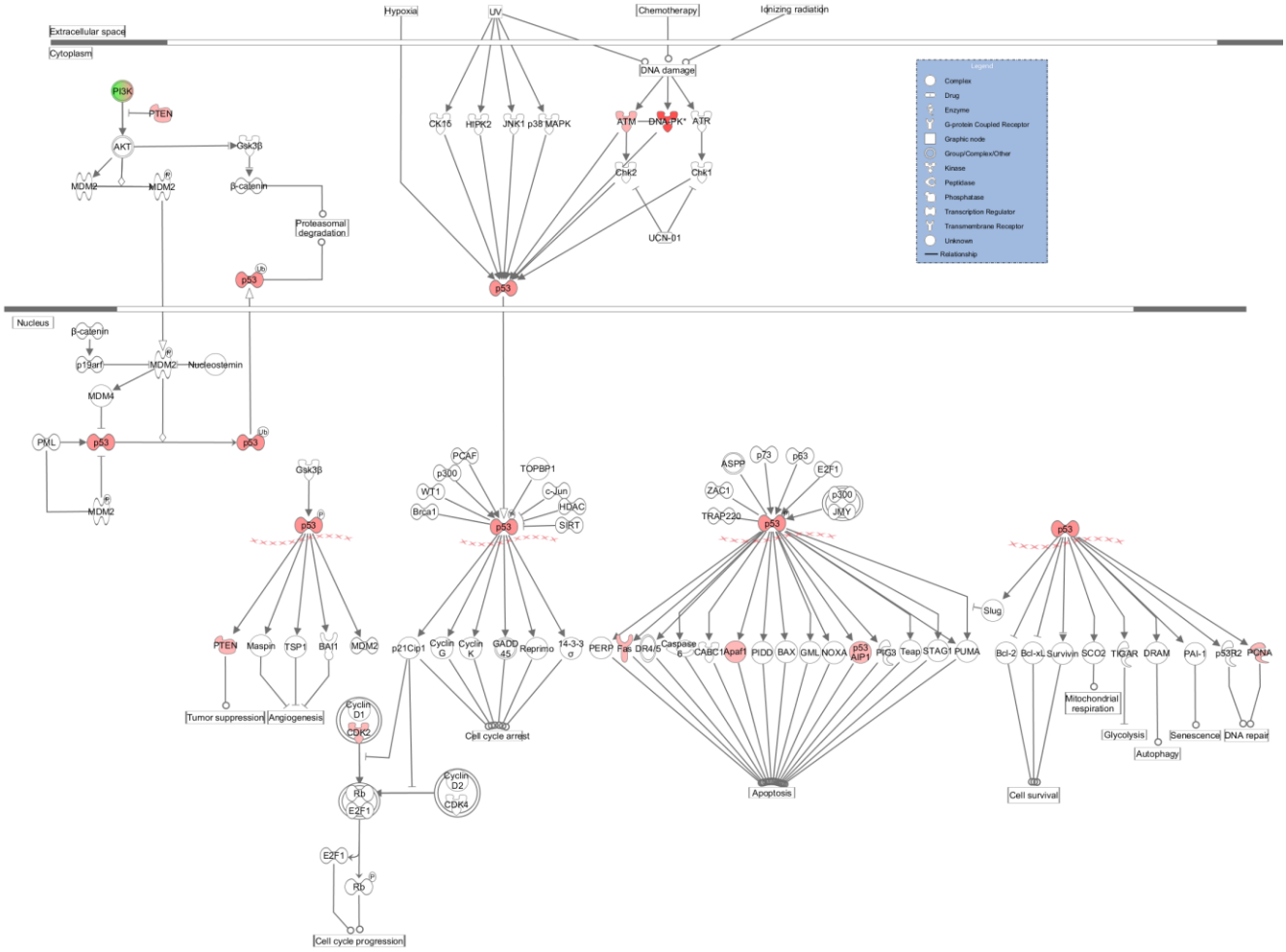


Figure 5.1. p53 signaling network. Differential gene expressions of molecules involved in p53-dependent signaling pathways were identified by canonical pathway analysis using IPA (Ingenuity systems). Shown is a graphical representation of several p53 canonical signaling pathways with dataset molecules that meet our threshold cut-offs (fold-change≥2, p≤0.05) represented in red (upregulated) and green (downregulated). Molecules that are coloured in both red and green were detected as being both up-and down-regulated by microarray analysis.

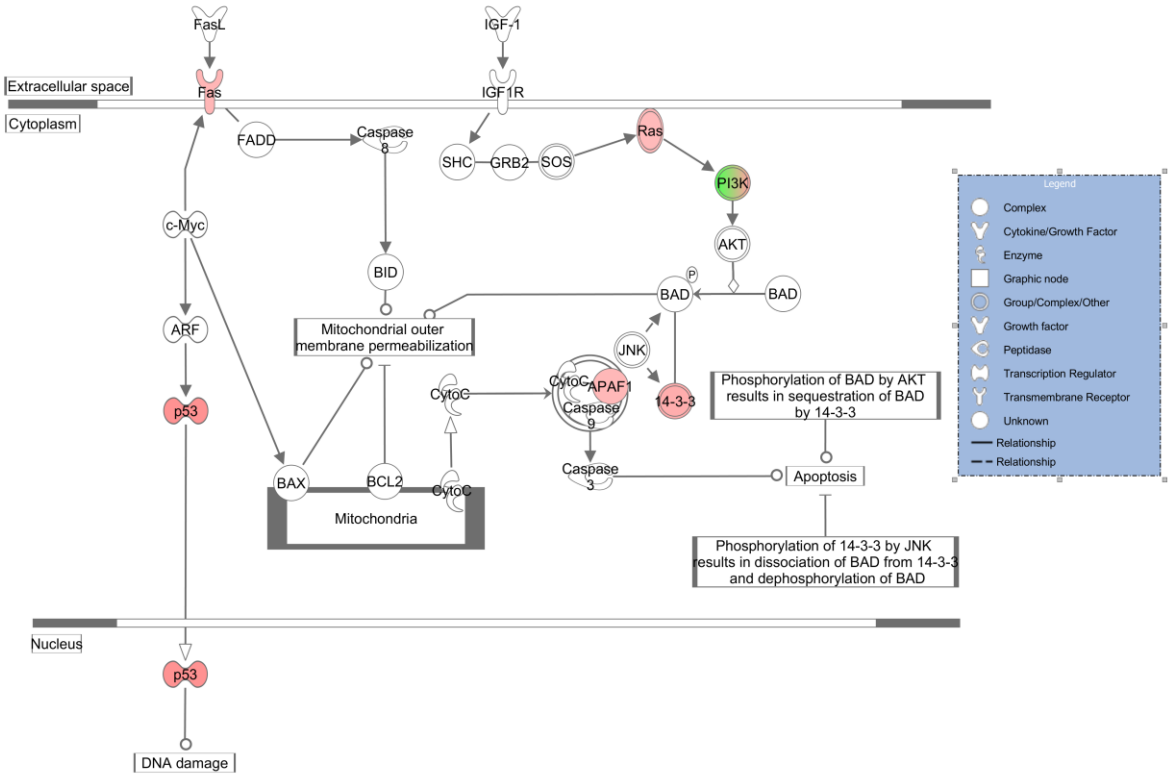


Figure 5.2. Myc-mediated apoptosis. Differential gene expression of molecules involved in Myc-mediated apoptotic signaling was identified by canonical pathway analysis using IPA (Ingenuity systems). Shown is a graphical representation of this signaling pathway with dataset molecules that meet our threshold cut-offs (fold-change≥2, $p \leq 0.05$) represented in red (upregulated) and green (downregulated). Molecules that are coloured in both red and green were found to be both up-and down-regulated by microarray analysis.

signaling pathways were also found to be elevated with wild type MOAP-1 overexpression including proteins for the pathways involving hypoxia-inducible factor 1, alpha subunit (HIF1α, Fig. 5.3) and the G2/M DNA damage checkpoint (Fig. 5.4). Whether or not MOAP-1 may be involved in the cellular responses to hypoxia or DNA damage are interesting areas of possible further investigation.

5.2.1.2. Potential alternative mechanisms of MOAP-1-mediated growth inhibition

In addition to the tumor suppressor p53 and several proteins involved in p53-dependent signaling pathways, a large number of other molecules were also differentially expressed in the presence of overexpressed wild type MOAP-1 in a manner that would favour growth suppression (Table 5.1). Many of these proteins are involved in more than one growth regulatory pathway and therefore may inhibit cell proliferation through a number of different mechanisms. For simplicity, each molecule shown in Table 5.1 is only represented under a single biological function.

DNA Repair

Defects in DNA repair pathways can lead to genomic instability and thereby promote carcinogenesis [425]. Data from our microarray study indicate that several DNA repair proteins are upregulated in the presence of wild type MOAP-1. Ataxia telangiectasia mutated (ATM) kinase is over twofold upregulated in

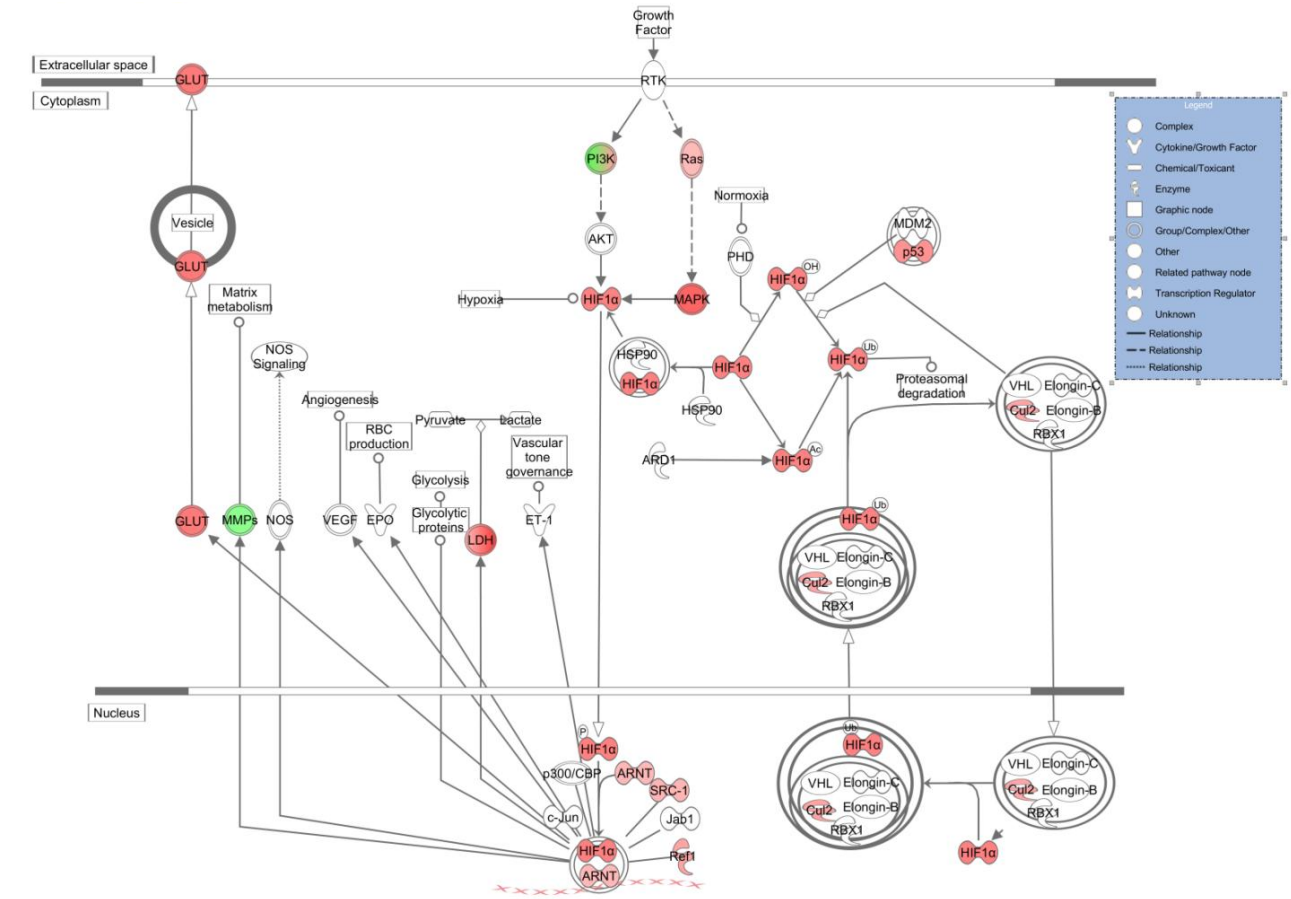


Figure 5.3. HIF1α signaling. Differential gene expression of molecules involved in HIF1α signaling was identified by canonical pathway analysis using IPA (Ingenuity systems). Shown is a graphical representation of this signaling pathway with dataset molecules that meet our threshold cut-offs (fold-change≥2, p≤0.05) represented in red (upregulated) and green (downregulated). Molecules that are coloured in both red and green were detected as being both up-and down-regulated by microarray analysis.

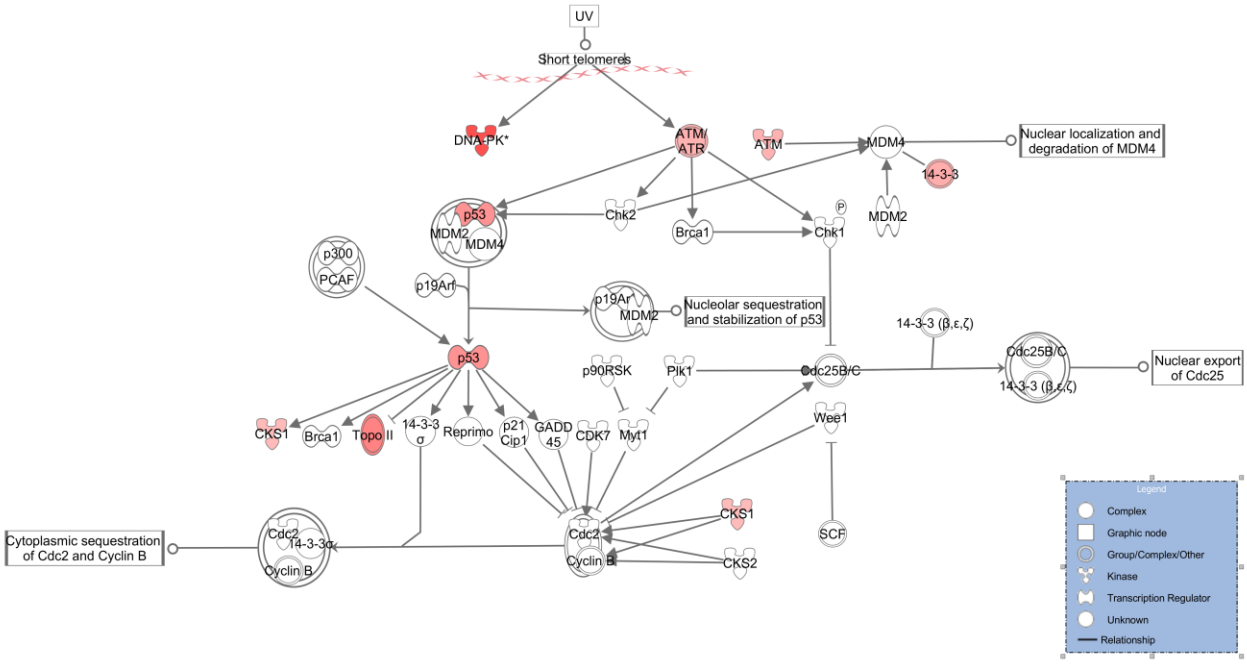


Figure 5.4. G2/M DNA damage checkpoint signaling. Differential gene expression of molecules involved in G2/M DNA damage checkpoint signaling was identified by canonical pathway analysis using IPA (Ingenuity systems). Shown is a graphical representation of this signaling pathway with dataset molecules that meet our threshold cut-offs (fold-change≥2, p≤0.05) represented in red (upregulated) and green (downregulated). Molecules that are coloured in both red and green were detected as being both up-and down-regulated by microarray analysis.

wild type MOAP-1 xenografts relative to vector (Table 5.1) and has well-established roles as a cell cycle checkpoint protein and DNA double strand break repair enzyme [426]. ATM can promote growth arrest and DNA repair through several different pathways involving HIF1α [427], DNA double strand break repair by non-homologous end-joining [428] and p53 signaling [429] as some examples (Table 5.2). Additional DNA repair proteins that were elevated in the presence of wild type MOAP-1 include: the poly(ADP-ribose) polymerases 1 (PARP1) and 3 (PARP3) that play key roles in the detection and repair of DNA strand breaks [402]; excision repair cross-complementing rodent repair deficiency, complementation group 3 (ERCC3); and the nonhomologous end-joining factor 1 (NHEJ1). The increase in multiple DNA repair proteins in the presence of wild type MOAP-1 suggests a possible role for this protein in the maintenance of the cellular genome, which would be necessary to prevent the propagation of DNA mutations and is a well-known mechanism of tumor suppression [327].

Cell Death

Several cell death proteins were also expressed at higher levels with WT MOAP-1 overexpression and are therefore consistent with its proapoptotic function (Table 5.1, 5.2). These proteins include the mitochondrial protein voltage-dependent anion channel 1 (VDAC1), apoptotic peptidase activating factor 1 (APAF1, Fig. 5.2) and the stress activated kinase serine/threonine kinase 4 (STK4/MST1) that functions as a key component in the RASSF regulated Hippo signaling pathway (Tab1e 5.1). While VDAC1 and APAF1 are both

components involved in the mitochondrial-dependent pathway of apoptosis [134], STK4/MST1 is activated by both intrinsic and extrinsic signals of cell death [430, 431]. Interestingly, expression of the gene *GNB2L1* was also upregulated by approximately twofold and encodes a homolog of the beta subunit of G proteins known as the receptor for activated C-kinase 1 (RACK1). RACK1 is involved in the regulation of multiple biological processes including apoptosis where it inhibits the expression of anti-apoptotic proteins Bcl-2 and Bcl-X_L [226] and promotes Bax oligomerization by causing its dissociation from Bcl-X_L [225]. Prior to this microarray study, our lab had identified and also confirmed by co-immunoprecipitation a novel protein association between RACK1 and MOAP-1 (discussed in chapter 6). The finding that RACK1 levels are upregulated with wild type MOAP-1 overexpression in xenograft tumors is of significant interest and raises the possibility that it may be involved in MOAP-1-mediated tumor suppressor function. In contrast, the mitotic cell death molecule microtubule-associated protein 1S (MAP1S) that is also required for RASSF1A-mediated inhibition of APC activity during mitosis [73] was downregulated by over twofold (Table 5.1). Recently, MAP1S and RASSF1A have been implicated in autophagic cell death [76] and, together, these results suggest that either autophagy or its RASSF1A-dependent function may not be important for the growth inhibition induced by MOAP-1. Conversely, this result may also suggest autophagy inhibition as a mechanism of MOAP-1-dependent cell growth regulation.

Gene Expression

The regulation of gene expression is a common function shared by both oncoproteins and tumor suppressors and may have important consequences for determining rates of cell growth and proliferation [432]. Multiple gene expression regulatory molecules were differentially expressed in our wild type MOAP-1 dataset and include various different transcription factors (Table 5.1). The most highly differentially expressed molecule encodes the serum response factor (SRF) that binds to serum response elements (SREs) in the promoter region of target genes and regulates the expression of multiple proteins involved in the cell cycle, apoptosis, and cell proliferation and differentiation [433]. Additionally, SRF also functions as a key regulator of the actin cytoskeleton [434] and is activated downstream of EGF signaling (Table 5.2). Interestingly, proapoptotic RACK1 contains an SRE within its gene promoter [435] and thus it is plausible that upregulation of this protein in the presence of wild type MOAP-1 may be partially attributed to increased levels of SRF (Table 5.1). Other upregulated transcription factors include the transcriptional activator Forkhead box J2 (FOXJ2), transcriptional repressor SMAD family member 2 (SMAD2) and cyclic adenosine monophosphate (cAMP)-responsive activating transcription factors 2 and 3 (ATF2, ATF3).

Given the upregulation of several transcription factors, it is not surprising that a number of translational and chromatin remodelling proteins are also increased with wild type MOAP-1 overexpression. Some examples include the

eukaryotic translation initiation factors 4 gamma 2 (EIF4G2) and 2B delta subunit (EIF2B4); and SWI/SNF family members AT rich interactive domain 1A (ARID1A) and SMARCA2 (SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 2) (Table 5.1, 5.2). These results suggest several mechanisms for wild type MOAP-1-induced changes in gene expression involving localized alterations in chromatin structure, elevated transcription factor-mediated gene expression and increased protein translation. These changes in gene expression regulatory molecules may be important for the regulation of genes encoding proteins that may be involved in MOAP-1 biology, including processes related to its tumor suppressor function.

Cell proliferation and cell-to-cell signaling

Lastly, our results also indicate multiple changes in the expression of genes directly involved in the regulation of cell growth and proliferation and cell-to-cell signaling and interaction (Table 5.1). Of these proteins, the most highly upregulated at over tenfold greater than vector control is the matrix glycoprotein thrombospondin 2 (THBS2), which functions as a potent inhibitor of tumor growth and angiogenesis [436]. Although its molecular mechanism of tumor suppression remains poorly understood, overexpression of an N-terminal THBS2 fragment has been shown to induce apoptosis in a CD36-dependent manner and inhibits vascular endothelial growth factor (VEGF)-induced tube formation [437]. Several other extracellular matrix proteins including alpha 1 type V collagen (COL5A1) and laminin gamma 2 (LAMC2) and alpha 2 (LAMA2) were also

more highly expressed in the presence of wild type MOAP-1 versus vector control (Table 5.1), suggesting that MOAP-1 may be involved in the regulation of cell migration and adhesion. Additionally, it is possible that MOAP-1 may also cooperate with other tumor suppressor proteins to inhibit malignant growth given the upregulation of Wnt signaling inhibitor axin 2 (AXIN2) [438], microtubule associated tumor suppressor 1 (MTUS1) [439] and phosphatase and tensin homolog (PTEN) [440] (Table 5.1). Overall, our microarray results suggest multiple mechanisms of growth suppression that may be influenced by wild type MOAP-1. The results presented in this section suggest several exciting starting points for further investigation and additional post-analyses will undoubtedly be required in order to ascertain the exact mode(s) of tumor suppression mediated by MOAP-1.

5.2.1.3. Venn analysis of WT MOAP-1 and RASSF1A regulated genes

Previous studies have clearly demonstrated the importance of RASSF1A for the ability of MOAP-1 to induce death receptor-dependent apoptosis [84, 85]. Surprisingly, though, our data indicate that MOAP-1 may possess additional biological functions that are independent from its role in extrinsically activated apoptosis (Fig. 4.5, 4.6). Given that RASSF1A is a bona fide tumor suppressor protein with close functional relationship to MOAP-1, we were interested in investigating potential similarities and differences in their mechanisms of growth inhibition. To gain insight into how MOAP-1 may compare with RASSF1A in its biological activities, we decided to examine the gene expression profiles of HCT 116 xenografts from cells that overexpressed either MOAP-1 or RASSF1A.

Remarkably, our results indicate a substantial seventeen-fold increase in the number of MOAP-1 versus RASSF1A-regulated genes (Fig. 5.5). Each of these gene expression changes were at least twofold greater than vector control ($p \leq 0.05$). In the presence of overexpressed MOAP-1, microarray expression analysis detected 1434 differentially expressed genes. In contrast, only 82 gene expression changes were detected at comparable levels due to RASSF1A overexpression. Although the number of RASSF1A-disregulated genes is dramatically lower than that observed upon MOAP-1 over-expression, this finding is in agreement with a previous RASSF1A microarray study in non-small cell lung cancer and neuroblastoma cells that identified sixty-six differentially expressed genes at the same threshold cut-off [441]. Consequently, our results suggest that MOAP-1 may have an enormous influence on cellular biology and signal transduction pathways and perhaps to a greater extent than what is currently known for RASSF1A [364]. However, it is also possible that MOAP-1 and RASSF1A may cooperate in regulating certain cellular processes in addition to extrinsically activated apoptosis given that twenty-six differentially expressed genes are common to both of these proteins. Considering the large number of differentially expressed genes in this dataset comparison, I will only highlight a few key findings of particular interest. It is also worth mentioning that each of the molecules previously presented in Table 5.1 are among the 1408 gene expression changes that are exclusively induced by MOAP-1 overexpression.

As discussed previously, results from our microarray expression analysis suggest a potential p53-dependent mechanism of MOAP-1 tumor suppressor

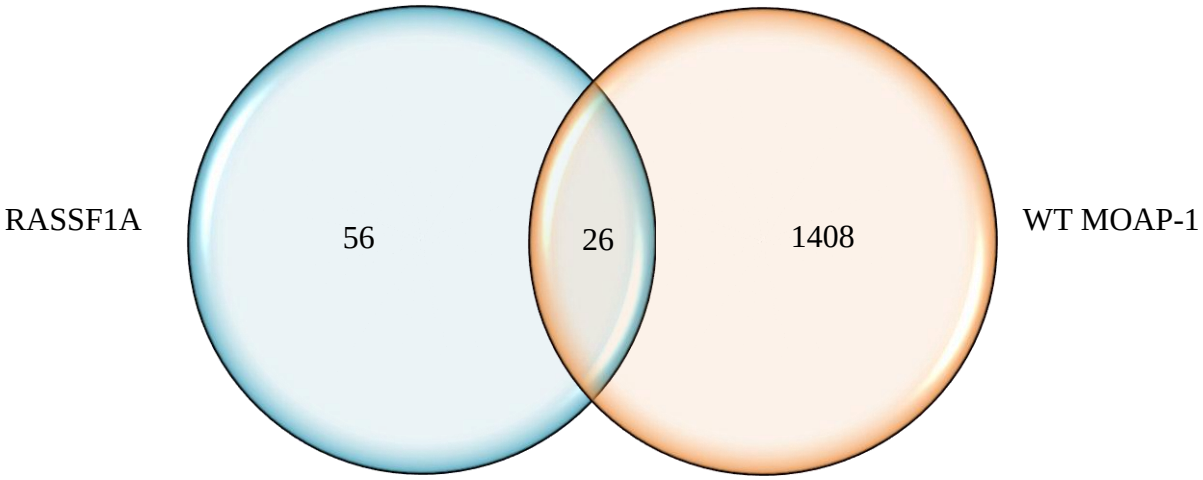


Figure 5.5. Venn diagram of differentially expressed genes by tumor suppressor proteins WT MOAP-1 and RASSF1A relative to vector. Venn analysis was performed for gene expression profiles of HCT 116 xenografts overexpressing RASSF1A or WT MOAP-1. Fold changes relative to vector are greater than or equal to two ($p \leq 0.05$).

function considering the significant upregulation of p53 and several of its associated regulatory molecules and target genes (Table 5.1). Interestingly, upregulation of p53 is exclusively observed in the presence of MOAP-1 and is unaffected by RASSF1A overexpression relative to vector control (Table. 5.3). Elevated expression of the p53-related kinase (TP53RK) and target genes p53-regulated apoptosis-inducing protein 1 (TP53AIP1), ATM and PCNA were also detected only with MOAP-1 overexpression. This suggests that RASSF1A may not be involved in p53 regulation or at least to a lesser degree than MOAP-1. It should be noted, however, that RASSF1A is phosphorylated on ser131 by the p53 target ATM kinase in response to DNA damage where it is involved in the stabilization of p73 [442]. Therefore, it is possible that MOAP-1 may regulate the DNA damage response pathway upstream of RASSF1A at the level of p53. This hypothesis is further supported by the number of DNA repair and p53-inducible genes upregulated by MOAP-1 overexpression (Table 5.1).

A second protein of significant interest to our work, RACK1, is encoded by the *GNB2L1* gene and is also specifically upregulated in MOAP-1 xenografts relative to vector (Table 5.1, 5.3). Although RACK1 is involved in several different biological processes [443], we have recently identified it as a novel MOAP-1 interacting protein and hypothesize that it may be important for MOAP-1 ubiquitination considering its role in PKC regulation [207]. That MOAP-1 ubiquitination is important for its tumor suppressor function also suggests that RACK1 may be involved in its growth inhibition. Preliminary results from our lab suggest that RASSF1A may actually impede the interaction between MOAP-1

Table 5.3. A selection of differentially expressed genes by WT MOAP-1 and RASSF1A relative to Vector

				WT MOAP-1		RASSF1A	
	Gene Symbol	**Description**	**GenBank accession no.**	**p-value**	**Avg. fold change**	**p-value**	**Avg. fold change**
WT MOAP-1	TP53RK	TP53 regulating kinase	NM_033550	1.6E-04	2.8		
	TP53	tumor protein p53	NM_000546	4.3E-03	3.2		
	TP53AIP1	tumor protein p53 regulated apoptosis inducing protein 1	NM_022112	9.3E-04	2.1		
	TP53INP2	tumor protein p53 inducible nuclear protein 2	NM_021202	1.5E-03	3.5		
	ATM	ataxia telangiectasia mutated	NM_000051	2.6E-03	2.2		
	PCNA	proliferating cell nuclear antigen	NM_002592	6.8E-03	2.0		
	EGFR	epidermal growth factor receptor	NM_201283	3.1E-03	-2.2		
	FAS	Fas (TNF receptor superfamily, member 6)	NM_000043	6.3E-04	2.1		
	GNB2L1	guanine nucleotide binding protein (G protein), beta polypeptide 2-like 1	NM_006098	2.3E-02	2.0		
	CDK8	cyclin-dependent kinase 8	NM_001260	1.8E-04	2.5		
	HIF1A	hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)	NM_181054	1.8E-03	3.7		
	PTEN	phosphatase and tensin homolog	NM_000314	2.3E-03	2.2		
RASSF1A	PEG3	paternally expressed 3	NM_006210			3.4E-03	3.5
	RAPGEF5	Rap guanine nucleotide exchange factor 5 (Guanine nucleotide exchange factor for Rap1)	AL833195			4.7E-02	3.3
	MMP3	matrix metallopeptidase 3 (stromelysin 1, progelatinase)	NM_002422			4.9E-02	2.5
	PCDH8	protocadherin 8	NM_002590			9.2E-03	2.4
	HIST1H1T	histone cluster 1, H1t	NM_005323			3.2E-02	2.2
	HIST1H1B	histone cluster 1, H1b	NM_005322			1.5E-02	2.1
	DNALI1	dynein, axonemal, light intermediate chain 1	NM_003462			4.3E-02	2.1
	CABP2	calcium binding protein 2	NM_016366			1.0E-02	2.0
	C12orf48	chromosome 12 open reading frame 48	BC018903			1.5E-02	2.0
	PRKCB	protein kinase C, beta	NM_002738			2.9E-02	-2.1

and RACK1 (Kwek and Baksh, unpublished observations) and, thus, it is not surprising that increased levels of RACK1 protein are only detected with MOAP-1 overexpression (Table 5.3). However, the significance of these results with respect to the tumor suppressor function of MOAP-1 remains to be further investigated. Together, these results suggest two potential mechanisms of specific MOAP-1-mediated tumor suppression - involving p53 and/or RACK1 - that warrant further investigation.

Gene expression profiling of RASSF1A xenografts revealed significantly fewer gene expression changes than MOAP-1, relative to vector. Surprisingly, out of the 56 genes that are exclusively disregulated by this protein, not one molecule is an interacting partner of RASSF1A (Table 5.3). Nonetheless, several of the disregulated genes from this dataset are involved in the regulation of cell growth and proliferation. Interesting examples include: a threefold upregulation of the Rap guanine nucleotide exchange factor 5 (RAPGEF5) that is involved in ras activation [444]; downregulation of the conventional PKCβ enzyme that functions as both a RACK1-interacting protein and a proapoptotic kinase [207, 399]; and upregulation of histone H1 protein histone cluster 1, H1b (HIST1H1B) and H1t (HIST1H1T) that are involved in DNA condensation and gene expression. These gene expression changes propose novel functions for the potential ras effector RASSF1A [89] in the regulation of ras signaling and gene transcription and also introduces new possibilities regarding its potential inhibitory role in RACK1/MOAP-1 biology.

Surprisingly, the majority of the proteins that were differentially expressed by both MOAP-1 and RASSF1A were regulated in the same fashion (Table 5.4). For instance, if a gene was upregulated by MOAP-1 overexpression then it was quite often also increased in the presence of exogenous RASSF1A. This suggests potential overlapping functions between these two tumor suppressor proteins in addition to their cooperative roles in death receptor-dependent cell death. Several proteins involved in immune functions were similarly regulated by MOAP-1 and RASSF1A including interferon epsilon (IFNE), chemokine (C-X-C motif) ligand 11 (CXCL11), CD160 antigen (CD160) and cytokine inducible SH2-containing protein (CISH) (Table 5.4), thereby suggesting roles for these proteins in immune regulation. Expression of the integral membrane protein claudin 11 (CLDN11) was downregulated by almost threefold by both MOAP-1 and RASSF1A overexpressions (Table 5.4). Similarly, decreased expression of the Wnt signaling molecule catenin (cadherin-associated protein), delta 1 (CTNND1) was also detected in the presence of overexpressed MOAP-1 and RASSF1A (Table 5.4). However, one molecule that was oppositely regulated by MOAP-1 and RASSF1A is IQ motif and Sec7 domain 3 (IQSEC3) that serves as both a protein trafficking molecule and a guanine nucleotide exchange factor for ADP-ribosylation factor 1 (ARF1) [445, 446] (Table 5.4). Given that ARF1 is involved in activation of the PI3K pathway and has also been implicated in breast cancer cell migration, proliferation and invasion [447, 448], it is possible that MOAP-1 and RASSF1A may differ in at least one growth regulatory function through their effects on ARF1 expression.

Table 5.4. A selection of genes differentially expressed by both WT MOAP-1 and RASSF1A relative to Vector

			WT MOAP-1		RASSF1A	
Gene Symbol	**Description**	**GenBank accession no.**	**p-value**	**Avg. fold change**	**p-value**	**Avg. fold change**
FAM131B	family with sequence similarity 131, member B	NM_001031690	7.4E-03	4.9	4.1E-02	2.4
IFNE	interferon, epsilon	NM_176891	1.8E-02	3.8	4.3E-02	2.3
CXCL11	chemokine (C-X-C motif) ligand 11	NM_005409	3.2E-02	3.4	9.6E-03	2.3
FAM170B	family with sequence similarity 170, member B	NM_001164484	5.0E-04	2.8	2.0E-03	2.3
FGB	fibrinogen beta chain	NM_005141	1.7E-02	2.6	4.5E-02	2.8
RPS6KA5	ribosomal protein S6 kinase, 90kDa, polypeptide 5	NM_182398	2.0E-02	2.6	1.5E-02	2.1
PRX	periaxin	NM_181882	4.8E-02	2.4	1.8E-02	2.2
PPP4R1L	protein phosphatase 4, regulatory subunit 1-like	NR_003505	3.9E-02	2.1	3.4E-03	2.4
HOXD13	homeobox D13	NM_000523	1.9E-02	2.0	7.0E-03	2.2
OR6X1	olfactory receptor, family 6, subfamily X, member 1	NM_001005188	9.9E-03	2.0	4.1E-02	2.8
LCN6	lipocalin 6	NM_198946	1.7E-02	-2.0	1.3E-03	-2.4
CD160	CD160 molecule	NM_007053	6.3E-03	-2.1	2.6E-02	-2.4
ZNF44	Zinc finger protein 44	XM_002345144	6.9E-03	-2.4	6.7E-03	-2.5
CISH	cytokine inducible SH2-containing protein	NM_145071	8.0E-03	-2.4	4.6E-02	-3.4
BHMT	betaine-homocysteine methyltransferase	NM_001713	1.4E-02	-2.4	3.5E-02	-2.6
CTNND1	catenin	NM_001085461	8.0E-04	-2.6	3.0E-02	-2.2
C19orf75	chromosome 19 open reading frame 75	NM_173635	3.3E-03	-2.6	2.2E-03	-2.9
RNF148	ring finger protein 148	NM_198085	4.7E-02	-2.6	4.2E-02	-2.0
LPXN	leupaxin	NM_004811	7.6E-03	-2.8	2.2E-02	-4.4
C14orf34	chromosome 14 open reading frame 34	NR_026796	1.8E-02	-2.8	3.0E-02	-2.6
CLDN11	claudin 11	NM_005602	3.8E-02	-2.9	1.2E-02	-2.8
IQSEC3	IQ motif and Sec7 domain 3	NM_015232	4.6E-03	-4.0	1.4E-02	5.4
GPR182	G protein-coupled receptor 182	NM_007264	1.7E-03	-4.5	4.6E-02	-2.6
JAKMIP2	janus kinase and microtubule interacting protein 2	NM_014790	7.7E-03	-4.7	4.7E-02	-3.5
WBP2NL	WBP2 N-terminal like	NM_152613	1.0E-03	-4.8	2.8E-02	-2.1
BICC1	bicaudal C homolog 1	NM_001080512	6.7E-03	-5.2	3.7E-02	-2.9

Overall, the results from our wild type MOAP-1 versus vector control dataset support a highly influential role for MOAP-1 in cell biology that may mediate its tumor suppressor function through regulation of several cellular process including: DNA repair, cell-to-cell signaling, cell adhesion, gene expression, or apoptosis. Interestingly, our data also strongly support a p53-dependent mechanism and also implicate the novel MOAP-1 binding protein RACK1 in its own growth suppression through upregulation of this protein.

5.2.2. An investigation of the significance of K264 and K278 in MOAP-1's tumor suppressor function

Over the course of study, we have identified a form of post-translational modification that is absolutely essential to MOAP-1-mediated tumor suppressor function (Fig. 4.7, 4.8, 4.9). In response to PKC activation, MOAP-1 is ubiquitinated in a process involving lysine residues K264 and K278 (Fig. 4.3). Although K278 may serve as the actual site of MOAP-1 ubiquitination, we have discovered that K264 also plays an important role in this modification. Genetic change at K278 in MOAP-1 results in a loss of ubiquitination and incites oncogenic growth. On the other hand, MOAP-1 mutation at K264 leads to delayed ubiquitination and impairs tumor suppressor function (Fig 4.3, 4.7, 4.8, 4.9). These results provide a foundation for further delineating the regulation of MOAP-1's growth regulatory function. However, we are foremost interested in further investigating a more fundamental question regarding the importance and biological significance of MOAP-1 ubiquitination. By evaluating transcriptional

differences and corresponding potential signaling pathways that may be affected between wild type, K264R and K278R MOAP-1, we hope to gain insight into how ubiquitination may be critical for MOAP-1-mediated tumor suppression. Following is a summary and discussion of our HCT 116 xenograft microarray results from cells overexpressing K264R or K278R MOAP-1 with gene expression changes presented relative to wild type MOAP-1.

5.2.2.1. Overview of transcriptional changes caused by K264R and K278R MOAP-1 mutations

In contrast to our wild type MOAP-1 versus vector control comparison (Table 5.1), transcriptional profiling results indicate fewer gene expression differences in the presence of K264R and K278R MOAP-1 mutants relative to wild type protein. In total, our microarray analysis identified 1073 and only 121 differentially expressed results for K264R and K278R MOAP-1, respectively, when compared to wild type MOAP-1. These results are based on p-values less than or equal to 0.05 and a reduced cut-off threshold of a 1.5-fold change in expression in order to accommodate the much smaller K278R MOAP-1 dataset. Data analysis was performed using Ingenuity pathway analysis (IPA, Ingenuity systems) in order to examine our results with reference to biological function and signal transduction pathways. Interestingly, gene expression profiling revealed only few similarities between the changes induced by K264R and K278R MOAP-1.

In comparison to wild type MOAP-1, the most significantly disregulated genes in our K264R MOAP-1 dataset are associated with cell growth and proliferation (149 molecules with p-values 2.53E-08 to 9.95E-03), cellular development (166 molecules with p-values 4.10E-07 to 1.05E-02), cell movement (101 molecules with p-values 1.19E-05 to 1.03E-02), cell-to-cell signaling and interaction (86 molecules with p-values 2.76E-05 to 1.08E-02) and cell death (166 molecules with p-values 3.20E-05 to 9.68E-03). These top five biological functions that best describe the genetic alterations induced by K264R MOAP-1 suggest several possibilities in how this lysine mutation may influence and promote tumorigenesis. In contrast, the IPA results that we retrieved for our K278R MOAP-1 dataset were less revealing into its potential mechanisms of oncogenicity. Relative to wild type MOAP-1, the biological functions that are most strongly associated with the disregulated genes induced by K278R MOAP-1 involve cellular function and maintenance (5 molecules with p-values 5.48E-05 to 4.20E-05), cell morphology (4 molecules with p-values 4.43E-04 to 3.51E-02), cell growth and proliferation (16 molecules with p-values 1.89E-03 to 4.88E-02), amino acid metabolism (2 molecules with p-values 3.56E-03 to 1.42E-02) and drug metabolism (3 molecules with p-values 3.56E-03 to 2.82E-02). Therefore, it appears that the majority of the most highly significant K278R MOAP-1-induced gene expression changes are not involved in what are typically considered cancer-associated biological processes, with the exception of the changes in genes for cell growth and proliferation.

As previously discussed the results from our wild type MOAP-1 versus vector control comparison reveal a significant upregulation of p53 expression and suggest a plausible p53-dependent mechanism of MOAP-1 tumor suppressor function (Table 5.1, Fig. 5.1). In the presence of overexpressed K264R or K278R MOAP-1, however, we did not observe any alterations in p53 expression when compared to wild type MOAP-1. This finding does not negate the potential role for p53 in MOAP-1-mediated growth inhibition but, rather, suggests that the mechanism of p53 upregulation does not require PKC-dependent MOAP-1 ubiquitination. However, whether or not the activation of p53-dependent signaling pathways is actually similar in the presence of either wild type or lysine mutants of MOAP-1 remains to further investigated. A similar theory also applies to the novel MOAP-1-interacting protein RACK1 whose levels are upregulated by approximately twofold in the presence of overexpressed wild type MOAP-1 and whose expression remains elevated even with K264R and K278R mutations. Together, our microarray analysis of K264R and K278R MOAP-1 xenografts indicates multiple changes in genes with relevance to tumorigenesis and supports the understanding that MOAP-1 ubiquitination involving K264 and K278 is important for its tumor suppressor function.

5.2.2.2. Summary of results from microarray expression analysis of K278R MOAP-1 xenografts

Surprisingly, gene expression profiling of wild type and K278R MOAP-1 xenografts revealed relatively few gene expression differences between the

overexpression of these two constructs. This was unexpected considering the oncogenic growth and striking loss of MOAP-1 ubiquitination induced by mutation at K278 in MOAP-1 (Fig. 4.3, 4.7, 4.8, 4.9). Although we were initially anticipating a larger number of transcriptional changes for genes encoding growth regulatory proteins, it is important to keep in mind that significant biological changes may still take place at the molecular level without any necessary alterations in gene expression. This possibility may help to explain the results that we observe with K278R MOAP-1. Upon closer examination of the differentially expressed genes in this dataset, however, we did discover several interesting findings with established cancer associations. For instance, the beta isoform of protein phosphatase 2, regulatory subunit A (PPP2R1B) is a tumor suppressor protein [449] that is downregulated by almost threefold in the presence of K278R MOAP-1 relative to wild type protein (Table 5.5). PPP2R1B is implicated in the regulation of cell growth and proliferation and is commonly altered in multiple human malignancies including colorectal, lung, breast and parathyroid cancers [450-453]. Additionally, cAMP-specific phosphodiesterase 4D (PDE4D) is present at elevated levels with K278R MOAP-1 overexpression and has also been shown to be upregulated in multiple prostate cancer cell lines where it promotes cell growth and proliferation [454] (Table 5.5). In melanoma, PDE4D inhibits cAMP signaling and consequently allows c-RAF-dependent mitogen activated protein kinase (MAPK) signaling during melanocyte transformation [455]. It is possible that these cancer-associated changes may also be affected by mutation at K278 in MOAP-1

Table 5.5. Genes differentially expressed by K278R MOAP-1 relative to WT MOAP-1[a]

Biological association[b]	GenBank accession no.	Probe Name	Gene Symbol	Description	Avg.[c] fold change	p-value
Cell growth and proliferation	BX648604	A_33_P3240552	PDE4D	phosphodiesterase 4D, cAMP-specific	2.0	4.1E-02
	NM_000185	A_33_P3241511	SERPIND1	serpin peptidase inhibitor, clade D (heparin cofactor), member 1	1.6	4.0E-02
	NM_007053	A_23_P354341	CD160	CD160 molecule	1.6	3.6E-03
	NM_002089	A_23_P315364	CXCL2	chemokine (C-X-C motif) ligand 2	-1.5	3.8E-02
	NM_032415	A_23_P82324	CARD11	caspase recruitment domain family, member 11	-1.6	2.9E-02
	NM_020070	A_24_P239076	IGLL1	immunoglobulin lambda-like polypeptide 1	-1.9	2.4E-02
	NM_145259	A_33_P3327404	ACVR1C	activin A receptor, type IC	-2.1	3.9E-02
	NM_002716	A_24_P98762	PPP2R1B	protein phosphatase 2 (formerly 2A), regulatory subunit A, beta isoform	-2.9	2.0E-02
Cell cycle	NM_001553	A_23_P353035	IGFBP7	insulin-like growth factor binding protein 7	1.7	2.4E-02
	NM_176891	A_23_P302060	IFNE	interferon, epsilon	-2.7	1.5E-02
Cell-to-cell signaling and interaction	NM_000371	A_23_P130333	TTR	transthyretin	1.7	1.7E-02
	NM_000501	A_24_P186943	ELN	elastin	-1.5	2.1E-02
	NM_006847	A_33_P3267799	LILRB4	leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 4	-1.9	3.3E-02
	NM_176875	A_23_P162010	CCKBR	cholecystokinin B receptor	-2.9	3.3E-02
	NM_001944	A_33_P3348747	DSG3	desmoglein 3 (pemphigus vulgaris antigen)	-5.1	2.3E-02
Gene expression	NM_017508	A_33_P3233916	SOX6	SRY (sex determining region Y)-box 6	4.3	1.2E-02
	NM_000209	A_23_P65189	PDX1	pancreatic and duodenal homeobox 1	-2.9	4.7E-02
DNA replication, recombination, and repair	NM_001002006	A_32_P421816	NT5C1B	5'-nucleotidase, cytosolic IB	2.2	2.7E-02

[a]Genes differentially expressed in K278R MOAP-1 relative to vector control HCT 116 xenografts.
[b]Biological associations were assigned by functional analysis in IPA. Each molecule is represented by only one biological association in cases where more than one may be applicable.
[c]Average (Avg.) fold change. Negative values denote downregulation, positive values indicate upregulation.

leading to deregulation of protein phosphatase activity or impairment of cAMP signaling during neoplasia.

It is worth mentioning that several differentially expressed genes in our K278R versus WT MOAP-1 dataset are also known to be involved in hematopoietic cancers. The CD160 cell surface glycoprotein is upregulated in the presence of K278R MOAP-1 and is expressed by a subset or natural killer (NK) and T lymphocyte cells [456] (Table 5.5). Although normal B cells do not typically display the CD160 antigen, this protein is aberrantly expressed during chronic lymphocytic leukemia (CLL) and promotes cell survival through phosphoinositide-3 kinase (PI3K) signaling [457]. More recently, CD160 expression has also been documented in a wider range of B cell malignancies and is now considered to be a tumor-specific antigen with novel therapeutic potential [458]. A second protein that was upregulated by K278R MOAP-1 overexpression is the insulin-like growth factor binding protein 7 (IGFBP7) that stimulates prostacyclin production and promotes cellular adhesion [459, 460] (Table 5.5). Elevated expression of IGFBP7 in T-cell acute lymphoblastic leukemia results from DNA hypomethylation insulin-like growth factor binding protein 7 [461] and is correlated with a high-risk subtype, treatment failure, and decreased patient survival [462]. Given that the gene expression changes in this dataset were discovered in HCT 116 colon carcinoma cells, it will be interesting to examine if the same findings still hold true with overexpression of K278R MOAP-1 in leukemic cells. Of particular relevance is the finding that - in contrast to leukemia - IGFBP7 is epigenetically silenced specifically in colorectal cancers and is also a p53-

responsive gene [463]. This finding is interesting for several reasons. Firstly, loss of IGFBP7 expression due to DNA hypermethylation is commonly observed in colon cancer cell lines [464] yet we are able to observe upregulation of this protein by K278R MOAP-1. This suggests that K278R MOAP-1 may be involved in the epigenetic regulation of certain genes or may promote the activity of demethylating enzymes. Secondly, re-expression of IGFBP7 in colon cancer cells has been shown to inhibit their growth and induce cell cycle arrest and apoptosis [465]; however, increased levels of this protein in the presence of K278R MOAP-1 is associated with oncogenic growth. Lastly, p53 expression levels are comparable between wild type and K278R MOAP-1 but expression of the p53 target gene *IGFBP7* is elevated with the latter protein. Together, these findings suggest that upregulation of IGFBP7 by K278R MOAP-1 in HCT 116 cells may contribute to tumorigenic growth similar to its role in leukemia and, furthermore, supports the likelihood that certain gene expression changes in our dataset may also be extended to other cancer types.

Given the sparse number of K278R MOAP-1-induced gene expression changes associated with growth regulatory functions, it is not surprising that there were also relatively few canonical pathways suggested by IPA as being potentially disregulated (Table 5.6). The vast majority of the suggested signaling deregulations were also not statistically significant due to the small number of differentially expressed molecules involved. However, one particular pathway that is significantly associated with the changes in this dataset involves Wnt/β-catenin signal transduction. The Wnt/β-catenin signaling pathway is highly

Table 5.6. Potential canonical pathways disregulated by K278R MOAP-1 relative to WT MOAP-1[a]

Ingenuity Canonical Pathways[b]	-log(p-value)[c]	Ratio[d]	Molecules[e]
Wnt/β-catenin Signaling	1.7	1.7E-02	SOX6, PPP2R1B, ACVR1C
G-Protein Coupled Receptor Signaling	9.7E-01	7.6E-03	CMKLR1, GPR101, PDE4D, CCKBR
Cell Cycle Regulation by BTG Family Proteins	9.3E-01	2.8E-02	PPP2R1B
Glutamate Metabolism	9.0E-01	1.3E-02	LGSN
Alanine and Aspartate Metabolism	8.4E-01	1.1E-02	AGXT2
Glycine, Serine and Threonine Metabolism	6.3E-01	6.7E-03	AGXT2
Ceramide Signaling	6.1E-01	1.2E-02	PPP2R1B
Cyclins and Cell Cycle Regulation	6.1E-01	1.1E-02	PPP2R1B
TGF-β Signaling	5.9E-01	1.1E-02	ACVR1C
CDK5 Signaling	5.7E-01	1.1E-02	PPP2R1B
FAK Signaling	5.7E-01	9.8E-03	CAPN11
Apoptosis Signaling	5.7E-01	1.1E-02	CAPN11
IGF-1 Signaling	5.3E-01	9.4E-03	IGFBP7
Glycolysis/Gluconeogenesis	5.1E-01	6.9E-03	ADH6
PI3K/AKT Signaling	4.6E-01	7.2E-03	PPP2R1B
Fatty Acid Metabolism	4.4E-01	5.3E-03	ADH6
AMPK Signaling	4.3E-01	6.1E-03	PPP2R1B
mTOR Signaling	4.0E-01	6.2E-03	PPP2R1B
Pyrimidine Metabolism	3.7E-01	4.3E-03	NT5C1B
NF-κB Signaling	3.5E-01	5.7E-03	CARD11
ILK Signaling	3.2E-01	5.2E-03	PPP2R1B
Integrin Signaling	2.9E-01	4.8E-03	CAPN11
cAMP-mediated signaling	2.8E-01	4.6E-03	PDE4D
Actin Cytoskeleton Signaling	2.7E-01	4.2E-03	SLC9A1

[a,b]Ingenuity canonical signaling pathways that were most significant to the K278R MOAP-1 versus vector control HCT 116 xenografts dataset were assigned from the IPA library of canonical pathways.
[c] -log(p-value) $\geq$1.3 is equivalent to p-value $\leq$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.

involved in regulating cell proliferation, cell polarity and cell fate determination and has important implications in cancer development [466]. Mutations in its pathway components are frequently observed during malignancy and typically result in enhanced β-catenin-dependent transcription of target genes. Our results indicate decreased expression of the TGFβ receptor family member activin A receptor, type 1C (ACVR1C), and upregulation of the β-catenin binding protein and transcriptional regulator sex determining region Y-box 6 (SOX6) (Table 5.4, 4.5). Given that upregulation of SOX6 is approximately twofold greater than the downregulation of its upstream receptors, it is possible that heightened activity of the Wnt/β-catenin signaling pathway may possibly contribute to the oncogenicity of K278R MOAP-1. However, it is also likely that multiple other signaling deregulations take place in the presence of K278R MOAP-1 and contribute to its pro-proliferative effects but which may not be detectable through our analysis of gene expressions changes.

5.2.2.3. Summary of results from microarray expression analysis of K264R MOAP-1 xenografts

It is interesting to note that the effects of K264 mutation in MOAP-1 are not as detrimental as genetic change at K278 with respect to either MOAP-1 ubiquitination or loss of its tumor suppressor function. However, gene expression profiling of K264R MOAP-1 xenografts revealed nearly nine times greater the number of gene expression changes than K278R MOAP-1, relative to wild type MOAP-1. This suggests that although K264 does not serve as the site of MOAP-1

ubiquitination, it likely possesses an important regulatory function, which may explain how ectopic expression of K264R MOAP-1 results in delayed ubiquitination and also a loss of growth inhibition (Fig. 4.3, 4.7). Although K264 and K278 are both important for the PKC-dependent ubiquitination and tumor suppressor function of MOAP-1, discrepancies in both the type and number of gene expression changes between K264R and K278R MOAP-1 xenografts suggest that these two lysine residues may serve different purposes.

Cell growth and proliferation

Overexpression of K264R MOAP-1 in HCT 116 cells resulted in alterations in the levels of multiple growth regulatory proteins relative to wild type MOAP-1. Expression of the *KRAS* oncogene was upregulated in the presence of K264R MOAP-1 and has well established roles in cell growth, differentiation and survival [467] (Table 5.7). Deregulation of Ras signaling is frequently observed in human cancers and may result from activating mutations, overexpression or enhanced upstream activation. Additionally, increased levels of the signal transducer and transcriptional regulator Smad1 was also detected with K264R MOAP-1 overexpression (Table 5.7). Together, K-Ras and Smad1 are both involved in transforming growth factor-beta (TGFβ) signaling that regulates several cellular processes including cell growth, differentiation and apoptosis [468-470] (Table 5.7). Although the receptors bone morphogenetic protein receptor, type IB (BMPR1B) and activin A receptor, type IC (ACVR1C) were both downregulated in K264R MOAP-1 xenografts, downstream runt-related

Table 5.7. Genes differentially expressed by K264R MOAP-1 relative to WT MOAP-1[a]

Biological association[b]	GenBank accession no.	Probe Name	Gene Symbol	Description	Avg.[c] fold change	p-value
Cell Growth and Proliferation	NM_006080	A_24_P192301	SEMA3A	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3A	2.1	3.8E-02
	NM_001013442	A_24_P205994	EPGN	epithelial mitogen homolog (mouse)	1.9	3.4E-02
	NM_033360	A_23_P306507	KRAS	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	1.7	1.5E-02
	NM_005470	A_33_P3622802	ABI1	abl-interactor 1	1.7	2.5E-02
	NM_012238	A_23_P98022	SIRT1	sirtuin (silent mating type information regulation 2 homolog) 1 (S. cerevisiae)	1.6	2.0E-02
	NM_005900	A_24_P71938	SMAD1	SMAD family member 1	1.6	2.4E-02
	NM_012484	A_23_P70007	HMMR	hyaluronan-mediated motility receptor (RHAMM)	1.6	5.1E-03
	NM_001432	A_23_P41344	EREG	epiregulin	1.6	1.5E-02
	NM_005843	A_24_P62860	STAM2	signal transducing adaptor molecule (SH3 domain and ITAM motif) 2	1.5	2.8E-02
	NM_003012	A_23_P10121	SFRP1	secreted frizzled-related protein 1	-1.5	2.0E-03
	NM_001085377	A_33_P3288246	MCC	mutated in colorectal cancers	-1.5	6.4E-03
	NM_144497	A_23_P214897	AKAP12	A kinase (PRKA) anchor protein 12	-1.8	4.9E-02
	NR_024549	A_33_P3381943	DMTF1	cyclin D binding myb-like transcription factor 1	-1.9	4.8E-02
	NM_003013	A_23_P81103	SFRP2	secreted frizzled-related protein 2	-3.0	3.0E-02
DNA replication, recombination, and repair	NM_002485	A_24_P278126	NBN	nibrin	1.6	5.5E-03
	NM_006265	A_23_P20463	RAD21	RAD21 homolog (S. pombe)	1.5	1.5E-02
Cell-To-Cell Signaling and Interaction	NM_021033	A_24_P81965	RAP2A	RAP2A, member of RAS oncogene family	2.4	1.1E-02
	NM_001663	A_24_P472455	ARF6	ADP-ribosylation factor 6	2.0	1.6E-02
	NM_000210	A_23_P210176	ITGA6	integrin, alpha 6	1.8	5.4E-03
	NM_015520	A_33_P3369530	MAGI1	membrane associated guanylate kinase, WW and PDZ domain containing 1	1.8	1.5E-02
	NM_012120	A_32_P155811	CD2AP	CD2-associated protein	1.7	1.6E-02
	NM_000212	A_24_P318656	ITGB3	integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)	1.7	2.3E-03
	NM_002740	A_33_P3294578	PRKCI	protein kinase C, iota	1.6	4.1E-02
	NM_003816	A_33_P3340769	ADAM9	ADAM metallopeptidase domain 9 (meltrin gamma)	1.6	2.0E-02
	NM_001076682	A_23_P1740	NCAM1	neural cell adhesion molecule 1	-1.6	1.9E-03
	NM_001166109	A_33_P3423979	PALLD	palladin, cytoskeletal associated protein	-1.7	8.4E-03
	NM_001400	A_23_P404481	S1PR1	sphingosine-1-phosphate receptor 1	-2.6	2.0E-02

Table 5.7 *continued*. Genes differentially expressed by K264R MOAP-1 relative to WT MOAP-1[a]

Biological association[b]	GenBank accession no.	Probe Name	Gene Symbol	Description	Avg.[c] fold change	p-value
Cell-To-Cell Signaling and Interaction	NM_000165	A_24_P55295	GJA1	gap junction protein, alpha 1, 43kDa	-3.4	4.1E-02
	NM_001944	A_33_P3348747	DSG3	desmoglein 3 (pemphigus vulgaris antigen)	-5.4	3.8E-02
Cell Death	NM_033012	A_33_P3503029	TNFSF11	tumor necrosis factor (ligand) superfamily, member 11	1.7	5.5E-03
	NM_052932	A_24_P309415	TMEM123	transmembrane protein 123	1.7	2.2E-02
	NM_006281	A_33_P3260669	STK3	serine/threonine kinase 3 (STE20 homolog, yeast)	1.6	8.4E-03
	NM_018947	A_24_P376556	CYCS	cytochrome c, somatic, nuclear gene encoding mitochondrial protein	1.6	3.1E-03
	NM_016542	A_23_P32414	MST4	serine/threonine protein kinase MST4	1.6	1.8E-03
	NM_004330	A_33_P3227467	BNIP2	BCL2/adenovirus E1B 19kDa interacting protein 2	1.5	1.8E-02
	NM_032974	A_33_P3255075	CASP10	caspase 10, apoptosis-related cysteine peptidase	1.5	3.1E-02
	NM_022161	A_23_P79769	BIRC7	baculoviral IAP repeat-containing 7	-1.5	1.7E-02
	NM_001030311	A_33_P3355538	CERKL	ceramide kinase-like	-1.5	1.7E-02
	NM_001001786	A_24_P50368	BLID	BH3-like motif containing, cell death inducer, nuclear gene encoding mitochondrial protein	-1.6	4.4E-03
Cell Cycle	NM_004060	A_23_P58606	CCNG1	cyclin G1	1.9	2.1E-02
	NM_004336	A_23_P124417	BUB1	budding uninhibited by benzimidazoles 1 homolog (yeast)	1.5	5.2E-03
Gene Expression	NM_004348	A_32_P161762	RUNX2	runt-related transcription factor 2	1.6	3.8E-02
	NM_006713	A_32_P170444	SUB1	SUB1 homolog (S. cerevisiae)	1.5	1.4E-02
	NM_032682	A_23_P155257	FOXP1	forkhead box P1	-1.9	1.9E-02
	NM_000209	A_23_P65189	PDX1	pancreatic and duodenal homeobox 1	-3.6	3.4E-02

[a]Genes differentially expressed in K264R MOAP-1 relative to vector control HCT 116 xenografts.
[b]Biological associations were assigned by functional analysis in IPA. Each molecule is represented by only one biological association in cases where more than one may be applicable.
[c]Average (Avg.) fold change. Negative values denote downregulation, positive values indicate upregulation.

transcription factor 2 (RUNX2) was expressed at elevated levels (Table 5.7). Considering that altered TGFβ signaling is commonly associated with cancer development [471], it is possible that the increased expression of its downstream components K-Ras, Smad1 and Runx2 may lead to increased transcription of growth promoting genes (Table 5.8, Fig. 5.6). Therefore, it is possible that increased TGFβ signaling may contribute to K264R MOAP-1 oncogenicity. Notwithstanding, upregulation of K-Ras may also affect several other signaling pathways [472], including that of cdk5. Cdk5 is implicated in cancer progression and cell migration, invasion and proliferation [473, 474]. In addition to K-Ras, several other proteins involved in cdk5 signaling are also differentially expressed in the presence of K264R MOAP-1 and consequently may also contribute to deregulated cell growth (Table 5.8, Fig. 5.7).

Potential impacts on p53-mediated tumor suppression

Expression of the NAD^+-dependent deacetylase sirtuin 1 (SIRT1) was also present at elevated levels with K264R MOAP-1 overexpression (Table 5.7). SIRT1 functions as an antagonist of p53-mediated tumor suppression by deacetylating p53 at K382 and reducing its transcriptional activity [475]. SIRT1 has also been shown to impair the ability of p53 to induce cellular senescence [476] and DNA damage-induced apoptosis [477]. Considering that p53 expression is significantly upregulated in the presence of wild type MOAP-1 relative to vector control (Table 5.1), it is possible p53 signaling may be undermined by the loss or alteration in MOAP-1 ubiquitination caused by K264R and K278R MOAP-1

Table 5.8. Potential canonical pathways disregulated by K264R MOAP-1 relative to WT MOAP-1[a]

Ingenuity Canonical Pathways[b]	-log (p-value)[c]	Ratio[d]	Molecules[e]
TGF-β Signaling	1.39	7.9E-02	BMPR1B, RUNX2, KRAS, SMAD1, ACVR1C, INHBB, INHBA
CDK5 Signaling	1.30	7.5E-02	ADCY2, LAMA1, ITGA6, PPP1R12A, PPP1CB, KRAS, PPP2R1B
Wnt/β-catenin Signaling	1.22	6.4E-02	GJA1, TCF4, SFRP2, SOX1, WNT7B, AKT3, SFRP1, PPP2R1B, WNT8B, ACVR1C, LRP1
Myc Mediated Apoptosis Signaling	1.12	8.2E-02	IGF1, PIK3R5, AKT3, CYCS, KRAS
DNA Double-Strand Break Repair by Homologous Recombination	1.00	1.2E-01	ATRX, NBN
ERK/MAPK Signaling	1.00	5.4E-02	PRKCI, PLA2G2F, DUSP6, PIK3R5, PPP1R12A, PPP1CB, TLN1, KRAS, PPP2R1B, EIF4E, KSR1
mTOR Signaling	9.9E-01	5.6E-02	PRKCI, IRS1, PIK3R5, AKT3, VEGFC, KRAS, HIF1A, PPP2R1B, EIF4E
Lymphotoxin β Receptor Signaling	8.1E-01	6.6E-02	PIK3R5, AKT3, CYCS, IKBKE
IGF-1 Signaling	7.4E-01	5.6E-02	PRKCI, IGF1, IRS1, PIK3R5, AKT3, KRAS
EIF2 Signaling	6.5E-01	5.0E-02	PIK3R5, AKT3, PPP1CB, KRAS, EIF4E
PTEN Signaling	6.4E-01	4.9E-02	BMPR1B, MAGI1, PIK3R5, AKT3, IKBKE, KRAS
VEGF Signaling	6.1E-01	5.1E-02	PIK3R5, AKT3, VEGFC, KRAS, HIF1A
RhoA Signaling	6.0E-01	5.3E-02	IGF1, RDX, PPP1R12A, RAPGEF6, ANLN, GNA13
Growth Hormone Signaling	5.7E-01	5.3E-02	PRKCI, IGF1, IRS1, PIK3R5
Protein Kinase A Signaling	4.1E-01	4.0E-02	AKAP12, ADCY2, TCF4, CALM1 (includes others), PDE12, PPP1CB, PDE4D, PRKCI, GLI3, RHO, PPP1R12A, PDE8B, GNA13
JAK/Stat Signaling	3.8E-01	4.7E-02	PIK3R5, AKT3, KRAS
ERK5 Signaling	3.7E-01	4.7E-02	KRAS, MEF2C, GNA13
NF-κB Signaling	3.3E-01	4.0E-02	TLR4, BMPR1B, TNFSF11, PIK3R5, AKT3, KRAS, CARD11
PI3K/AKT Signaling	3.2E-01	3.6E-02	AKT3, IKBKE, KRAS, PPP2R1B, EIF4E
SAPK/JNK Signaling	3.1E-01	3.9E-02	IRS1, PIK3R5, KRAS, GNA13

[a,b]Ingenuity canonical signaling pathways that were most significant to the K264R MOAP-1 versus vector control HCT 116 xenografts dataset were assigned from the IPA library of canonical pathways.
[c]-log(p-value) $\geq$1.3 is equivalent to p-value $\leq$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.

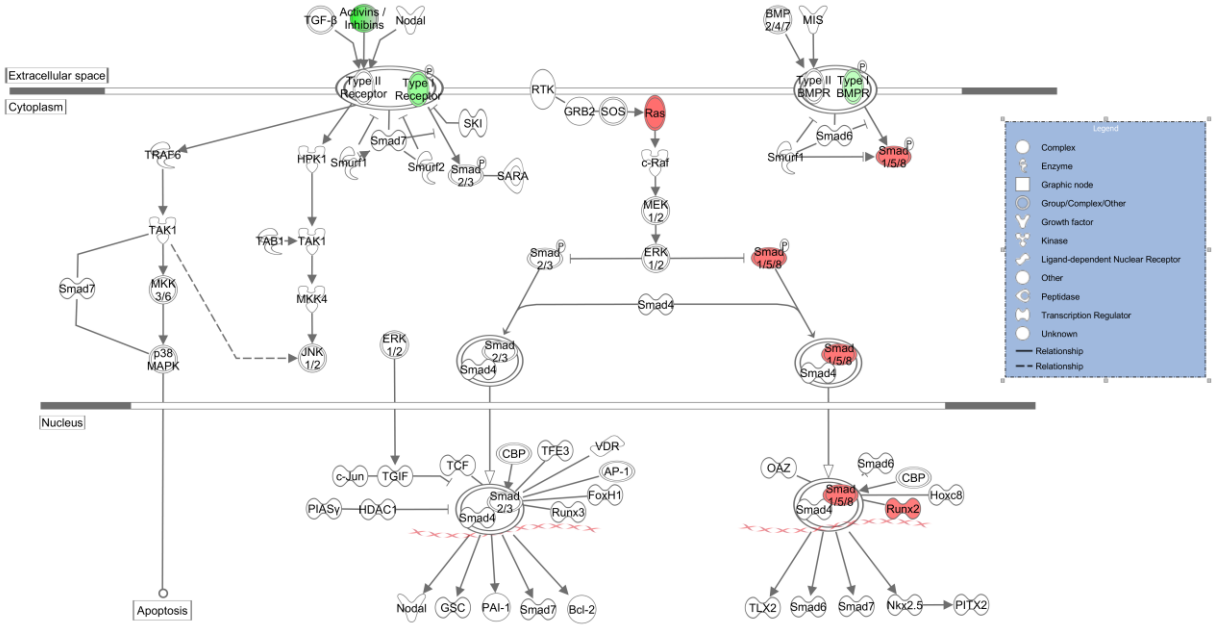


Figure 5.6. TGFβ signaling. Differential gene expression of molecules involved in TGFβ signaling was identified by canonical pathway analysis using IPA (Ingenuity systems). Shown is a graphical representation of this signaling pathway with dataset molecules that meet our threshold cut-offs (fold-change≥2, p≤0.05) represented in red (upregulated) and green (downregulated).

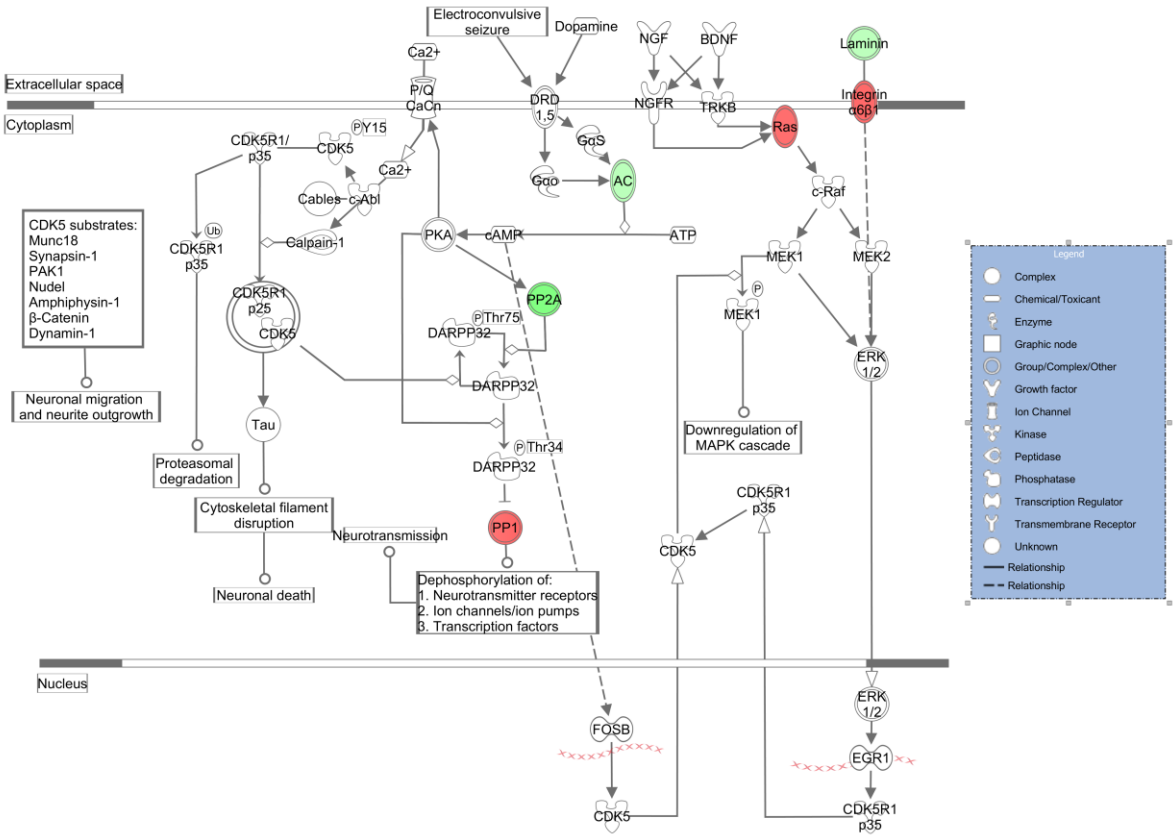


Figure 5.7. Cdk5 signaling. Differential gene expression of molecules involved in cdk5 signaling was identified by canonical pathway analysis using IPA (Ingenuity systems). Shown is a graphical representation of this signaling pathway with dataset molecules that meet our threshold cut-offs (fold-change≥2, p≤0.05) represented in red (upregulated) and green (downregulated).

mutations. In the case of K264R MOAP-1, increased SIRT1 expression may contribute to the potential loss of p53-mediated tumor suppression. Interestingly, SIRT1 activity is also modulated by the tumor suppressor protein hypermethylated in cancer 1 (Hic1), whose loss of expression in cancer promotes tumorigenesis through activation of SIRT1 expression and leads to the attenuation of p53 function [477]. Therefore, K264 in MOAP-1 may also be important for inhibiting SIRT1 expression. In further support of potential p53 deregulation due to K264R MOAP-1, the transcription factor cyclin D binding myb-like protein 1 (DMTF1) was also downregulated in this dataset (Table 5.7). DMTF1 expression is normally activated in response to oncogenic Ras signaling and functions as a tumor suppressor protein by initiating cell cycle arrest through the ARF/p53-dependent pathway [478]. Thus, K264 in MOAP-1 may influence p53 activation through a number of different possible ways that may be important for MOAP-1's tumor suppressor function.

Influences on PKC-dependent and Wnt signaling pathways

In addition to the changes in TGFβ and p53-associated proteins, we also observed differences in the expression of several other growth regulatory molecules. These changes include downregulation of A kinase anchor protein 12 (AKAP12) and Wnt signaling inhibitors secreted frizzled-related proteins 1 (SFRP1) and 2 (SFRP2) (Table 5.7). AKAP12 possesses tumor suppressor properties and is epigenetically silenced in several humans cancers [479-481]. As a scaffolding protein, AKAP12 expression can be induced by phorbol ester

stimulation and is involved in coordinating the subcellular compartmentalization of both PKA and PKC [482]. Given that MOAP-1 ubiquitination is dependent on PKC activation (Fig 4.3), the downregulation of AKAP12 may impair its post-translational modification in the presence of K264 mutation. It is possible that residue K264 may be important for facilitating MOAP-1 ubiquitination at K278 by influencing PKC localization through AKAP12, which may be important for activation of the MOAP-1 E3 ligase. Interestingly, increased expression of the atypical protein kinase C, iota (PRKCI) was also detected in this dataset but is not likely to be involved in MOAP-1 ubiquitination given its insensitivity to phorbol ester stimulation [483] (Table 5.7). On the other hand, decreased expression of SFRP1/2 may result in oncogenic activation of the Wnt signaling pathway that is associated with cell transformation and invasion [484]. Interestingly, gene expression changes in Wnt/β-catenin signaling molecules were also detected with K278R MOAP-1 overexpression (Table 5.5) and were also more significantly associated with the Wnt signaling pathway than the gene expression changes detected in our K264R MOAP-1 dataset (Table 5.6). This suggests that K278 may be more highly involved in the regulation of the Wnt/β-catenin signaling pathway and that its mutation may have greater effects on Wnt-mediated cell growth than K264 in MOAP-1. However, the mechanism through which MOAP-1 may be involved in this process remains to be further investigated.

Cell death

Surprisingly, our microarray expression analysis detected transcriptional changes in several cell death proteins even though we did not observe any differences in the abilities of wild type and K264R MOAP-1 to induce TNFR1-dependent apoptosis (Fig. 4.5, 4.6). These gene expression changes include upregulation of the transmembrane protein 123 (TMEM123) that is involved in oncotic cell death [485] and increased expression of the serine/threonine kinase MST4 that is implicated in cell transformation [486] and prostate cancer progression [487] (Table 5.7). Unexpectedly, increased levels of cytochrome C (CYCS) and caspase 10 (CASP10) were also upregulated with K264R MOAP-1 overexpression. A possible explanation for these results is that increased levels of cytochrome c and caspase 10 may simply accumulate in non-apoptotic cells, or conversely, that elevated levels of these proteins in the presence of K264R MOAP-1 may induce higher rates of mitochondrial-dependent cell death but are insufficient for suppressing tumorigenic growth. Therefore, it may be important to compare the abilities of wild type, K264R and K278R MOAP-1 to induce intrinsically activated cell death as part of a future investigation. Additionally, increased expression of TMEM123 may facilitate necrotic oncosis, which is typically associated with an inflammatory response; in turn, this may further promote tumor growth in the presence of K264R MOAP-1 [488, 489]. Overall, gene expression profiling of K264R MOAP-1 xenografts revealed a large number of gene expression changes relative to wild type protein. In comparison to K278R MOAP-1, these results provide greater mechanistic insights into the potential

importance of ubiquitination for MOAP-1's tumor suppressor function and support an important regulatory function for K264.

5.2.2.4. Venn analysis of differentially expressed genes between K264R and K278R MOAP-1 relative to WT MOAP-1

Thus far, we have identified K278 as the site of PKC-dependent MOAP-1 ubiquitination and have discovered that this modification is important for MOAP-1's tumor suppressor function (Fig. 4.3, 4.7). However, we are currently uncertain about the significance of K264 in MOAP-1 and its relation to ubiquitination at K278. Although K264 is not essential for MOAP-1 ubiquitination, mutation at this site results in altered kinetics of post-translational modification and a loss of tumor suppressor function (Fig. 4.3, 4.7). This suggests that K264 may play an important regulatory role during both ubiquitination and MOAP-1-mediated growth suppression. To briefly investigate the potential similarities between the roles of K264 and K278 in MOAP-1 biology, we carried out Venn analysis of the gene expression profiles for K264R and K278R MOAP-1 xenografts and compared these results to our wild type MOAP-1 dataset.

As mentioned previously, microarray expression analysis detected 1073 and 121 gene expression differences, respectively, for K264R and K278R MOAP-1 xenografts relative to wild type MOAP-1. Thirty-five of these gene expression differences are common to both K264R and K278R MOAP-1 and, interestingly, it appears that these lysine mutants may share similar influences on gene expression given that 34 out of 35 genes were differentially expressed in the same manner

and to comparable extents (either up- or down-regulated) (Fig. 5.8). This supports a common biological function that is likely to be shared by both K264 and K278 in MOAP-1. Among this common set of disregulated genes is one member of the TGFβ family of receptors, ACVR1C, which is downregulated in the presence of K264R and K278R MOAP-1 (Table 5.9). Although these receptors function as upstream activators of Wnt/β-catenin signaling [466], it is important to note that additional gene expression changes in Wnt signaling proteins makes it difficult to predict the effects of ACVR1C downregulation. For instance, downregulation of Wnt signaling inhibitors SFRP1 and SFRP2 and upregulation of the transcriptional regulator SOX6 were also detected but are specific to only K264R or K278R MOAP-1 (Table 5.5, 5.7). Therefore, further investigation would be necessary in order to determine how these gene expression changes may affect cell proliferation and Wnt/β-catenin signaling and whether or not K264 and K278 share a similar purpose in relation to this pathway.

One considerable gene expression change that was detected for both K264R and K278R MOAP-1 is seen for the transmembrane protein 71 (TMEM71) that is downregulated by eighteen and seventeen-fold, respectively, relative to wild type (Table 5.9). Although TMEM71 is a currently uncharacterized protein with unknown function, the sizable change in its expression is particularly intriguing and suggests a potential importance for MOAP-1 biology. Consequently, it would be interesting to further investigate its biological function considering that our data also propose a connection to

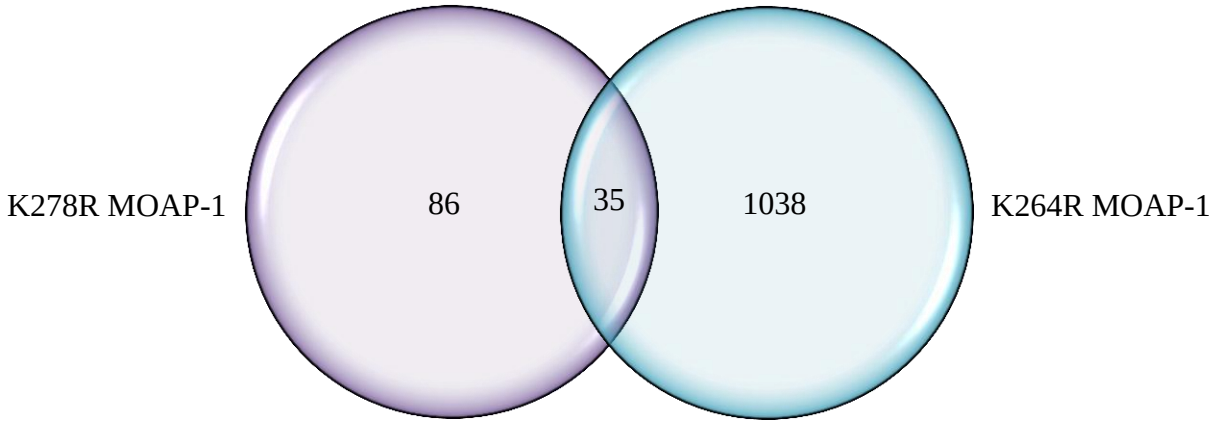


Figure 5.8. Venn diagram of differentially expressed genes by K264R and K278R MOAP-1 relative to WT MOAP-1. Venn analysis was performed for gene expression profiles of HCT 116 xenografts overexpressing K264R or K278R MOAP-1. Fold changes relative to WT MOAP-1 are greater than or equal to 1.5 ($p \leq 0.05$).

Table 5.9. Genes differentially expressed by both by K264R and K278R MOAP-1 relative to wild type MOAP-1

			K264R MOAP-1		K278R MOAP-1	
Gene Symbol	**Description**	**GenBank accession no.**	**p-value**	**Avg. fold change**	**p-value**	**Avg. fold change**
LGSN	lengsin, lens protein with glutamine synthetase domain	NM_016571	4.4E-02	2.7	1.3E-02	2.1
AP1AR	adaptor-related protein complex 1 associated regulatory protein	NM_018569	1.1E-02	2.3	4.7E-02	1.6
IFT57	intraflagellar transport 57 homolog (Chlamydomonas)	NM_018010	4.6E-03	2.2	2.7E-02	1.7
TTPA	tocopherol (alpha) transfer protein	NM_000370	1.2E-02	2.2	4.2E-02	1.7
C3orf57	chromosome 3 open reading frame 57	NM_001040100	2.0E-04	2.0	2.1E-03	1.8
SLC30A10	solute carrier family 30, member 10	NM_018713	1.1E-03	2.0	1.4E-03	1.7
RIC3	resistance to inhibitors of cholinesterase 3 homolog (C. elegans)	NM_024557	4.0E-02	1.8	4.3E-02	1.7
LOC128322	PREDICTED: similar to nuclear transport factor 2	XM_001716411	2.5E-02	1.7	4.8E-02	1.6
PCDH15	protocadherin 15	NM_001142770	1.5E-02	1.6	4.4E-02	1.7
ELN	elastin	NM_000501	8.4E-03	-1.7	2.1E-02	-1.5
ZFATAS	ZFAT antisense RNA (non-protein coding)	NR_002438	6.3E-03	-1.7	2.6E-02	-1.5
NFS1	NFS1 nitrogen fixation 1 homolog (S. cerevisiae)	NM_021100	3.0E-02	-1.7	2.5E-02	-1.7
PKD1L2	polycystic kidney disease 1-like 2	NM_052892	1.5E-02	-1.8	2.7E-02	-1.6
NHE1	Sodium/hydrogen exchanger 1	BC012121	1.4E-02	-1.8	7.9E-03	-2.0
PCDH21	Protocadherin-21 Precursor	BC038799	4.1E-02	-1.9	2.1E-03	-2.0
CARD11	caspase recruitment domain family, member 11	NM_032415	6.5E-03	-1.9	2.9E-02	-1.6
TNKS1BP1	tankyrase 1 binding protein 1, 182kDa	NM_033396	2.5E-03	-2.0	4.5E-02	-1.8
AGXT2	alanine-glyoxylate aminotransferase 2	NM_031900	1.0E-02	-2.0	2.9E-02	1.7
FRMPD4	FERM and PDZ domain containing 4	NM_014728	3.1E-03	-2.1	1.7E-02	-1.7
IFNE	interferon, epsilon	NM_176891	1.8E-02	-2.2	1.5E-02	-2.7
C21orf34	chromosome 21 open reading frame 34	NR_027791	3.7E-02	-2.4	2.1E-02	-2.2
TTLL2	tubulin tyrosine ligase-like family, member 2	NM_031949	1.9E-02	-2.4	4.4E-02	-1.7
ACVR1C	activin A receptor, type IC	NM_145259	9.7E-03	-2.5	3.9E-02	-2.1
CYorf15A	chromosome Y open reading frame 15A	NM_001005852	1.4E-02	-2.9	2.7E-02	-2.4
LILRB4	leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 4	NM_006847	1.1E-02	-2.9	3.3E-02	-1.9
PPP2R1B	protein phosphatase 2 (formerly 2A), regulatory subunit A, beta isoform	NM_002716	7.2E-03	-3.0	2.0E-02	-2.9
PLD5	phospholipase D family, member 5	NM_152666	4.0E-02	-3.2	4.1E-02	-2.8
PRX	periaxin	NM_181882	2.3E-03	-3.3	1.4E-02	-2.5
PDX1	pancreatic and duodenal homeobox 1	NM_000209	3.4E-02	-3.6	4.7E-02	-2.9
CCKBR	cholecystokinin B receptor	NM_176875	1.5E-02	-4.3	3.3E-02	-2.9
ADH6	alcohol dehydrogenase 6 (class V)	NM_000672	9.6E-03	-4.5	2.8E-02	-3.0
ITB2	Integrin beta-2 Precursor	NM_001165324.1	1.2E-02	-5.1	9.7E-03	-4.4
DSG3	desmoglein 3 (pemphigus vulgaris antigen)	NM_001944	3.8E-02	-5.4	2.3E-02	-5.1
KIAA1632	KIAA1632	NM_020964	4.0E-02	-5.5	2.3E-02	-5.3
TMEM71	transmembrane protein 71	NM_144649	7.4E-03	-18.0	4.0E-03	-17.1

MOAP-1 ubiquitination. Of interest, many transmembrane proteins also function as signaling receptors and transport proteins and therefore MOAP-1-mediated regulation of this protein may have other biological implications.

Additional proteins that were differentially regulated by both K264R and K278R MOAP-1 include the cellular adhesion proteins desmosome glycoprotein DGS3, integrin beta-2 precursor (ITB2) and protocadherins 15 (PCDH15) and 21 (PCDH21) (Table 5.9). Downregulation of the autophagy-associated protein KIAA1632 was also detected at almost fivefold lower than wild type MOAP-1 (Table 5.9). Together, these gene expression changes propose novel roles for MOAP-1 in the regulation of cell-to-cell interactions and potentially autophagic cell death.

Overall, the results from our microarray expression analysis of K264R and K278R MOAP-1 xenografts support the notion that these lysine residues may cooperate with one another to influence several processes in cell biology. However, given the greater number of gene expression changes that are induced by K264R MOAP-1 overexpression, we believe that K264 may fulfill an important regulatory function in MOAP-1 biology. In contrast, ubiquitination at K278 may be important for MOAP-1's tumor function through its involvement in cellular signaling processes that are not as highly dependent on downstream gene expression changes. We anticipate that further investigation and a more thorough analysis of our transcriptional profiling results will glean insight into more specific functions for lysine residues K264 and K278 in MOAP-1 biology.

5.3. FUTURE DIRECTIONS

Transcriptional profiling of wild type, K264R and K278R MOAP-1 xenografts generated a wealth of data that will certainly require further post-analysis and validation. Through careful interpretation of our microarray results, we hope to elucidate the mechanism(s) of MOAP-1-mediated tumor suppression and, additionally, the significance of K264 and K278 in its biological function. Currently, it has been difficult to interpret the gene expression profiles of our K264R and K278R MOAP-1 xenografts given the sheer number of possibilities for MOAP-1's tumor suppressor function and the lack of specific direction. Consequently, a logical approach to better understanding our microarray results may first begin by determining the mode of MOAP-1-mediated growth suppression. By doing so, we will then have a better understanding of which signaling pathways to focus our attention towards.

In overview, our microarray results suggest that MOAP-1 may be involved in regulating several different biological processes including gene expression, cell death, DNA repair, cell adhesion and cell-to-cell signaling (Fig. 5.9). While we currently know that MOAP-1 functions as an important modulator of extrinsically activated cell death, we hypothesize that at least some of the above mentioned functions may also be important for MOAP-1-mediated tumor suppression. Although there were a large number of interesting results from this analysis, perhaps of greatest personal interest are the potential connections between MOAP-1-dependent tumor suppression and signaling pathways involving the well-known tumor suppressor protein p53, the novel MOAP-1 interacting protein

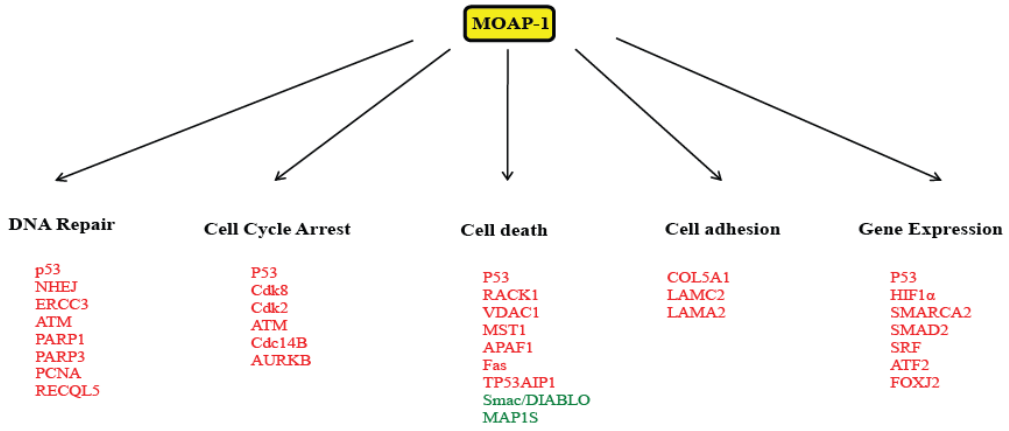


Figure 5.9. Summary of potential MOAP-1-mediated tumor suppressor functions. Gene expression profiling results of MOAP-1-overexpressing HCT 116 xenografts suggest that MOAP-1 may mediate its tumor suppressor function(s) through several different processes including regulation of DNA repair, cell cycle arrest, cell death, cell adhesion or gene expression. Selected genes dysregulated in the presence of overexpressed MOAP-1 are displayed in red (upregulated) or green (downregulated) under each biological process. All gene expression changes are made relative to vector (fold change≥2, $p \leq 0.05$).

Table 5.10. A selection of genes for potential immediate further investigation

Gene Symbol	Description	WT MOAP-1 (relative to vector)	
		Avg. fold change	p-value
HIF1A	hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)	3.7	1.8E-03
TP53	tumor protein p53	3.2	4.3E-03
ATM	ataxia telangiectasia mutated	2.2	2.6E-03
TP53AIP1	tumor protein p53 regulated apoptosis inducing protein 1	2.1	9.3E-04
GNB2L1/RACK1	guanine nucleotide binding protein (G protein), beta polypeptide 2-like 1	2.0	2.3E-02

Table 5.11. A selection of potential MOAP-1-regulated pathways for immediate further investigation

Canonical Pathways[a]	-log (p-value)[b]	Molecules from this dataset[c]
HIF1α Signaling	3.25	TP53, MAPK6, PIK3R5, KRAS, HIF1A, SLC2A3, LDHB, ARNT, PIK3R3, CUL2, NCOA1, PIK3CB, APEX1, MMP17, LDHA, ATM
p53 Signaling	1.9	PIK3R3, TP53, PRKDC, TP53AIP1, PCNA, APAF1, PIK3R5, PIK3CB, FAS, CDK2, PTEN, ATM
DNA Double-Strand Break Repair by Non-Homologous End Joining	2.1	PRKDC, DCLRE1C, PARP1, ATM
Apoptosis Signaling	1.3	TP53, RELA, APAF1, KRAS, DIABLO, NAIP, FAS, DFFA, MCL1, PARP1

[a]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.
[b]-log(p-value) ≥1.3 is equivalent to p-value ≤0.05
[c]Molecules from the dataset that meet cut criteria and are involved in the corresponding signaling pathway.

RACK1 and intrinsically activated apoptosis which so far has not been thoroughly studied with respect to MOAP-1. Therefore, our immediate future directions may involve focusing on the validation and investigation of p53 and RACK1 and some of their associated pathways, as well as non-death receptor-dependent apoptosis (Table 5.10, 5.11). In addition to these possibilities, however, there are also a large number of other exciting prospects. Unquestionably, the results from this microarray expression analysis have allowed us to generate meaningful hypotheses for further investigation.

5.4. CONCLUSION

Our gene expression profiling data suggest that MOAP-1 may mediate its tumor suppressive effects through its ability to influence a large number of cellular processes and signaling pathways including DNA repair, cell cycle, cell adhesion, gene expression, or apoptosis. Through further analysis and validation of our results, we hope to unravel the mechanisms that contribute to MOAP-1 biological activity and tumor suppressor function.

CHAPTER 6

Identification and validation of a novel MOAP-1 protein interaction involving RACK1, an intracellular receptor for PKC

6.1. INTRODUCTION

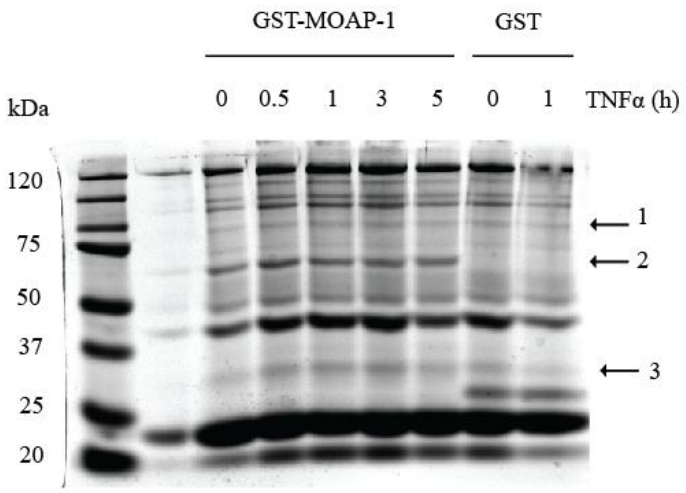
MOAP-1 is a proapoptotic molecule with a limited set of known interacting partners. To date, the group of established MOAP-1 associating proteins includes TNF-R1, TRAIL-R1, RASSF1A [84], Bax, Bcl-$X_{L,}$ Bcl-2 [187] and TRIM 39 [193]. During death receptor-dependent apoptosis, MOAP-1 is recruited to receptors TNF-R1 or TRAIL-R1 and associates downstream with RASSF1A and Bax [84, 85]. MOAP-1 also basally interacts with anti-apoptotic proteins Bcl-X_L and Bcl-2 under experimental overexpression conditions and may associate with Bax during intrinsically activated cell death [189, 190]. During apoptosis, MOAP-1 stabilization is mediated by the RING domain-containings protein TRIM39. Through our research, we have identified MOAP-1 as a novel tumor suppressor protein and results from transcriptional profiling of MOAP-1-overexpressing HCT 116 xenografts reveals an enormous influence of this protein on gene expression changes and potential signaling pathways. To help identify new biological roles for MOAP-1 that may be important for its tumor suppressor function, we decided to perform a protein interaction study through a glutathione S-transferase (GST) pull-down assay followed by mass spectrometry. Analysis findings suggest that, in addition to apoptosis, MOAP-1 may participate in regulating actin dynamics and protein translation that may be important in cell migration and the control of gene expression. Most notably, this study has also led to the identification of a novel MOAP-1 protein interaction involving RACK1 that may have important implications for its PKC-dependent ubiquitination and tumor suppressor function.

6.2. RESULTS

6.2.1. Identification of potential MOAP-1 interacting proteins

TNFα is a multifunctional cytokine that plays a key role in the induction of inflammatory responses and may also initiate apoptosis under the appropriate circumstances when NF-κB signaling is blocked [490]. During TNF-R1-mediated cell death, MOAP-1 serves a pivotal role through its ability to promote Bax conformational change and, subsequently, mitochondrial outer membrane permeabilization [84, 85]. Considering that TNFα has the ability to stimulate MOAP-1 biological function and may also activate non-apoptotic signaling pathways [174], we decided to investigate if MOAP-1 may possibly associate with any other downstream molecules that would suggest new biological functions for this protein. To assess this possibility, we chose to perform a GST pull-down assay in order to help identify currently unknown MOAP-1 interacting proteins. HT-29 colorectal adenocarcinoma cells were transiently transfected with either pEBG-GST or pEBG-GST-MOAP-1 and treated with TNFα two days post-transfection. Cell lysates were subsequently incubated with glutathione sepharose beads in order to precipitate GST control or GST-MOAP1 and any associated protein complexes, prior to resolution by SDS-PAGE and coomassie blue staining (Fig 6.1A). Protein bands that demonstrated a sustained, loss or increase in MOAP-1 association upon TNFα-stimulation were excised from SDS-PAGE gel and sent for liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis by Jack Moore (Research technician, U of A Institute for Biomolecular Design). As a measure of control, we also ensured that corresponding protein bands from non-

A)



B)

Band	Molecular weight (kDa)	Protein Name	Function
1	85	LIM domain and actin binding protein 1 isoform b	A cytoskeletal protein that inhibits actin depolymerisation.
2	64	GST-MOAP-1	Bait used for GST pull-down.
3	36	Receptor for activated C kinase-1 (RACK1)	Intracellular receptor for activated PKC. Structural component of 40S ribosome. Associates with Bax and Bcl2. Associates with TRIM63 E2 ubiquitin ligase
3	26-31	40S/60S ribosomal proteins L7A, L8, S2 and S6	Molecules involved in protein translation.

Figure 6.1. Identification of MOAP-1 interacting proteins. (A) Coomassie blue stain of a GST-MOAP-1 pull-down. HT29 colorectal adenocarcinoma cells were transiently transfected with pEBG-GST or pEBG-GST-MOAP-1. Two days later, cells were stimulated with TNFα (20 ng/ml) prior to harvest, lysis and overnight incubation with glutathione sepharose beads. Associated protein complexes were separated by SDS-PAGE and commassie blue stained. Association of proteins with MOAP-1 was identified by LC-MS/MS. Numbered arrows correspond to the migration of bands in table below. (B) Table of MOAP-1 interacting proteins identified by gst-pull down in (A) followed by mass spectrometry.

stimulated and pEBG-GST control samples were analysed by the same methods in order to have a basis for comparison when interpreting our results.

Several proteins were identified through our GST-MOAP-1 pull down assay as potential TNFα-induced MOAP-1 interacting molecules. The highest molecular weight protein that was detected at 4% peptide coverage is the 85 kDa LIM domain and actin binding protein 1 (LIMA1/EPLIN) that associates with GST-MOAP-1 at one and three hours following TNFα stimulation (Fig. 6.1B). EPLIN is a cytoskeletal protein that regulates actin dynamics through the stabilization and cross-linking of filaments and is frequently downregulated in oral, prostate and breast cancer cell lines [491, 492]. EPLIN also inhibits Rac-induced membrane ruffling and suppresses cell proliferation when overexpressed [492]. Consequently, it has been suggested that decreased expression of this protein during cancer may contribute to cell motility and invasiveness [491].

Several different ribosomal proteins were also pulled down by GST-MOAP-1 at 30 minutes post-TNFα treatment in this study at peptide coverages ranging from 11% to 22% including 60S components L7A (RPL7A) and L8 (RPL8), and 40S proteins S2 (RPS2) and S6 (RPS6) (Fig. 6.1B). In addition to coordinating protein translation, some of these proteins may have other cellular functions. L7A inhibits gene transactivation by nuclear thyroid hormone and retinoic acid receptors [493] and frequently undergoes gene rearrangement with the Trk proto-oncogene to form an activated Trk-2h oncogenic protein [494]. As well, RPS6 serves as the major substrate of ribosomal protein kinases [495, 496] and may

also be involved in the regulation of cell growth and proliferation through the selective translation of particular mRNAs [497].

Interestingly, mass spectrometry analysis also identified at 7% peptide coverage the 36 kDa guanine nucleotide-binding protein subunit beta-2-like 1 (GNB2L1) as a GST-MOAP-1 interacting molecule 30 minutes following TNFα stimulation (Fig. 6.1B). GNB2L1 is more commonly known as the receptor for activated C-kinase 1 (RACK1) and serves as an adaptor and shuttling protein for PKC [443]. Considering the wide range of PKC-mediated functions, it is not surprising RACK1 is also involved in regulating a variety of different cellular processes including cell growth and proliferation [219, 221], modulation of integrin signaling [222], protein translation [224], neuronal functions [227] and apoptosis [225, 226]. Because of RACK1's involvement in PKC regulation and the fact that non-degradative MOAP-1 ubiquitination occurs in a PKC-dependent manner, we found this GST pull-down result particularly intriguing. Consequently, we decided to further investigate RACK1 as a potential MOAP-1 interacting protein.

6.2.2. RACK1 is highly expressed in most transformed cell lines

Previous studies have demonstrated an upregulation of RACK1 expression in human cancers that is associated with angiogenesis and carcinogenesis [247]. To investigate and verify RACK1 expression for ourselves, immunoblotting was performed for a selection of various human cancer cell lines (Fig. 6.2). In accordance with published findings, we were able to detect high levels of RACK1 expression in the majority of transformed cell lines tested. Notable exceptions

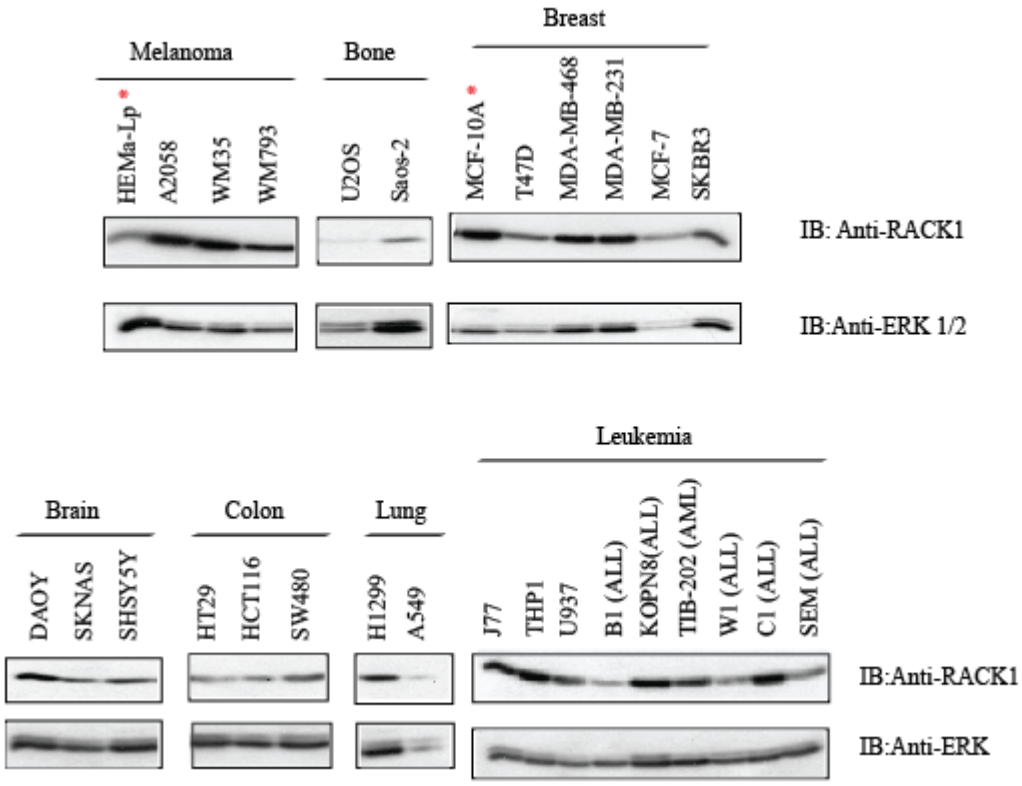


Figure 6.2. RACK1 is highly expressed in most transformed cell lines. Western blotting for endogenous RACK1 expression in a selection of human cancer cell lines. Red asterisk signifies a non-transformed control cell line of the same tissue origin. THP1 is also known as TIB-202. ALL: acute lymphoblastic leukemia; AML: acute myeloid leukemia.

were A549 lung carcinoma and U2OS osteosarcoma cells in which RACK1 expression was surprisingly low. In contrast, RACK1 expression was present at elevated levels in MCF-10A non-transformed immortalized mammary epithelial cells relative to the other breast cancer cell lines tested. Taking into account the discrepant findings regarding RACK1's role in breast cancer [250-252], these results support the research of Al-Reefy et al. that has demonstrated higher RACK1 expression in normal versus cancerous breast tissue [250]. Overall, the high levels of RACK1 expression detected in most cancer cell lines stands in contrast to the decrease or loss in MOAP-1 expression in many of these cell types (Fig. 3.3, 3.4). However, in HCT 116 colon carcinoma cells, RACK1 was expressed at moderate levels compared to many of the other cell lines (Fig. 6.2). Considering that a significant portion of our MOAP-1 research had been carried out in this cell line, we decided to pursuit the remainder of our RACK1 investigation in HCT 116 cells.

6.2.3. MOAP-1 colocalizes with RACK1 in cells

RACK1 functions as an intracellular receptor and shuttling protein for multiple PKC isozymes and, upon activation, is involved in their translocation to the appropriate intracellular locations [443]. Under non-stimulated conditions, however, RACK1 is localized to different cellular sites and is not associated to a specific organelle [212]. To investigate the subcellular localization of RACK1 relative to MOAP-1 under non-stimulated conditions, HCT 116 cells were transiently co-transfected with Myc-MOAP-1 and GFP-RACK1 prior to

immunofluorescence staining and analysis by confocal microscopy using sequential viewing of MOAP-1 and RACK1. Unexpectedly, MOAP-1 colocalized with RACK1 in the absence of cell stimulation (Fig. 6.3A). This was unanticipated considering that MOAP-1 interaction with RACK1 was shown to be induced by TNFα stimulation in our GST-MOAP-1 pull-down experiment (Fig. 6.1). However, we must also keep in mind that protein colocalization merely demonstrates that RACK1 and MOAP-1 are present in close spatial proximity to one another and is not sufficient to conclude a true physical interaction [498].

Results from immunoblotting previously demonstrated that MOAP-1 undergoes non-degradative ubiquitination in a process involving K264 and K278 (Fig. 4.3). Therefore, we decided to evaluate if PKC-dependent MOAP-1 ubiquitination was required for its RACK1 colocalization. HCT 116 cells were co-transfected with GFP-RACK1 and either K264R or K278R Myc-MOAP1 and prepared for immunofluorescence confocal microscopy. Similar to wild type MOAP-1, K264R and K278R MOAP-1 were both able to colocalize with RACK1 under non-stimulated cellular conditions (Fig. 6.3B, C). Therefore, MOAP-1 ubiquitination involving K264 and K278 is not required for RACK1 colocalization and is in accordance with previous results demonstrating that these mutations do not alter the localization of MOAP-1 (Fig. 4.2). Nonetheless, it will be interesting to investigate how the subcellular localizations of wild type, K264R and K278R MOAP-1 change with the addition of TNFα or PMA with respect to RACK1 and relative to one another.

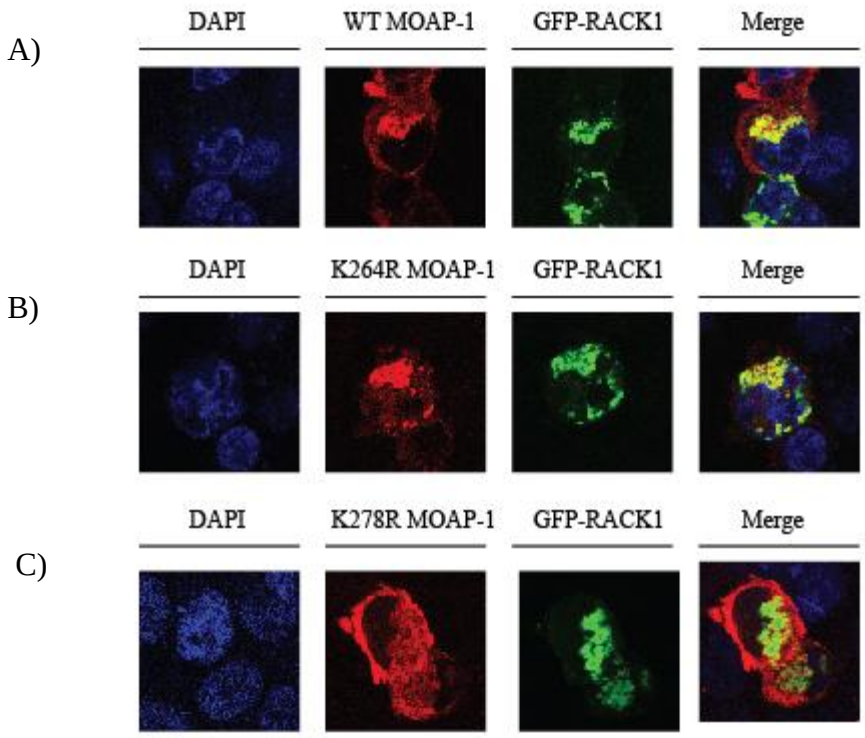


Figure 6.3. MOAP-1 colocalizes with RACK1 under non-stimulated conditions. HCT 116 cells were co-transfected onto square coverslips with GFP-RACK1 and Myc-tagged (A) WT MOAP-1, (B) K264R MOAP-1 or (C) K278R MOAP-1. Two days post-transfection, cells were immunostained with mouse anti-Myc antibody followed by anti-mouse Alexa 555 secondary antibody. Cellular localization of RACK1 and MOAP-1 was visualized by confocal microscopy using a Leica SP5 microscope by sequential acquisition.

6.2.4. MOAP-1 association with RACK1 coincides with its non-degradative ubiquitination

RACK1 is well-known for its PKC-associated functions [443]. However, more recent findings have demonstrated a role for this protein in TNFR1 signaling where it has been implicated as a modulator of neutral sphingomyelinase activation [499]. Results from our GST-MOAP-1 pull down assay indicate that RACK1 may also associate with MOAP-1 downstream of TNFR1 (Fig. 6.1) and, therefore, may possess other currently unknown functions. To verify that RACK1 and MOAP-1 do interact with each other in response to TNFα stimulation, a co-immunoprecipitation experiment was performed. HCT 116 cells were transiently transfected with Myc-tagged MOAP-1 and evaluated two days later for association with endogenous RACK1 protein following cell treatment with TNFα. Western blotting results confirm that MOAP-1 associates with RACK1 within one hour following TNFα stimulation and that this protein interaction increases by three hours post-treatment (Fig. 6.4A). Surprisingly, we were also able to detect non-degradative ubiquitination of MOAP-1 during RACK1 association. Thus, it appeared that perhaps RACK1 was potentially involved in MOAP-1's ubiquitination. This introduced the question as to whether RACK1 association with MOAP-1 could also be induced by PKC activation considering that this proteasome-independent form of MOAP-1 ubiquitination was previously detected in response to PMA addition to cells (Fig. 4.3). Subsequently, a second similar co-immunoprecipitation experiment was performed in transiently transfected Myc-MOAP-1-expressing HCT 116 cells. In place of TNFα, cells were stimulated with

the PKC activator PMA. Results from immunoblotting verified our suspicion that PKC activation could also induce MOAP-1 interaction with RACK1 (Fig. 6.4B). MOAP-1 strongly associated with RACK1 after one hour following PMA addition and this was coincident with its non-degradative ubiquitination as predicted. That either TNFα or PMA can induce MOAP-1 association with RACK1 as well as its ubiquitination suggests that RACK1 may facilitate PKC-mediated MOAP-1 modification downstream of TNFR1. This possibility is supported by the observation that PMA cell stimulation results in earlier MOAP-1 ubiquitination than with TNFα treatment (Fig. 6.4), possibly due to more efficient PKC activation. In addition to HCT 116 cells, we have also been able to detect an interaction between RACK1 and overexpressed Myc-MOAP-1 in other cell lines including HEK 293T, COS-7 and H1299 (Law, unpublished observations). Work by other members of the Baksh lab has demonstrated endogenous associations in response to PMA or TNFα stimulation in TIB-202 leukemia and BL2 lymphoma cells (Kwek and Baksh, unpublished observations). Interestingly, we are also able to detect a protein interaction between endogenous RACK1 and overexpressed K264R and K278R MOAP-1 mutants that are impaired in their ability to undergo PMA-induced MOAP-1 ubiquitination (Fig. 4.3). Although the kinetics of their association remain to be further investigated, these results suggest that non-degradative MOAP-1 ubiquitination likely occurs downstream of RACK1 association. Overall, our results validate a novel RACK1/MOAP-1 protein interaction that can be induced by either TNFα or PMA. We also

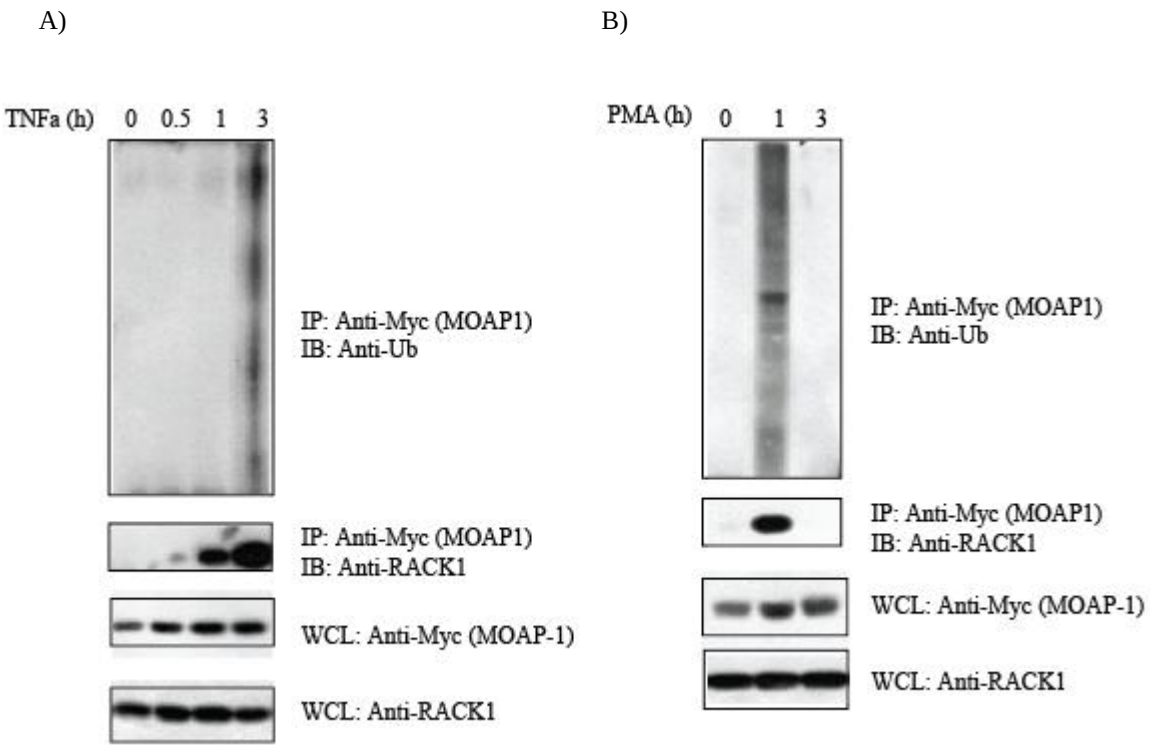


Figure 6.4. MOAP-1 association with RACK1 coincides with its non-degradative ubiquitination. HCT 116 cells were transiently transfected with pXJ40 Myc-MOAP-1 and stimulated with (A) TNFα (20 ng/ml) or (B) PMA (150 ng/ml) two days post-transfection. Immunoprecipitation (IP) was performed using an anti-Myc antibody and followed by Western blotting (IB) for endogenous RACK1 association and Myc-MOAP-1 ubiquitination. WCL: whole cell lysate.

hypothesize that RACK1 may be implicated in the non-degradative ubiquitination of MOAP-1 and may possibly function to recruit an E3 ligase in order to ubiquitinate MOAP-1. Consequently, further studies will be required in order to determine the role of RACK1 in MOAP-1 biology and its ubiquitination.

6.3. DISCUSSION AND FUTURE DIRECTIONS

6.3.1. Subcellular colocalization of MOAP-1 and RACK1

Western blotting results indicate that MOAP-1 association with RACK1 is stimulated by TNFα or PMA addition to cells (Fig. 6.3A, B). However, these proteins appear to colocalize in certain areas under normal cellular conditions and in the absence of external stimuli (Fig. 6.3). Therefore, an important question to ask is whether or not the basal colocalization of MOAP-1 and RACK1 is biologically significant. Previous studies have demonstrated that RACK1 is a highly mobile protein that localizes at different sites and does not contain any consensus sequence motifs for anchoring to specific subcellular regions [212]. Although RACK1 basally associates with different proteins including the integrin beta subunit [500] and Src kinase [219], we do not detect an interaction between RACK1 and MOAP-1 in non-stimulated cells (Fig. 6.3A, B). Given the significant amount of protein colocalization present, we would expect that a physical interaction between RACK1 and MOAP-1 should be detectable by immunoblotting if it were truly present. Consequently, we hypothesize that RACK1 and MOAP-1 simply reside in close spatial proximity to one another in

non-stimulated cells and do not actually physically associate in the absence of TNFα or PMA. In support of this possibility is an interesting point of fact that even at high resolution the volume of a single pixel is significantly larger than the volume of even one GFP molecule and therefore cannot be used as proof of a protein interaction [498]. Nonetheless, protein colocalization is commonly indicative of a shared structural or functional characteristic [501]. Thus, it is possible that the colocalization of RACK1 and MOAP-1 may have an underlying biological significance. Importantly, it will also be interesting to study the dynamics of colocalization between RACK1 and MOAP-1 in response to TNFα or PMA stimulation that may provide valuable clues about the order of events in the MOAP-1 ubiquitination process.

6.3.2. Potential RACK1 involvement in the PKC-dependent ubiquitination and tumor suppressor function of MOAP-1

Non-degradative MOAP-1 ubiquitination occurs in response to PKC activation (Fig. 4.3) and is required for its tumor suppressor function (Fig. 4.7). Through our GST-MOAP-1 pull down assay, we have identified a novel MOAP-1 protein interaction with the PKC adaptor RACK1 that may be important for facilitating its PKC-dependent ubiquitination, and thus activation of its growth inhibitory function. Surprisingly, our results demonstrate that MOAP-1 ubiquitination occurs during RACK1 association and in response to both TNFα and PMA stimulation. This suggests that RACK1 may participate in the PKC-mediated MOAP-1 modification downstream of TNFR1. Our hypothesis is

strengthened by the finding that MOAP-1 ubiquitination occurs more efficiently with direct PMA-induced PKC activation than with TNFα stimulation alone (Fig. 6.4).

Based on our data, we hypothesize that RACK1 may promote MOAP-1 ubiquitination through a number of different possible mechanisms. Given RACK1's established role as an intracellular receptor and shuttling protein for PKC, we hypothesize that RACK1 may mediate the translocation of PKC either to phosphorylate MOAP-1 and to signal for its ubiquitination or to activate the appropriate enzymes for MOAP-1 ubiquitination. Both possibilities seem probable when considering that substrate phosphorylation frequently precedes ubiquitination [410] and the fact that PKC is a known regulator of protein ubiquitination [406-409]. Additionally, we have also been able to detect a phosphorylated form of MOAP-1 in C1 and SEM leukemia cell lines (Fig. 3.4) and, thus, a future investigation may involve a direct evaluation of the ability of PKC to phosphorylate MOAP-1. In either case, our data suggest that RACK1 forms a complex with MOAP-1 prior to or during its ubiquitination (Fig. 6.4A, B). Therefore, it is possible that RACK1 may also facilitate the recruitment of MOAP-1's E3 ubiquitin ligase. Indeed, RACK1 is involved in the recruitment of several E3 ligases including the Elongin C/B-ubiquitin ligase complex that degrades HIF-1α [502] and the Fbw7 E3 ligase that targets c-Jun for degradation [503]. RACK1 also interacts with the E3 ligase TRIM63/MURF1 in neonatal rat ventricular myocytes and it appears that this protein interaction is involved in the inhibition of PKC epsilon activation [504]. Therefore, it is possible that RACK1 may

also be involved in the recruitment of other ubiquitin ligases such as TRAF2, an E3 ligase that is involved in death receptor dependent cell death and for which MOAP-1 contains two potential binding sites. Consequently, it will be interesting to investigate what other proteins may be part of the RACK1/MOAP-1 protein complex during the ubiquitination process in order to gain further insight into the events surrounding its post-translational modification. Based on the assumption that RACK1 is important for the PKC-dependent ubiquitination of MOAP-1, we expect that it may also be required for MOAP-1's tumor suppressor function. As part of our future work, we would also like to evaluate the absolute requirement of RACK1 in MOAP-1 mediated functions and anticipate that the use of siRNA against RACK1 as previously employed by Zhang et al. [505] may reveal interesting results. Thus, further investigations into the contribution of RACK1 to MOAP-1-mediated growth inhibition are expected.

6.3.3. Additional biological implications for MOAP-1 function

In addition to RACK1, mass spectrometry results from our GST-MOAP-1 pull down assay have identified several other potential MOAP-1-interacting proteins. These include the cytoskeletal protein EPLIN and ribosomal components RPL7A, RPL8, RPS2 and RPS6 (Fig. 6.1A, B). The finding that MOAP-1 may associate with the actin regulatory protein, EPLIN, suggests that MOAP-1 may participate in the modulation of actin dynamics. Reorganization of the actin cytoskeleton has important implications in carcinogenesis and cell motility [506, 507] and, thus, its proper regulation is of extreme importance. In addition to inhibiting

actin depolymerization and stabilising actin filaments [491], however, EPLIN may also function as a growth inhibitory protein [492]. Given that its expression is frequently downregulated in human cancers, it is possible that the combined loss of MOAP-1 and EPLIN may have potential clinical significance. Thus, it will be interesting to investigate if MOAP-1 is involved in EPLIN-mediated actin regulation.

That MOAP-1 may interact with four different ribosomal proteins (RPL7A, RPL8, RPS2 and RPS6) strongly suggests that it may have a role in protein translation. Supportive of this are results from gene expression profiling of MOAP-1-overexpressing xenografts that indicate the upregulation of multiple translation initiation factors and RNA polymerase components in its presence (Table 5.1). Interestingly, the newly identified MOAP-1-interacting protein, RACK1, is also an important regulator of eukaryotic translation [224]. As an actual component of the ribosome, RACK1 recruits PKC to phosphorylate and activate initiation factor 6 in order to stimulate protein translation. Therefore, RACK1 serves to couple signal transduction events directly to the ribosome. It is possible that RACK1 and MOAP-1 may cooperate during protein translation particularly since GST-MOAP-1 pull down of RACK1 and ribosomal proteins RPL7A, RPL8, RPS2 and RPS6 occurred at the same time point thirty minutes following TNFα stimulation. Thus, future work may include an examination of MOAP-1's ability to regulate protein translation and how RACK1 may also be involved in the process. Although these putative MOAP-1 protein interactions remain to be fully

validated, results from our GST pull-down assay provide an exciting platform for future research.

6.4. CONCLUSION

Collectively, we have demonstrated a novel protein interaction between MOAP-1 and RACK1 that can be induced by TNFα or PKC activation. The finding that MOAP-1 undergoes non-degradative ubiquitination while in contact with RACK1 suggests a role for the latter protein in this modification process. Given the crucial importance of ubiquitination to MOAP-1's tumor suppressor function, we also hypothesize that RACK1 may be involved in MOAP-1-mediated growth inhibition. Although MOAP-1 and RACK1 colocalize under non-stimulated cell conditions, we hypothesize that these proteins do not physically interact in the absence of stimuli and await future studies to determine the biological importance of their spatially close cellular localizations. Lastly, preliminary results from our GST-MOAP-1 pull down assay have identified additional MOAP-1-interacting proteins that suggest novel biological functions for MOAP-1 and remain to be further investigated.

CHAPTER 7

Final Summary

7.1. Summary of results

Through this research study, we have identified MOAP-1 as a novel tumor suppressor protein whose loss of expression is observed in multiple human cancer types. Given that the MOAP-1-binding protein RASSF1A is also epigenetically silenced in a large number of human cancers [364], it is possible that the combined loss of MOAP-1 and RASSF1A during carcinogenesis may result in the inhibition of extrinsically-activated cell death signaling pathways in cancer cells. However, in addition to apoptosis, our data suggest that MOAP-1 may also possess additional biological functions that may be important for its tumor suppressor function (Fig. 3.9, 3.10, 3.10). This possibility is similar to RASSF1A, which exerts its growth inhibitory function through its ability to regulate multiple biological processes including the cell cycle, microtubule dynamics, apoptosis and cell migration [1]. Therefore, it will be interesting to further elucidate which biological processes MOAP-1 may be involved in and that may also contribute to its tumor suppressor function. Moreover, it is also possible that MOAP-1 may possess RASSF1A-independent functions and, thus, a future study dissecting the role of RASSF1A in MOAP-1-mediated processes warrants investigation.

Surprisingly, we have also discovered a mode of MOAP-1 regulation that is essential to its tumor suppressor function. In the absence of PKC-dependent MOAP-1 ubiquitination, we observe a loss in the ability of MOAP-1 to suppress tumor formation (Fig. 4.7). Most peculiarly, non-degradative MOAP-1 ubiquitination occurs at K278 but involves a process through which K264 is also important but not required (Fig. 4.3, 4.7). Although impaired in their ability to

inhibit tumor growth, MOAP-1 mutants containing K264R and K278R mutations retain their ability to promote TNFα-induced apoptosis and therefore lend further support to the notion that MOAP-1 possesses additional non-apoptotic functions that may govern its tumor suppressor function such as the regulation of DNA repair, cell cycle arrest, cell adhesion or gene expression (Fig. 4.5, 4.6). Indeed, further studies will be required in order to determine the exact importance of MOAP-1 ubiquitination involving K264 and K278 in its tumor suppressor function. However, gene expression profiling of tumor xenografts overexpressing wild type, K264R or K278R MOAP-1 suggest several proteins and molecular pathways that may be important for MOAP-1-mediated tumor suppression and provide an excellent starting point for future investigations.

Over the course of study, we have also validated a novel protein interaction between MOAP-1 and the PKC adaptor receptor for activated C-kinase 1 (RACK1). Our data indicate that the protein association between MOAP-1 and RACK1 can be induced with both TNFα and PMA and also reveals the presence of non-degradative MOAP-1 ubiquitination during the span of their interaction. Therefore, we hypothesize that RACK1 may be important for facilitating PKC-dependent MOAP-1 ubiquitination and, consequently, may have important implications for its tumor suppressor function. Although a number of questions still remain unanswered, our data suggest a model in which RACK1 may contribute to the tumor suppressor function of MOAP-1 (Fig. 7.1). In response to TNFα stimulation or PKC activation, MOAP-1 forms a complex with RACK1. We hypothesize that RACK1 may facilitate MOAP-1 ubiquitination by

recruiting an E3 ligase such as TRAF2, the RACK-1 interacting protein TRIM63 [504] or the MOAP-1-binding molecule TRIM39 [193]. Upon PKC-dependent ubiquitination at K278, MOAP-1 may acquire its ability to mediate tumor suppression. At this time, our model remains merely speculative. However, we anticipate that future investigations will resolve the validity of our theory and foresee a growth in the study of MOAP-1 biology as new cellular functions are discovered for this protein.

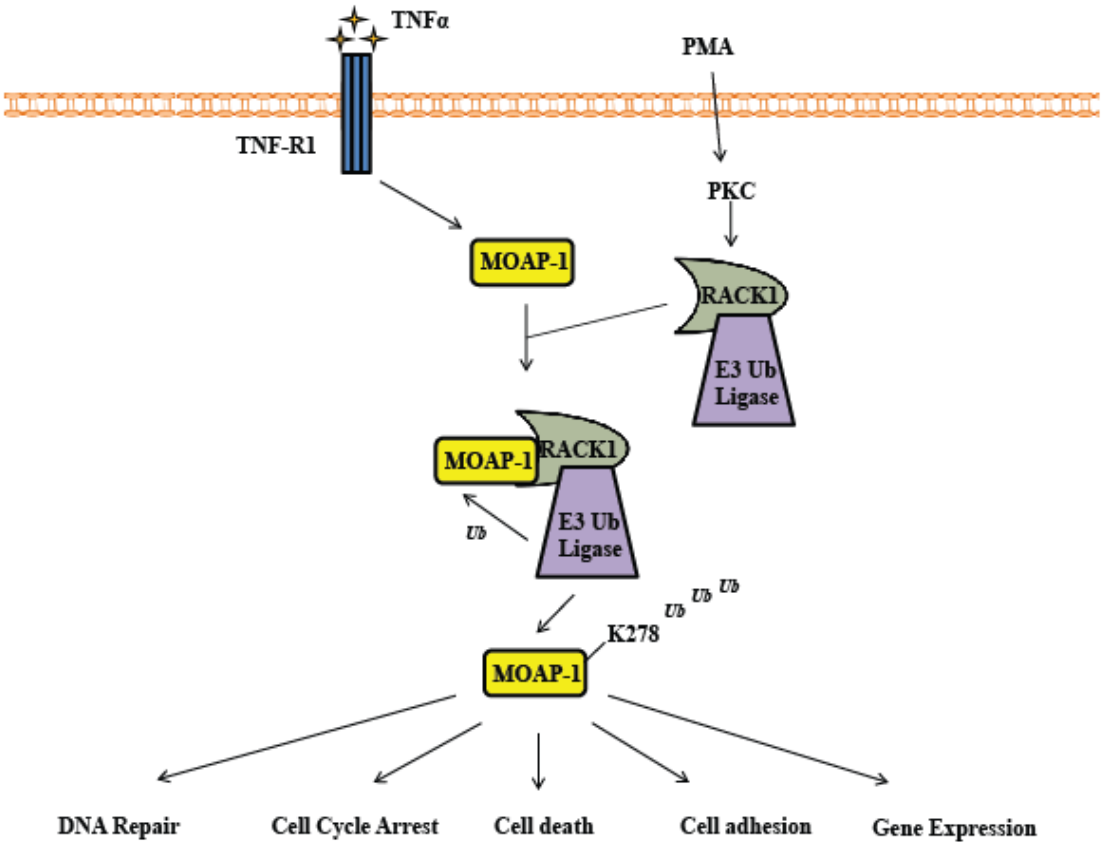


Figure 7.1. A working hypothesis for the role of RACK1 in MOAP-1-mediated tumor suppression. In response to TNFα stimulation or PKC activation, MOAP-1 associates with the PKC adaptor, RACK1. RACK1 may function to recruit the MOAP-1 E3 ligase that results in the non-degradative ubiquitination of MOAP-1 at K278. Downstream of this modification, MOAP-1 may function as a tumor suppressor through the regulation of multiple biological processes including DNA repair, cell cycle arrest, cell death, cell adhesion or gene expression.

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