THE ROLE OF TAPASIN AND ITS ISOFORMS IN ANTIGEN PRESENTATION
AND TUMOR IMMUNITY

by

ROBYN PATRICIA SEIPP

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Abstract

Major Histocompatibility Complex (MHC) Class I molecules present peptides to CD8+ T cells and are essential for most adaptive immune responses. The first described-spliced tapasin (“isoform 1”) plays a critical role in MHC-I antigen presentation by facilitating peptide loading onto MHC-I molecules in the endoplasmic reticulum (ER). This thesis examines the expression, localization and function of two novel, alternatively-spliced isoforms of human tapasin that lack exon 7 (“isoform 2”) or both exons 6 and 7 (“isoform 3”). Isoform 1 contains a di-lysine ER-retention motif; the two novel isoforms encode different carboxy (C) termini that lack this motif. It was hypothesized that isoforms 2 and 3 would function in MHC-I cross-presentation of exogenous antigens in non-ER compartments. Isoform 2, like isoform 1, was found to be mainly ER-localized; however, both these isoforms were also found to co-localize in smaller amounts with the trans Golgi network and endo/lysosomes by confocal microscopy. Isoform 3 lacks a transmembrane domain and was found to be secreted from cells as well as being found within the ER. All isoforms were widely expressed at the RNA level in many tissues and cell types; however, mature dendritic cells (DCs) expressed the highest levels of all three isoforms, consistent with the high cross-presenting abilities of DCs. Both isoform 1 and 2 stabilized the transporters associated with antigen processing (TAP) in murine tapasin−/− cells, but isoform 3 did not due to its missing transmembrane domain. Isoform 1 and 2 mediated very similar effects on endogenous MHC-I presentation of self and viral peptides, on surface MHC-I thermostability, and on MHC-I maturation rates. Isoform 3 was found to decrease loading of exogenous peptides onto MHC-I. None of the isoforms
influenced cross-presentation of the soluble antigen ovalbumin in a mouse dendritic cell line.

This thesis also examines the effect of antigen presentation machinery (APM) re-expression in MHC-I-deficient tumor cell lines, B16F10 and CMT.64, which are deficient in TAP and tapasin. Virally-driven TAP1 and Tapasin expression increased MHC-I expression in the tumor cell lines, augmented tumor cell immunogenicity, and decreased tumor growth \textit{in vivo} due to increased tumor cell elimination by the immune system.
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List of Abbreviations

ABC : ATP-binding cassette
Ad : adenovirus
APC : antigen presenting cell
APM : antigen processing machinery
ANOVA : Analysis of Variance
ATCC : American Type Culture Collection
ATP : adenosine triphosphate
β2m : beta-2-microglobulin
bp : base pair
BFA : Brefeldin A
BSA : bovine serum albumin
C-terminus : carboxy terminus
CAR : coxsackie and adenovirus receptor
CD : cluster of differentiation
cDNA : complementary deoxyribonucleic acid
CLIP : MHC Class II-associated invariant chain peptide
CMV : cytomegalovirus
CNS : central nervous system
CTL : cytotoxic T lymphocyte
DC : dendritic cell
DMEM : Dulbecco’s Modified Eagle Media
DMSO : dimethyl sulfoxide
DNA : deoxyribonucleic acid
dNTP : deoxyribonucleotide triphosphate
dT : 2’-deoxy-thymidine
EBV : Epstein-Barr Virus
Endo H : endoglycosidase H
ER : endoplasmic reticulum
FACS : fluorescent-activated cell sorting
FBS : fetal bovine serum
FWD : forward (for PCR primer)
GAPDH : glyceraldehyde-3-phosphate dehydrogenase
GFP : green fluorescent protein
GM-CSF : granulocyte-macrophage colony-stimulating factor
HEPES : 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
HLA : human leukocyte antigen
HRP : horseradish peroxidase
IFN : interferon
IHC : immunohistochemistry
Ig : immunoglobulin
II : invariant chain of MHC Class II
IL-2 : interleukin-2
i.p. : intraperitoneal
IRES : internal ribosomal entry site
kb : kilobase
kDa : kilodalton
LMP : low-molecular-weight protein
LPS : lipopolysaccharide
mAb : monoclonal antibody
MHC : major histocompatibility complex
MIIC/CIIV : MHC Class II compartment/Class II-associated vesicle
MOI : multiplicity of infection
mRNA : messenger ribonucleic acid
N-terminus : amino terminus
NF-AT : nuclear factor of activated T cells
NK : natural killer
OVA : chicken egg ovalbumin
PAGE : polyacrylamide gel electrophoresis
PBS : phosphate-buffered saline
PCR : polymerase chain reaction
PE : phycoerythrin
Poly(A) : poly-adenylated
PVDF : polyvinylidene fluoride
REV : reverse (for PCR primer)
RNA : ribonucleic acid
RPMI : Roswell Park Memorial Institute
RT-PCR : reverse transcriptase-polymerase chain reaction
S15 : small ribosomal subunit rRNA used a housekeeping gene control
SIINFEKL : amino acid sequence of the immunodominant H-2K\textsuperscript{b}-binding peptide from ovalbumin
s.c. : subcutaneous
SD : standard deviation
SEM : standard error of the mean
SDS : sodium dodecyl sulfate
TAA : tumor associated antigen
TAP : transporter associated with antigen processing
TCA : Trichloroacetic acid
TCR : T cell receptor
TGF-\beta : transforming growth factor beta
TGN : trans Golgi network
TIL : tumor infiltrating lymphocyte
TLR : toll-like receptor
TMD : transmembrane domain
TNF-\alpha : tumor necrosis factor alpha
Tpn : tapasin
Tpn-R : tapasin-related
UTR : untranslated region
VSV : vesicular stomatitis virus
WT : wild-type
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During the course of my Ph.D., I also ate a fair number of take-out Chinese food dinners while simultaneously developing a bit of a superstitious streak when it came to tricky experiments. I therefore paid careful attention to the contents of the fortune cookie at the end of each meal to see if they held any promise for future experimental outcomes and related issues. If, to my delight, the message seemed promising, I taped the good news above the lab bench to remind me of the fact that there was hope. As I feel, in a sense, that they are somewhat responsible for encouraging me in producing this thesis through some trying moments, it seems only fair that they share in the final outcome as well.
Dedication

Having pursued my formal education to the highest possible degree is, I believe, a credit to the quality of teaching I have received along the way. I would like to dedicate this thesis to my teachers, formal and not, who, along the way, provided the key ingredient in reaching this point: inspiration.
Co-Authorship Statement

The tapasin isoforms were originally identified and cloned by Dr. Alexander Moise (Michael Smith Laboratories, University of British Columbia, Vancouver, BC) and Siri Lok (Michael Smith Laboratories, University of British Columbia, Vancouver, BC). Alexander Moise and Siri Lok generated the recombinant retrovirus plasmids encoding tapasin isoforms 1 and 2. I made the recombinant retrovirus plasmid expressing isoform 3 and generated the recombinant retroviruses used in all experiments. Dr. Yuanmei Lou and Bing Cai generated the AdhTAP1 and AdhTpn (isoform 1) adenoviruses and subcloned tapasin isoforms 2 and 3 into the adenoviral shuttle plasmid. I generated the recombinant adenoviruses expressing tapasin isoforms 2 and 3.

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In Chapter 5, the experiments to generate Figure 5.1 were performed by myself and Xiao-Lin Li (Michael Smith Laboratories, University of British Columbia, Vancouver, BC). The flow cytometry in Figure 5.3 was performed by Xiao-Lin Li and Dr. Qian-Jin Zhang (Michael Smith Laboratories, University of British Columbia, Vancouver, BC). The flow cytometry in Figure 5.4 was performed by Yuanmei Lou (Michael Smith Laboratories, University of British Columbia, Vancouver, BC) and Bing Cai (Michael Smith Laboratories, University of British Columbia, Vancouver, BC). The CTL assays in Figure 5.5, Figure 5.6 and Figure 5.7 were performed by Susan Chen (Department of Zoology, University of British Columbia, Vancouver, BC), Xiao-Lin Li and Qian-Jin Zhang. The ELISPOT assay in Figure 5.8 was performed by Xiao-Lin Li.
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All other figures and tables in this thesis were generated from experiments performed solely by me.
Chapter 1: General Introduction

The immune system has evolved to protect us from a constant barrage of potentially deadly invaders. Interactions with different pathogens have shaped and selected this system. This has resulted in an incredibly complex network of interrelated host defense components capable of targeting potential threats with exquisite specificity, and of recognizing and responding to subsequent attacks by the same agent even more efficiently. The host is constantly evolving, and many components of its defense system are amongst the most diverse in the human genome and cooperate to counteract threats in complex ways that are only beginning to be understood. In some instances, it is the pathogens that emerge with the upper hand. In others, the host immune system prevails, but undesirable side effects, such as autoimmunity, may result. Tight control of the immune system is required to carry out responses robust enough to eliminate disease, while at the same time not harming the host beyond repair. Understanding the mechanisms governing these responses is critical for the development of new therapies that promise to harness the inherent power of the immune system and to direct or re-direct it to benefit human and animal health.

1.1 Mechanisms and regulation of RNA splicing

In eukaryotes, virtually all RNA is normally spliced to remove introns, leaving only the protein-encoding exons and some regulatory elements such as the 5’ and 3’ untranslated regions (UTRs). However, RNA can also be alternatively spliced in a variety of different ways, including the inclusion or exclusion of alternative exons, exon-
skipping, intron retention, or alternative use of 5' and 3' splice sites to elongate or shorten exons as depicted in Figure 1.1 (1).

![Figure 1.1 Several types of alternative splicing occur in eukaryotes](image)

Constitutive sequences present in all final mRNAs are gray boxes. Alternative RNA segments that may or may not be included in the mRNA are hatched boxes. (A) Exon-skipping. (B) Mutually-exclusive exon incorporation. (C, D) Alternative 5' and 3' splice sites change the length of a given exon. (E, F) Alternative promoters and alternative poly(A) sites alter the starting or ending exons. (G) Intron-retention. (H) Compound phenotype with multiple different splicing events often used in combination. Reproduced from (1) with permission.

The physical mechanism of RNA splicing is well understood; however, the regulation of alternative splicing remains poorly defined. Splicing often begins almost immediately after transcription of the RNA by RNA polymerases. Specialized RNA-protein complexes known as snRNPs (small nuclear ribonucleoproteins, denoted U1, U2, U4, U5 and U6) assemble at exon-intron boundaries, recognizing conserved splice acceptor and donor sites via their small nuclear RNA components (snRNAs), which are
complimentary to the sequences of the splice donor and acceptor sites. Generally, the U1 snRNP binds the 5’ donor splice site, and the U2 snRNP binds at the site of the nucleophilic adenosine residue responsible for the splicing reaction within the intron to be removed (Figure 1.2). This is followed by binding of the U2, U4, U5 and U6 snRNPs and assembly of the spliceosome in an ATP-dependent process. The actual splicing event then takes place via nucleophilic attack of the adenosine hydroxyl group bound by U2 on the phosphate group at the 5’ exon-intron boundary donor site, and subsequent attack of the 5’ hydroxyl group thus formed on the phosphate at the 3’ intron-exon boundary acceptor site. This results in neat excision of the intron through a branched-lariat intermediate structure (Figure 1.3) (2).

**Figure 1.2** Recognition of 5’ and 3’ exon-intron boundaries and adenosine nucleophile by the complimentary RNA components of snRNPs U1 and 2 initiates the splicing reaction to remove intron sequences.

Ψ = pseudouridine. Binding of U2 to the RNA template helps create a bulge that assists in promoting nucleophilic attack of the adenosine 2’-OH group (pink) on the 5’ exon-intro phosphorus group. Reproduced from (2) with permission.
Figure 1.3 Spliceosome assembly is a multi-step process involving the binding of several snRNPs.

After initial binding of U1 and U2, U4/U6 and U5 join the complex to form an inactive spliceosome, which is activated by an ATP-dependent internal rearrangement involving ejection of U1 and U4, and U6 binding to both U2 and the 5’ splice site. Catalysis of the splicing reaction involves nucleophilic attack of the hydroxyl group of the key adenosine residue (shown in red) with the 5’ splice site, followed by subsequent nucleophilic attack of the hydroxyl group thus formed on the 3’ splice site to join the exons together while removing the intron through a branched-lariat intermediate structure. Reproduced from (2) with permission.
Regulation of RNA splicing, including the choice of alternative splicing sites, is poorly understood, but it seems to result from the cumulative effects of the binding of both splicing enhancer and suppressor proteins to the RNA. Sites within the 5’ or 3’ splice sites, or other regions within the RNA, have been dubbed exonic or intronic splicing enhancers (ESEs or ISEs) and silencers (ESSs or ISSs), and bind these regulatory proteins to control selection of the splice sites (3). ESEs are known to bind SR proteins, which contain both RNA and protein binding regions, and are believed to promote interactions across exons, thereby influencing the actions of the spliceosome (4). ESSs and ISSs are recognized by heterogeneous nuclear ribonucleoproteins (hnRNPs), which also contain RNA and protein binding domains, and function to repress splicing and/or exon selection (3). However, the nucleotide sequences of these RNA regions are highly degenerate, and furthermore are influenced by many extrinsic factors. With the exception of a few well-understood examples of splicing regulation, factors influencing alternative splicing remain a major unknown area of biology.

1.2 The role of RNA splicing in the generation of biological diversity

The current estimates suggest that the human genome is comprised of about 35,000 genes (5;6). This is astonishingly few genes for an organism as complex as Homo sapiens. This only roughly doubles the approximately 19,000 (7) and 14,000 genes (8) of the less complex organisms Caenorhabditis elegans and Drosophila melanogaster, respectively. However, it has become apparent that alternative splicing of genes offers an economical way for increasing protein diversity and complexity from a relatively small pool of starting templates. Several other mechanisms also exist for generating protein
diversity, including the use of multiple transcription start sites, alternative polyadenylation, pre-mRNA editing, and post-translational modifications; however, alternative splicing is considered to be amongst the most prevalent and important of these mechanisms. It is interesting that this process is particularly common in vertebrates (3) and suggests a great importance for splicing in higher and more complex animals. It is estimated that between 39 to 59% of human genes undergo some form of alternative splicing, though this may be an underestimate (9-11). In some cases, the number of possible transcripts generated by splicing can be staggeringly large; for example, the *Drosophila* gene *Dscam*, which plays a role in neural axon guidance during development, has 95 alternatively-spliced exons, with the possibility of generating over 38,000 different isoforms of the Dscam protein (12;13) - approximately double the total number of genes in the organism’s entire genome!

### 1.2.1 Alternative splicing in the immune system

In humans, alternative splicing appears to be particularly important in the central nervous system (CNS), especially in neurons (1;3). It is perhaps not surprising that such a complex organ, and arguably one of the main components responsible for our own complexity as a whole, requires a large number of differentially-spliced mRNAs giving rise to diverse proteins interacting in intricate networks to function. However, while more diffuse in nature, the immune system, composed of many different interacting tissues, cells and their molecular products, can also be regarded as a complex “organ” that must deal with many different challenges and handle the delicate task of balancing defense from the outside world with maintenance and protection of the inside one. To cope with the challenges of dealing with constantly evolving pathogens, the immune system has
developed some of the most impressive mechanisms of generating protein diversity known to molecular biologists. For example, the receptors of B cells and T cells are created during development by rearrangement of germline-encoded DNA regions and by somatic hypermutation to potentially generate up to $\sim 10^{12}$ different B cell clones and $\sim 10^{18}$ different T cell clones of distinct specificities, capable of recognizing almost any pathogen with which the host comes into contact (14). However, diversity in the immune system is not limited only to B and T cell receptors. Increasingly, alternative splicing of other immune-related transcripts is being discovered and the functions of the alternative proteins they encode deduced. For example, MHC Class I molecules have been shown to be alternatively spliced at their C termini, generating proteins with potentially different signaling and/or intracellular trafficking properties (15-17). The natural killer (NK) cell NKG2D receptor has also been found to be alternatively-spliced, leading to differential downstream signalling (18). The transporters associated with antigen processing and presentation, or “TAP”, (responsible for transport of peptides into the ER for presentation to T cells), and the MHC Class II chaperone, invariant chain, have also been shown to be alternatively-spliced with unknown functional effects (19-21).

### 1.3 The adaptive immune system

Jawed vertebrates have evolved an adaptive immune system that allows for the specific recognition and targeted elimination of antigens derived from harmful agents both within the cells of the body and without (14). The adaptive immune system utilizes two main effector cell types, B and T lymphocytes, for this purpose. B lymphocytes express and secrete antibodies with a wide variety of affinities and effector functions to target and neutralize humoral antigens for destruction, primarily in extracellular body
fluids. T lymphocytes are further divided into two subgroups distinguished by their expression of either CD4 or CD8 molecules on their cell surfaces. Both T cell groups recognize peptides derived from infected or malignant cells via their T cell receptors (TCRs), and following this recognition carry out effector functions to assist in the elimination of compromised cells in the body. However, T cells and their TCRs do not directly gain access to the cell interior to survey for the presence of abnormalities. Rather, a sophisticated system has evolved in the majority of nucleated somatic cells to allow T cells to inspect cells from the outside using specialized molecules known as Major Histocompatibility Complex (MHC) molecules.

### 1.4 Major histocompatibility complex molecules

A key process in adaptive immune responses is the presentation of antigenic peptides on the surface of cells for surveillance by T cells via their T cell receptors (TCRs). Peptides derived from antigens are presented on the cell surface by MHC molecules, of which there are two subtypes: Class I and Class II. In humans, MHC molecules are known as Human Leukocyte Antigens or “HLA”. The TCRs of CD4⁺ T cells recognize peptide bound in MHC Class II molecules, while the TCRs of CD8⁺ T cells recognize peptide bound in MHC Class I molecules. In addition to the TCRs, the T cell CD4 and CD8 co-receptors interact with the target cell MHC II and MHC I molecules, respectively, assisting in ensuring the specificity of contact. The process of assembling peptides with MHC molecules for cell surface display is known as antigen processing and presentation. Although the mechanisms of antigen processing and presentation differ between MHC Class I and II, both involve the proteolysis of protein antigens to produce short peptides, binding of these peptides to MHC molecules (Figure
1.4) with assistance from chaperones, and transport of the resulting peptide-MHC complexes to the cell surface for presentation to T cells (22;23).

![Diagram of the overall structures of MHC Class I and Class II molecules, with the peptide binding groove indicated.](image)

Figure 1.4 Diagram of the overall structures of MHC Class I and Class II molecules, with the peptide binding groove indicated.
Reproduced from (24) with permission.

### 1.4.1 Structure of MHC Class I and Class II molecules

MHC Class I molecules consist of a 45 kDa heavy chain of three domains: α1, α2 and α3. The α3 membrane-proximal domain non-covalently binds a separate subunit, the 12kDa protein β2microglobulin (Figure 1.5a). Both the heavy chain and β2m subunits contain immunoglobulin (Ig) protein folding motifs. The heavy chain α1 and α2 domains rest above the α3 domain, forming a beta sheet base bordered by parallel alpha-helices that generate a groove in which the peptide binds through hydrogen bonding to conserved
“anchor” residues (Figure 1.5b) (25). The MHC I molecule is membrane-bound through a
single transmembrane domain in the heavy chain, followed by a short cytoplasmic tail.
This cytoplasmic tail contains a tyrosine addressin motif that is critical in routing the
MHC I molecule through various cellular compartments for proper immunologic
functioning (26), particularly in antigen cross-presentation, discussed below.

(a)       (b)

Figure 1.5 Structure of MHC Class I as determined by X-ray crystallography.
(a) Side view of the HLA-A2 molecule showing the three heavy chain domains α1
(yellow) and α2 (pink) - which together form the peptide binding groove - and α3 (dark
blue), which interacts with the β2m subunit (light blue) (b) Ribbon diagram of Kb viewed
from above. α1 domain is colored light cyan, the α2 domain is dark blue, and peptide is
gray. The yellow segment on the upper left side of the antigen-binding platform
demarcates the epitope detected by mAbs 64-3-7 and KU1 [residues 46–54]. Moving
clockwise alone the α1 helix, the green segments represent the epitopes for mAbs HC10
[residues 57–62], LA45 [62, 63], L31 [66–68], and HCA [77–84]. The magenta segment
in the lower right hand corner of the class I antigen-binding platform represents the
tapasin-interaction region [residues 128–136]. Reproduced from (27) and (28) with
permission.
MHC Class II molecules consist of two separate proteins that associate non-covalently: a 33 kDa alpha chain and a 28 kDa beta chain (Figure 1.6a). Both subunits contain transmembrane domains anchoring the molecule to the membrane, and each has two external immunoglobulin domains (α1 and α2, β1 and β2, respectively). The membrane-proximal α2 and β2 domains are analogous to the β2m/α3 domain of MHC I in that they create the platform upon which the α1 and β1 domains form the peptide-binding cleft, which is similarly characterized by anti-parallel beta-sheet “floor” and anti-parallel alpha-helical sides and looks quite similar to the MHC I peptide-binding groove (Figure 1.6b), despite having slightly different peptide-binding properties, discussed below.

![Figure 1.6](image)

**Figure 1.6 Structure of MHC Class II as determined by X-ray crystallography.** (a) Side view of the HLA-DR molecule, which was crystallized as a dimer. Peptide is indicated as a yellow ribbon bound in the peptide binding groove. (b) Ribbon diagram of I-E\(^k\) viewed from above. α1 domain is colored light cyan, the β1 domain is dark blue, and peptide is gray. The magenta segment on the upper left of the antigen-binding platform demarcates the region implicated in DM interaction. In an opposite corner on the class II
antigen-binding platform, the yellow segment represents residues [β50–69] detected by mAbs to open forms of class II. Reproduced from (28;29) with permission.

### 1.5 MHC Class I antigen presentation

MHC Class I antigen presentation occurs in most somatic nucleated cells of the body. In non-lymphoid tissues, its main purpose is to present endogenously-derived antigenic peptides on the cell surface for surveillance by CD8+ cytotoxic T cells (CTLs) (30;31). This allows the immune system to effectively sample the nature of the proteins being synthesized within the cells. Under normal circumstances, healthy cells present peptides derived from normal products of cellular metabolism on their cell surface. T cells, in the absence of autoimmune disease, do not respond to these normal self peptides, as all T cells with TCRs recognizing self-peptides in the context of self-MHC I molecules are eliminated during thymic selection, or become anergic in the periphery through tolerizing mechanisms. However, if a cell presents non- or altered-self peptide on the surface due to viral infection or malignant transformation, specific CTLs will mediate the killing of the cell (30;32).

The MHC I antigen processing and presentation pathway begins in the cytosol when endogenous antigen is degraded into peptides by the proteasome (33-36). The transporter associated with antigen processing (TAP) complex, consisting of two ATP-binding cassette (ABC) proteins, TAP1 and TAP2, then binds and translocates the peptides into the lumen of the endoplasmic reticulum (ER) (30;37;38). Imported peptides are loaded onto pre-assembled heterodimers of MHC I heavy chain and β2m by a peptide loading complex (PLC) consisting of calreticulin, ERp57 thioreductase and tapasin (39). Peptides bound by MHC I molecules are normally between 8 to 10 amino acids long, though the TAP transporter can import longer peptides whose N-termini can
subsequently trimmed by ER aminopeptidases such as ERAAP (40-42). MHC I molecules associated with peptide are then transported through the secretory apparatus to the cell surface (30;39), as depicted in Figure 1.7.

Figure 1.7 MHC Class I antigen presentation.
Endogenous proteins (for example, viral proteins) are degraded in the cytosol by the proteasome into peptides, which are imported into the ER through the heterodimeric TAP complex. Once in the ER lumen, the peptides are loaded onto MHC Class I molecules (called HLA molecules in humans) with the assistance of the peptide loading complex (tapasin, ERp57, calreticulin and PDI – not shown in figure) for transport to the cell surface and surveillance by CD8+ T cells. Reproduced from (24) with permission.

The MHC I heavy chain has many alleles encoded at three gene loci: HLA-A, B and C in humans, and H-2K, D, L in mice. Each allele has slightly different peptide-binding properties, and therefore each molecule binds a different subset of peptides. This provides protection against a wide variety of pathogens through the presentation of
peptides that vary depending on a given individual’s set of alleles (43). This diversity also protects the population as a whole since some alleles may be more effective in presenting the antigens of a given pathogen than others, increasing the odds that at least some individuals within the group will survive an outbreak (44).

1.6 MHC Class II antigen presentation

Under non-inflammatory conditions, MHC Class II antigen presentation occurs only in specialized immune system cells called “professional” antigen presenting cells (pAPCs). These include B cells, macrophages and dendritic cells (DCs) (45). One function of these cells is assisting in the initiation of immune responses by activating CD4+ T “helper” cells that then proliferate and secrete immune-activating cytokines. These cytokines, as well as direct cell-cell interactions, in turn help to activate B cells, cytotoxic T cells, macrophages and other cells that participate in the immune response (23;45). In contrast to MHC I molecules (which bind endogenously-derived peptides), MHC II molecules bind peptides derived from exogenous antigens taken up by pAPCs via endocytosis and phagocytosis (45;46). MHC II molecules are co-translationally inserted into the ER similarly to MHC I, but are prevented from binding endogenously-derived peptides imported by TAP by their association upon synthesis with the invariant chain (Ii) (45). Invariant chain bound to MHC II directs the molecules to a specialized compartment referred to as the MHC II compartment (MIIC) or Class II vesicles (CIIV). In this compartment, invariant chain is degraded by lysosomal proteases to a smaller fragment called Class II-associated invariant-chain peptide (CLIP) (45;47). Exogenous antigen taken up by the cells is also targeted to the MIIC/CIIV compartment and is digested by lysosomal proteases into peptides that replace CLIP in binding the MHC II
molecules. This peptide exchange is mediated by the chaperone HLA-DM (48). Generally, MHC II molecules bind slightly longer peptides than MHC I molecules, averaging between 13 and 18 amino acids in length. The peptide-MHC II complexes are then presented on the cell surface to CD4⁺ cells (49), as shown in Figure 1.8.

Figure 1.8 MHC Class II antigen presentation.
Exogenous antigens are endocytosed by antigen presenting cells (APCs) into lysosomal compartments where the antigens are degraded by lysosomal proteases of the cathepsin family. MHC Class II molecules are synthesized in ER and chaperoned to the antigen-containing lysosomal compartments by invariant chain. Once the MHC II molecules reach the ER, invariant chain is degraded and HLA-DM assists in loading exogenously-derived peptides onto the Class II molecules for transport to the cell surface and surveillance by CD4⁺ T cells. Reproduced from (24) with permission.
1.7 MHC Class I cross-presentation

More recently, the idea that peptides derived from endogenous antigens are only presented by MHC I and peptides derived from exogenous antigens are only presented by MHC II has been modified. Naïve T cells circulate throughout the lymph nodes, spleen and blood, but not all pathogens enter the body at these sites; therefore, naïve T cells will not encounter and be activated by all pathogens directly (22;50;51). Furthermore, it is now clear that naïve T cells of both CD4+ and CD8+ subsets require prior activation by pAPCs to carry out effector functions (22;51). It is now thought that naïve T cells rely primarily on DCs, the most potent of pAPCs, to present foreign antigens for the initiation of a T cell response and to provide the necessary co-stimulation for activation.

DCs capture antigens at the site of infection and then move to the draining lymph nodes where they undergo maturation and display the captured antigens on both MHC I and II molecules (22;51). Mature DCs also express a variety of co-stimulatory molecules to allow activation of both CD4+ and CD8+ naïve T cells. Activated T cells then proliferate, differentiate and leave the secondary lymphoid compartments to eliminate the invaders at the site of infection (22;50). Since DCs are ubiquitously distributed throughout the body, this system ensures that all invading pathogens - no matter their site of entry - can be captured and their antigens presented by DCs to select and activate those T cells with TCRs that recognize the pathogen. Other professional antigen presenting cells, such as a macrophages (52;53) and B cells (54-56), and even “innate” immune system cells such as neutrophils (57), have also been thought to perform this function under certain conditions but are not as efficient or as flexible in terms of the range of antigens they can present.
For MHC II-restricted immune responses, it is well documented how DCs capture and internalize exogenous antigens at sites of infection and present their peptides on MHC II to CD4⁺ T cells (51). However, for MHC I-restricted responses, it is less well-known how exogenously-captured antigens enter this normally-endogenous pathway (22). In some cases, DCs may themselves be infected by certain viruses, and MHC I peptides could therefore be derived from viral proteins synthesized within the DC for presentation to CD8⁺ T cells via the classical endogenous pathway (58). However, not all viruses can infect DCs, and in the case of malignant cells (which can be eliminated by MHC I-restricted CD8⁺ T cell immune responses (59;60)) it is difficult to envision a scenario by which malignant cells could “infect” DCs and have them synthesize tumor-associated antigens endogenously (61). Therefore, another mechanism has been proposed by which exogenous antigens are captured by DCs, processed and presented on MHC I. This mechanism has been called “cross-presentation” (22;50).

There appears to be several pathways of MHC Class I cross-presentation. With certain exogenous antigens, the protein is taken up in endosomes or phagosomes and is exported to the cytosol where it enters the “classical” endogenous MHC I pathway (i.e. degraded by the proteasome and imported into the ER by TAP). This is referred to as the endosome (or phagosome)-to-cytosol pathway (62-64). In other cases, it appears that post-Golgi MHC I molecules encounter the exogenously-derived antigenic peptides in a later compartment, such as the MIIC/CIV or an endolysosomal compartment (26;65-67). There, the original peptide bound by the MHC I molecule is exchanged for the exogenous peptide, and the complex is displayed on the cell surface. Some of the MHC I molecules involved in this process are believed to be recycled from the cell surface (68). This is
known as the “vacuolar” or “endolysosomal” pathway of cross-presentation. It has also been suggested that phagocytosed antigens access the MHC I antigen presentation machinery via fusion of the phagosomal membrane with the ER membrane, forming a compartment with all components necessary for antigen presentation (69;70). Most recently, gap junctions have been shown to allow transfer of antigenic peptides between infected cells and APCs, providing yet another possible route for exogenous peptide transfer to the cytosol of DCs for presentation on MHC I (71). These mechanisms are summarized in Figure 1.9.
**Figure 1.9 Proposed mechanisms of cross presentation.**

(a) The vacuolar or endolysosomal pathway. MHC I molecules are loaded with peptides that are generated in internalized vesicles such as phagosomes or endosomes by lysosomal cathepsin proteases in a TAP-independent manner. (b) Phagosome-to-cytosol-to-phagosome pathway. The phagosomal membrane fuses with the ER membranes during phagocytosis, such that the phagosome acquires MHC I, TAP, Sec61 and other ER molecules, making this compartment functional for MHC I presentation. Internalized antigen is exported (possibly by Sec61) to the cytosol, hydrolyzed by proteasomes and the resulting peptides are then re-imported into phagosomes by TAP, where they bind MHC I molecules. (c) Phagosome-to-cytosol pathway. Internalized antigens are exported to the cytosol where they are digested by the proteasome into that peptides are shuttled into the classical “endogenous” antigen presentation pathway by TAP in the ER membrane. (d) Gap junction pathway. APCs acquire peptides from other cells through Gap junctions, which are presented similar to the phagosome-to-cytosol pathway. (e) Endosome-to-ER pathway. Antigen in endosomes is transported back into the ER and then degraded in the cytosol by the ER associated degradation pathway (ERAD). The resulting peptides are transported to class I molecules in the ER by TAP. Reproduced from (72) with permission.

**1.8 The MHC Class I peptide loading complex**

The MHC Class I molecule heavy chain is co-translationally inserted into the ER membrane. Once synthesis is complete, a complex series of steps unfold prior to exit of the mature, peptide-bound form of MHC I from the ER. The MHC I heavy chain is first bound by the ER lectin chaperone calnexin (CNX), which assists in folding the heavy chain and assembling it with β2m. Calnexin has been shown to interact with the MHC I heavy chain through both its lectin and protein-binding sites (73;74); the former interaction is likely mediated by N-linked glycosylation of the MHC I heavy chain (75). Following association with β2m, the MHC I heterodimer is released from calnexin (at least in humans) and assembles the peptide loading complex (PLC) consisting of calreticulin (CRT), tapasin (Tpn), and ERp57 (30). Like calnexin, calreticulin is also a lectin chaperone but unlike calnexin is soluble rather than membrane-bound. Tapasin mediates assembly of the CRT/ERp57/MHC I/β2m protein complex and tethers it with
the TAP1/TAP2 heterodimer for easy access to imported peptides. Stoichiometric analyses have indicated that four tapasin/MHC I complexes (with other associated PLC proteins) bind to a single TAP heterodimer (76;77) (Figure 1.10). Protein disulfide isomerase (PDI) was also recently shown to interact with the peptide loading complex; it has been proposed to regulate oxidation of the disulfide bond within the MHC I peptide-binding groove, thereby influencing peptide selection and binding (78). Together these components assist in loading peptides onto the MHC I/β2m heterodimer (Figure 1.11).

Figure 1.10 Proposed model of the peptide loading complex as viewed from the endoplasmic reticulum (ER) lumen.
The TAP complex core transmembrane domains (TMD) form a ‘pore’ through which peptides are transported into the ER using the energy derived from ATP hydrolysis by the nucleotide binding domains (NBD). The N terminal accessory transmembrane domains of the TAP heterodimer lie outside of the pore and interact with tapasin (in turn associated with Class I molecules). Stoichiometric analyses indicate four tapasin-MHC I molecules bind a single TAP heterodimer. Calreticulin (not shown in figure) may be present at sub-stoichiometric levels. Reproduced from (79) with permission.
MHC Class I heavy chains co-translationally synthesized and glycosylated in the ER are first bound by calnexin (CNX). Upon assembly with β2m, CNX is released and the peptide loading complex is formed around the MHC I molecule, consisting of calreticulin (CRT), ERp57 and Tapasin (Tpn). Tapasin anchors the complex to the TAP transporter (TAP1 and TAP2), which imports peptides generated by the proteasome into the ER from the cytosol. Peptides may be further trimmed by ER peptidases such as ERAP. Peptides are loaded onto the Class I molecules by the peptide loading complex, resulting in a stabilized trimeric complex of MHC Class I heavy chain, β2m and peptide that is ready for transport to the cell surface for surveillance by CD8+ T cells. Reproduced from (80) with permission.

1.9 Tapasin

Tapasin ("TAP-associated glycoprotein") is a 48 kDa ER-localized glycoprotein with a critical role in MHC I antigen presentation and processing (81). It acts as a chaperone uniquely dedicated to antigen presentation (unlike calnexin and calreticulin), bridging the TAP1-TAP2 heterodimer and the MHC I heavy chain associated with β2m (77;82-84). Tapasin facilitates the loading of peptides imported by TAP onto the peptide-receptive MHC complex (77;85). In addition, tapasin stimulates peptide transport and MHC I peptide loading by stabilizing the TAP heterodimer (86-88), thereby increasing the local concentration of peptides available for MHC I binding (77). There is also evidence that tapasin retains empty and/or suboptimally-loaded MHC I molecules in the
ER (89;90) and quantitatively and qualitatively influences the peptide repertoire presented by MHC I molecules, favouring high-affinity peptides that best stabilize the MHC I/β2m heterodimer (87;91).

The tapasin protein consists of three domains: an N-terminal ER-lumenal domain, a transmembrane domain, and a cytoplasmic tail domain. The N-terminal domain consists of two immunoglobulin motifs (making tapasin a member of the immunoglobulin superfamily (92)), as well as a third domain with no known structural similarities. It has a potential glycosylation site at position 233 (human tapasin) and cysteines at positions 7 & 71, and 292 & 362 that may be disulfide linked (93) (Figure 1.12). Another cysteine at residue 95 is important for covalent linkage to ERp57. The N-terminal domain is also rich in proline residues, which is characteristic of many proteins that have several protein interaction partners, as tapasin does with MHC I and the other PLC components (81;85;86;94-96).
Figure 1.12 Structural organization of human tapasin.
The N terminal domain of tapasin contains two IgG domains (formed by disulfide bonding between cysteines 7 & 71 and 292 & 362) that interact with MHC I. Interactions with calreticulin may be mediated by N-linked glycosylation of an asparagine residue at position 233. ERp57 interacts with a conserved cysteine at position 95 of tapasin. The transmembrane domain interacts with TAP1 and TAP2. The C terminus contains a dilysine ER-retention motif (“KKAKE”). Reproduced from (93) with permission.

The transmembrane domain (TMD) of tapasin mediates its interactions with the N-terminal helices of both TAP1 and TAP2 (79;97-100). A conserved lysine residue at position 408 of human tapasin, located within the transmembrane/cytoplasmic domain, was found to influence surface MHC I expression and TAP stabilization (100). In mouse, changing this residue to an alanine did not abrogate tapasin stabilization of TAP2, but additionally mutating four residues hypothesized to form part of an alpha-helix did decrease tapasin’s ability to stabilize TAP2 (99). These studies indicate that while there are slight differences in tapasin from the two species, overall the TMD in both is important for TAP stabilization.
Finally, the short cytoplasmic C-terminal domain of tapasin contains a dilysine ER retention motif that results in greater egress of tapasin from the ER when mutated to alanine residues (101). Apart from this motif, the rest of the C-terminus does not contain any other known functional motifs and appears to be less conserved than the rest of the molecule (86).

The exact mechanism by which tapasin mediates its various effects is still a matter of some debate; however, it appears that tapasin binds preferentially to empty MHC I – an interaction that is inhibited \textit{in vitro} by peptide binding - suggesting that tapasin acts as a direct mediator of peptide binding and release of MHC I from the TAP heterodimer (102). Furthermore, tapasin was shown to widen the peptide-binding groove of a human MHC I allele, allowing faster peptide association and dissociation through the disruption of conserved C terminal hydrogen bonds within the peptide-binding groove (103). Together these results suggest that tapasin acts to prevent binding of peptides that are unable to dissociate it from MHC I, thereby selecting a repertoire of peptides that bind the MHC I groove with high enough affinity to cause the release of the MHC I molecule from tapasin and the PLC (102;103). Very recently, it has been shown that in order to function, tapasin must be covalently linked at a 1:1 ratio to ERp57 through disulfide bonding (104). Mutant forms of tapasin lacking the cysteine residues required for interactions with ERp57 were not capable of mediating MHC I peptide loading or editing the peptide repertoire (104). A large proportion of cellular ERp57 is normally bound to tapasin, between 15 and 30% in unstimulated cells and up to 80% in IFN-\(\gamma\)-treated cells (105-107). Recently it was shown that in the absence of tapasin, ERp57 reduces the MHC I \(\alpha_2\) disulfide bond, preventing peptide binding within the MHC I
groove (108). This demonstrates that yet another mechanism by which tapasin exerts its function is to sequester much of the cellular ERp57, thereby preventing it from reducing this critical disulfide bond and allowing MHC I molecules to remain in a peptide-receptive redox conformation (108). Nevertheless, the role of ERp57 in antigen presentation cannot be purely “destructive”, since mice deficient in ERp57 in the B cell compartment showed reduced MHC I surface expression and stability (106), and peptide loading complexes deficient in ERp57 tended to form nonfunctional aggregates (109). Further study will be required to ascertain the exact mechanisms of action of tapasin in relation to ERp57 in MHC I antigen presentation.

Interestingly, some MHC I alleles display a strong dependency on tapasin for optimal peptide loading, whereas other alleles appear to be almost entirely tapasin-independent and show very little quantitative or qualitative differences in peptide loading when expressed in either tapasin-expressing or tapasin-deficient cells (110-113). As tapasin itself is not significantly polymorphic (114), these differences appear to be mainly due to different amino acids encoded within the MHC I molecules. Residue 114 (of human MHC molecules) is located within the peptide binding groove and is naturally quite polymorphic (110). When this position is occupied by a basic histidine or arginine residue, the alleles are tapasin-independent, whereas the presence of an aspartic or glutamic acid residue confers tapasin-dependency (110). The strength of tapasin dependency also correlates with the degree of tapasin interaction with the MHC I complex. It was recently shown that these differences in tapasin-dependency were mirrored by the susceptibility of the MHC I alleles to reduction of the α2 disulfide bond by ERp57. Tapasin-independent alleles like HLA-B*4405 were found to be insensitive to
α2 disulfide bond reductions, while tapasin dependent alleles such as HLA-B*4402, which differ from B*4405 only by a Asp→Tyr substitution at position 116 (115), indicating this residue also influences tapasin dependency, were sensitive (108). It therefore appears that tapasin-dependency is directly linked to tapasin’s restraining effect on ERp57-mediated reduction of this particular disulfide bond of MHC I molecules, with susceptibility to reduction determined by the amino acids at positions 114 and 116 in HLA molecules (108;110;115). While these differences in tapasin-dependency have been better characterized in the more divergent human alleles of MHC I, it appears that mouse MHC I molecules also display variability in tapasin-dependency as well (116).

1.9.1 Models of tapasin-deficiency in humans and mice

Tapasin-deficient mice demonstrate greatly reduced cell surface expression of the murine MHC Class I molecules H-2K\textsuperscript{b} and H-2D\textsuperscript{b}, though this reduction is not as profound that observed in TAP1\textsuperscript{−/−} mice (38;117;118). In addition, the number of CD8\textsuperscript{+} T cells in the periphery of tapasin\textsuperscript{−/−} mice is reduced, likely due to impaired positive selection because of low MHC I expression in the thymus (117;118). The tapasin\textsuperscript{−/−} mice also exhibit deficient CTL responses to viruses and impaired cross-presentation of antigen by tapasin\textsuperscript{−/−} DCs (117). The phenotype of tapasin\textsuperscript{−/−} mice is consistent with that of human cells deficient in tapasin, such as the LCL 721.220 B lymphoblast cell line (“.220”) (119;120). Both human and mouse cells deficient in tapasin exhibit low, thermolabile MHC I expression due to impaired peptide loading (117;120). This phenotype can be reversed by the introduction of wild-type tapasin (77). The phenotype of mouse and human tapasin-deficient cells does differ somewhat however, in that human MHC I molecules seem to be retained to a greater extent in the ER in the absence of
tapasin (121;122), whereas mouse MHC I molecules escape the ER at higher rates in tapasin-deficient cells but are loaded with suboptimal peptides and quickly turn over at the cell surface (89;90;121;122) (see Figure 1.13). There has been one report of bare lymphocyte syndrome (BLS) in humans caused by a partial deletion of the patient’s TAPASIN gene. MHC I surface expression on the patient’s cells was greatly reduced, but not to the same extent as patients suffering from BLS caused by TAP defects (123). This is also consistent with the tapasin\(^{-/-}\) mouse phenotype.

**Figure 1.13 MHC Class I assembly in wild-type, tapasin- and TAP-deficient cells.**

In both human and mouse tapasin-deficient cells, surface MHC Class I expression is impaired. Interactions of MHC Class I with calreticulin, ERp57 and TAP are impaired, and TAP protein levels are less stable, reducing peptide transport into the ER. However, some differences between human tapasin-deficient cells (721.220) and mouse tapasin-deficient cells (from tapasin\(^{-/-}\) mice) have been observed. In human tapasin-deficient cells, Class I molecules accumulate in the ER and are eventually degraded via ERAD mechanisms. However, in mouse tapasin-deficient cells Class I molecules are transported to the cell surface but are unstable at 37°C due to loading of suboptimal peptides and turn over rapidly for degradation. TAP-deficient cells are more similar in mouse and humans: in both, very few Class I molecules leave in the ER due to the lack of peptides for binding. Class I molecules are still able to interact with tapasin, calreticulin and ERp57 but are eventually degraded because of their instability in the absence of peptide. Abbreviations: crt, calreticulin; ER, endoplasmic reticulum; HC, heavy chain of Class I; TAP, peptide transporter associated with antigen processing; tpn, tapasin. Reproduced from (122) with permission.
1.10 Identification of isoforms of human tapasin

We have identified two new isoforms of human tapasin that result from alternative splicing of the C-terminal end of the tapasin mRNA, generating novel C-termini that we predicted would result in a soluble form of tapasin and a non-ER membrane bound form of tapasin. Based on predicted protein sequence, it appears that these two previously-unknown tapasin isoforms are generated by an “exon-skipping” mechanism of RNA splicing. Normally-spliced tapasin has eight exons (124), whereas the two novel isoforms lack exon 7 (“isoform 2”) and exon 6 + exon 7 (“isoform 3”), respectively (see Figure 1.14, full sequences in Appendix A). This leads to a frame shift and novel C-terminal ends lacking the ER dilysine retention signal “KKAE” (82;125) and instead encoding 26 amino acids that form no known motifs. Isoform 2 retains the tapasin transmembrane domain and is predicted to localize to a non-ER membrane. Isoform 3 lacks a discernable transmembrane domain and is predicted to be soluble and likely secreted from the cell. Both novel isoforms encode the N-terminal ERP57- and MHC I-binding domain, and thus may be capable of recruiting other members of the peptide loading complex.
Figure 1.14 Protein sequence of the tapasin isoforms.
Isoform 1 (wild-type tapasin) contains eight exons. Isoform 2 lacks exon 7 and isoform 3 lacks both exons 6 and 7. Both encode novel C termini in exon 8 due to a frame-shift, which lack the “KKAEE” dilylsine ER-retention motif but do not contain any other predicted motifs. Isoform 3 is predicted to be soluble because it lacks the entire transmembrane domain encoded by exon 6 and part of exon 7. Isoform 2 is still predicted to contain a whole transmembrane domain and may localize to a non-ER membrane. Sequence based on (124).

1.11 Subversion of tapasin in disease

The importance of tapasin in CD8+ T cell mediated-immune responses can be inferred from the large number of pathogens and diseased states that have evolved molecular mechanisms to subvert tapasin’s actions.

1.11.1 Viruses

Several viruses have acquired or evolved inhibitors of tapasin, highlighting its importance in anti-viral immunity. For example, the human cytomegalovirus protein US3 binds tapasin directly and inhibits peptide loading onto tapasin-dependent alleles (126). Interestingly, however, tapasin-independent alleles are not affected by this mechanism, providing some evolutionary insight into why alleles of differing tapasin dependency might have evolved in the first place. The adenovirus E19 protein binds and retains MHC I in the ER; however, it also acts on tapasin by binding TAP and preventing tapasin/MHC
I association with the TAP complex, slowing trafficking of MHC I to the cell surface (127). These examples suggest that subversion of tapasin provides a selective advantage for the virus, allowing it to avoid detection by anti-viral CD8\(^+\) T cells and thus produce greater numbers of progeny virus.

### 1.11.2 Cancer

Tapasin expression has been shown to be downregulated in certain forms of cancer, particularly carcinomas (128-130). This downregulation is often accompanied by downregulation of many other APM components as well, including TAP1, TAP2 and the proteasome components LMP2 and 7, among others (131-135). The functional consequence of downregulation of some or all of these components is a lack of MHC I surface expression. Tumor cells often express tumor associated antigens (TAAs): proteins that are either mutated or not present in normal cells, such as fetal or tissue-specific proteins (136-138). Peptides derived from TAAs can be presented on the cell surface for surveillance and killing of the tumor cell by CD8\(^+\) T cells. This limits the progression and spread of the tumor in many cases. However, tumor cells that lack expression of APM components can avoid presenting TAA-derived peptides on the cell surface and therefore evade T cell recognition, allowing the tumor to grow unchecked by the immune system. Many types of tumors, particularly metastases, exhibit this MHC I-deficient phenotype (130;137;139-141). The mechanisms underlying this phenotype vary, and include deletions, mutations or downregulation of some or all of the APM components (142). The latter group displaying downregulation of APM components is very large, and has in many cases been traced to dysregulation of the expression of APM components at the transcriptional level, since the defects can often been overcome by treatment with IFN\(\gamma\).
GENERAL INTRODUCTION

(143). Several initiatives attempting to restore tumor antigen presentation in these types of tumors are underway in an effort to develop novel therapeutic approaches that take advantage of the immune system’s natural ability to eliminate cancer (144-148).

1.12 Project rationale

This thesis examines the expression, localization and function of the two alternatively-spliced forms of human tapasin in antigen presentation, and the role and restoration of normally-spliced tapasin and other APM components in tumors.

The two novel tapasin isoforms retain their N-terminal ERp57- and MHC I-binding domains (86), and could therefore act as scaffolds for peptide-loading complex assembly in cellular compartments other than the ER, such as at the cell surface or within endolysosomes. The presence of tapasin in other cell compartments and/or the cell surface could have very important roles in loading peptides onto MHC I molecules and generating immune responses, and could explain observed immunological phenomena. For example, peptides added to the extracellular media can bind MHC I molecules present on the surface of cells, apparently in the absence of the antigen processing machinery used to load peptides onto MHC I in the ER (67;149). The presence of tapasin at the cell surface could influence the loading and exchange of peptides bound in the ER with peptides at the cell surface. In addition, specialized antigen presenting cells such as dendritic cells can cross-present exogenously-derived antigenic peptides on MHC I molecules in an ER-independent fashion in endolysosomes, as described in section 1.6 (22;46;50). A non-ER retained form of tapasin could potentially be involved in the loading of these peptides onto MHC I molecules in the cellular compartment(s) where cross-presentation is believed to occur (26).
APM components, including tapasin, are often downregulated in tumor cells, which is believed to contribute to their escape from immune recognition and elimination. Restoration of APM component expression in tumor cells might allow presentation of tumor-associated peptides to be resurrected, allowing recognition and killing of the tumor by the body’s own T cells. Prior investigations in the Jefferies laboratory and others have indicated that TAP1 re-expression is key to restoring the antigen presentation pathway in tumor cells (146;147;150-154), but how TAP1 alone accomplishes this is not well understood. Furthermore, the effects of re-expression of other components such as tapasin have not been tested, nor has the effect of ectopically-expressing several APM components in combination. This thesis examines the effect of restoring TAP1 expression alone in a metastatic mouse melanoma cell line, B16F10, which is deficient in several APM components and surface MHC I expression (155). The effect of TAP1 re-expression on the expression of other APM components including tapasin, as well as the functional consequences on the immunogenicity and growth of the tumor cells both in vitro and in vivo is assessed. Furthermore, the effect of recombinant adenovirus vector-driven expression of human tapasin (isoform 1) in an MHC I-deficient metastatic murine lung carcinoma model, CMT.64, is tested and compared in mice with a combination treatment of human tapasin and human TAP1 in tumors formed from this cell line.

1.13 Thesis synopsis

- Chapter 3 describes the expression and subcellular localization of the tapasin isoforms. The expression of the tapasin isoforms at the mRNA level was examined in a variety of tissues and cell types since it was hypothesized that the isoforms would be overexpressed in tissues and cells involved in cross-
presentation. The subcellular localization of the isoforms was examined in both tapasin<sup>-/-</sup> fibroblasts and the DC2.4 dendritic cell line by immunofluorescence and confocal microscopy using a panel of organelle-specific antibodies. It was hypothesized that the novel isoforms would be non-ER localized due to their lack of a C-terminal ER-retention motif. Results demonstrate that the tapasin isoforms are expressed in patterns consistent with a role in cross-presentation, with high levels in mature DCs and lymphoid tissues. While the isoforms co-localized extensively with ER markers, some co-localization with trans-Golgi network and endo/lysosomal markers, indicative of compartments where cross-presentation could be occurring, was observed.

• Chapter 4 examines the functional characteristics of the tapasin isoforms in antigen presentation. It was hypothesized that the tapasin isoforms might be capable of restoring MHC I antigen presentation in tapasin<sup>-/-</sup> cells, but that their true role would be in MHC I cross-presentation of exogenous antigens. The function of the isoforms in antigen presentation was assessed in tapasin<sup>-/-</sup> fibroblasts and the DC2.4 dendritic cell line. It was found that isoforms 1 and 2 mediated very similar effects on endogenous MHC I presentation. Isoform 3 was not able to contribute to this pathway because it did not stabilize TAP, rendering endogenous (and exogenous) MHC I antigen presentation non-functional in tapasin<sup>-/-</sup> cells expressing only isoform 3. However, isoform 3 did appear to have a role in inhibiting the loading of exogenously-added peptides onto MHC I. Assays of cross-presentation ability in cells expressing the isoforms indicated no
major differences amongst the different isoforms in this pathway, and no dominant negative or synergistic effects were observed.

- Chapter 5 tests the immunological and anti-tumor effects of TAP1 expression in B16F10 melanoma cells and tapasin expression in CMT.64 lung carcinoma cells. The stabilization of other APM components, as well as the functional consequences of this expression on MHC I antigen presentation and tumor growth \textit{in vivo}, were examined. It was hypothesized that TAP1 expression would reconstitute antigen presentation in these tumor cells, leading to decreased tumor growth \textit{in vivo} due to increased immune recognition of tumor-specific antigens by CD8$^+$ T cells. It was not expected that tapasin alone could mediate the same effect. It was found that, indeed, TAP1 expression led to increased MHC I presentation of tumor-associated antigenic peptides on the surface of the tumor cells, making them more susceptible to CTL killing. Furthermore, TAP1 expression increased endogenous tapasin expression. These results also had \textit{in vivo} significance, as TAP-treated tumors exhibited decreased growth compared to non-TAP-expressing controls. Surprisingly, tapasin expression also mediated similar increases in MHC I surface expression and susceptibility to CTL killing in CMT.64 lung carcinoma cells. This occurred without the concurrent increase in protein expression of other APM components observed with TAP1 re-expression. Nevertheless, survival of tumor-bearing mice treated with adenoviruses expressing human tapasin was significantly improved over vector alone controls, and an increase in tumor-infiltrating lymphocytes and dendritic cells was also
observed in tumors from tapasin-treated mice. Combining Tapasin and TAP1 treatments led to maximal protection.

- The overall objective of this research was to contribute to our understanding of antigen processing and presentation in both normal and malignant cells at the molecular level, with special emphasis on the role of tapasin.
Chapter 2: Materials and Methods

2.1 Cell lines and stable transfections

Cell lines in were grown in either RPMI 1640 or DMEM medium supplemented with 10% heat inactivated fetal bovine serum (HyClone, Logan, UT, USA), 2mM L-glutamine, penicillin (100 IU/ml), streptomycin (100 μg/ml), and 20mM HEPES.

Table 2.1 Cell lines used in this thesis

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Description</th>
<th>Media</th>
<th>Source/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>721.221</td>
<td>Human lymphoblastoid B cell line</td>
<td>RPMI</td>
<td>(112;156) Dr. Peter Cresswell, Yale University, New Haven, CT, USA</td>
</tr>
<tr>
<td>721.220</td>
<td>Human lymphoblastoid B cell line deficient in tapasin protein</td>
<td>RPMI</td>
<td>(112;120) Dr. Peter Cresswell, Yale University, New Haven, CT, USA</td>
</tr>
<tr>
<td>B16F10</td>
<td>Tumor-forming mouse melanoma cell line, surface MHC I-deficient, C57BL/6 background</td>
<td>RPMI</td>
<td>(155)</td>
</tr>
<tr>
<td>B3Z</td>
<td>Mouse T cell hybridoma that produces β-galactosidase under the control of an NF-AT promoter in response to engagement with H-2K&lt;sup&gt;b&lt;/sup&gt;/SIINFEKL</td>
<td>RPMI</td>
<td>(157) Dr. Nilabh Shastri, UC Berkeley, Berkeley, CA, USA</td>
</tr>
<tr>
<td>BJA-B</td>
<td>Human B cell line</td>
<td>RPMI</td>
<td>(158)</td>
</tr>
<tr>
<td>C57BL/6 fibroblasts</td>
<td>Immortalized ear fibroblast cell line derived from a C57BL/6 mouse</td>
<td>DMEM</td>
<td>Dr. Luc Van Kaer, Vanderbilt University School of Medicine, Nashville, TN, USA</td>
</tr>
<tr>
<td>CMT.64</td>
<td>Tumor-forming mouse lung carcinoma cell line, surface MHC I-deficient, C57BL/6 background</td>
<td>DMEM</td>
<td>(159)</td>
</tr>
<tr>
<td>CMT.64/VSV-NP</td>
<td>CMT.64 transfected with vesicular stomatitis virus nucleocapsid protein (VSV-NP) minigene containing the immunodominant epitope from amino acids 52 to 59 presented on H-2K&lt;sup&gt;b&lt;/sup&gt;</td>
<td>DMEM</td>
<td>Jefferies Laboratory (146)</td>
</tr>
<tr>
<td>COS-1</td>
<td>African Green Monkey kidney cell line transformed with SV40 large T antigen</td>
<td>DMEM</td>
<td>ATCC #CRL-1650</td>
</tr>
<tr>
<td>CRE8</td>
<td>HEK293 cell line stably expressing CRE recombinase for generating recombinant adenovirus</td>
<td>DMEM</td>
<td>(160), Dr. Calvin Vary, Maine Medical Centre Research Institute, Maine, ME, USA</td>
</tr>
<tr>
<td>CV-1</td>
<td>African Green Monkey kidney cell line</td>
<td>DMEM</td>
<td>ATCC #CCL-70</td>
</tr>
</tbody>
</table>
### Table 1: Cell Lines and Media

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Type</th>
<th>Media</th>
<th>ATCC/Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daudi</td>
<td>Human B cell line, C37BL/6 background</td>
<td>RPMI</td>
<td>ATCC #CCL-213</td>
</tr>
<tr>
<td>DC2.4</td>
<td>Mouse dendritic cell line, C37BL/6 background</td>
<td>RPMI</td>
<td>Dr. Ken Rock, University of Massachusetts, MA, USA</td>
</tr>
<tr>
<td>HEK-293</td>
<td>Human embryonic kidney cell line</td>
<td>DMEM</td>
<td>ATCC #CRL-1573</td>
</tr>
<tr>
<td>HeLa</td>
<td>Human cervical carcinoma cell line</td>
<td>DMEM</td>
<td>ATCC #CCL-2</td>
</tr>
<tr>
<td>Jurkat</td>
<td>Human T cell line, clone E6-1</td>
<td>RPMI</td>
<td>ATCC #TIB-152</td>
</tr>
<tr>
<td>KG-1</td>
<td>Human monocyte/DC-like cell line</td>
<td>RPMI</td>
<td>ATCC #CCL-246</td>
</tr>
<tr>
<td>Raji</td>
<td>Human B cell line</td>
<td>RPMI</td>
<td>ATCC #CCL-86</td>
</tr>
<tr>
<td>Ramos</td>
<td>Human B cell line</td>
<td>RPMI</td>
<td>ATCC #CRL-1596</td>
</tr>
<tr>
<td>RMA</td>
<td>Mouse lymphoma, C37BL/6 background</td>
<td>RPMI</td>
<td>ATCC #CRL-1592</td>
</tr>
<tr>
<td>T1</td>
<td>Human B cell/T cell fusion cell line</td>
<td>RPMI</td>
<td>ATCC #CRL-1991</td>
</tr>
<tr>
<td>Tapasin⁺ fibroblasts</td>
<td>Immortalized ear fibroblast cell line derived from a Tapasin⁺ mouse on a C37BL/6 background</td>
<td>DMEM</td>
<td>Dr. Luc Van Kaer, Vanderbilt University School of Medicine, Nashville, TN, USA</td>
</tr>
<tr>
<td>TAP1⁻ fibroblasts</td>
<td>Immortalized ear fibroblast cell line derived from a TAP1⁻ mouse on a C37BL/6 background</td>
<td>DMEM</td>
<td>Dr. Luc Van Kaer, Vanderbilt University School of Medicine, Nashville, TN, USA</td>
</tr>
<tr>
<td>U937</td>
<td>Human monocyte cell line</td>
<td>RPMI</td>
<td>ATCC #CRL-1593.2</td>
</tr>
</tbody>
</table>

The clones of rat TAP1 (rTAP1) and vector-only transfectants of B16F10 cells were created by transfecting the cells with rTAP1 cDNA in the mammalian expression vector pβ(4pr-1neo) and were selected and maintained in 1 mg/ml Geneticin (Invitrogen, Burlington, ON, Canada) in RPMI-1640 medium. Two rTAP1-transfected clones were designated as B16/rTAP1 3-3 and B16/rTAP1 3-8, and a clone to control for the transfection vector was designated as B16/PHβ 1-1.

### 2.2 Molecular biology

#### 2.2.1 Identification and cloning of the tapasin isoforms

The tapasin isoforms were identified by RT-PCR of a human splenic cDNA library (Clontech Marathon-Ready cDNA, Mountain View, CA) using PCR primers
spanning exon 1 and exon 8 of human tapasin (see Table 2.2) and Pfu polymerase (Stratagene, La Jolla, CA). PCR conditions used were 95°C for 3 min, followed by 35 cycles of 95°C 1 min, 60°C 1 min, 72°C 2 min, with a final 10 min 72°C extension step. Human tapasin cDNA thus obtained by PCR was subcloned into the pCR-Blunt II-TOPO cloning vector (Invitrogen, Burlington, ON), transformed into TOP10 *E. coli* bacteria (Invitrogen) and plated on selective kanamycin LB agar plates. Individual bacterial colonies were screened for the tapasin cDNA insert by DNA sequencing, whereupon it was noted that while most sequences corresponded to full-length wild-type human tapasin cDNA encoding eight exons, a minor fraction of the clones differed at their C-termini, lacking either exon 7 alone or exon 6 and 7. These clones were isolated together with full-length tapasin cDNA and subcloned into the retroviral pMX-pie vector using restriction enzymes BamHI (New England Biolabs, Pickering, ON) and BglII (New England Biolabs) - converted from an XbaI site in the pCR-BluntII-TOPO vector with an oligonucleotide adaptor 5’-CTAGAGATCT-3’- to separate the cDNA fragment from the TOPO vector backbone. T4 DNA ligase (New England Biolabs) was used to ligate the gel-purified fragment into the BamHI site of pMX-pie. Sequence integrity and correct orientation within the vector was verified by DNA sequencing of the final pMXpie constructs.
Table 2.2 PCR primers used in this thesis (all synthesized by Sigma-Genosys). Primers sequences are underlined and in italics in the DNA sequence in Appendix A.

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Primer sequence (5’-3’)*</th>
<th>PCR product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exon 1 + Exon 8</td>
<td>F: GCCATGAAGTCCCTGTCTCTG R: GGGATTAGGAGCAGATGATAGGGTA</td>
<td>1448 (iso1) 1413 (iso2) 1323 (iso3)</td>
</tr>
<tr>
<td>Exon 5 + Exon 8</td>
<td>F: AGGTCACCCTGGAGGTAGCA R: GGGATTAGGAGCAGATGATAGGGTA</td>
<td>256 (iso1) 221 (iso2) 131 (iso3)</td>
</tr>
<tr>
<td>Exon 5 + Exon 7-8</td>
<td>F: CACCACCTGAGCAGCATGGGGCACGC R: TCACCTCTGCTTTCTTTTGAATCTTTTG</td>
<td>235</td>
</tr>
<tr>
<td>Exon 5 + Exon 6-8</td>
<td>F: CACCACCTGAGCAGCATGGGGCACGC R: TCACTCTGCTTTTCAGCCC</td>
<td>220</td>
</tr>
<tr>
<td>Exon 5 + Exon 5-8</td>
<td>F: CACCACCTGAGCAGCATGGGGCACGC R: TCACCTGCTTTTCGCTTA</td>
<td>110</td>
</tr>
<tr>
<td>S15</td>
<td>F: TTCCGCAAGTTTCACCTACC R: CGGGCCCGCCATGCTTTACG</td>
<td>361</td>
</tr>
<tr>
<td>Mouse TAP1</td>
<td>F: TGGCTCGTTGGGACCCCTCAA R: TCAGTCTGCGAGGAGCGGGAAGA</td>
<td>775</td>
</tr>
<tr>
<td>Mouse TAP2</td>
<td>F: GCTGTGGGGACTGCTAAAAG R: TATTGGGATTGAAAGGGAGC</td>
<td>665</td>
</tr>
</tbody>
</table>

* F = forward primer, R = reverse primer.

2.2.2 Isoform-specific PCR primers and conditions

PCR primers capable of amplifying all three isoforms simultaneously were designed to span exon 5 and exon 8 (Table 2.2), leading to a PCR product of 256 bp for isoform 1, 221 bp for isoform 2 and 131 bp for isoform 3 with the following conditions: 95°C 3 min, 35 cycles of 95°C, 60°C, 72°C with a final elongation step of 72°C for 10 min using Taq polymerase (Invitrogen). PCR primers were also designed to specifically amplify each isoform individually. A common forward primer within exon 5 was used for all three isoforms, while the isoform 1-specific reverse primer spanned exon 7 and 8, the isoform 2-specific primer spanned exon 6 and 8, and the isoform 3-specific primer spanned exon 5 and 8 (see Table 2.2 and Figure 3.1a). For all isoform-specific PCR
reactions, PCR conditions were 95°C 3 min, 30-40 cycles of 95°C, 60°C, 72°C with a final elongation step of 72°C for 10 min using Taq polymerase (Invitrogen) and a Stratagene Robocycler PCR machine. PCR bands were sequenced to confirm that the correct isoform was being amplified with each primer set.

2.2.3 RT-PCR

2.2.3.1 Human tissues

Total RNA from a panel of 21 different normal human tissues pooled from at least three donors (both male and female) per tissue was purchased from Ambion (Streetsville, ON). Using the SuperScript II kit with OligodT primers (Invitrogen) as per the manufacturer’s instructions, 2 μg of RNA was converted to cDNA, followed by RNase H (Invitrogen) digestion. In a 25 μl Taq PCR reaction, 1 μl of cDNA was used as template, except for isoform 3 where 2 μl were used, with primers specific for isoform 1, isoform 2, isoform 3 or S15 as a loading control (Table 2.2). PCR reactions were performed as described above, with 30 cycles for isoform 1, isoform 2 and S15 and 40 cycles for isoform 3. PCR products were separated on 2% agarose gels and visualized with SyberSafe (Invitrogen) on an AlphalImager (Alpha Innotech, San Leandro, CA). Spot densitometry was performed with the AlphaEaseFC software, version 6.0.0 (Alpha Innotech).

2.2.3.2 Human cell lines

Total RNA was isolated from cell lines used in Figure 3.3 with Trizol (Invitrogen) and DNase-1-digested (Fermentas, Burlington, ON). Superscript II and OligodT
(Invitrogen) were used to convert 1.5 μg of RNA to cDNA with, as per the manufacturer’s instructions, followed by RNase H (Invitrogen) digestion. RT-PCR was performed as described above for the human tissue panel with isoform 1-, isoform 2-, isoform 3- or S15-specific primers. PCR products were visualized and quantified by spot densitometry as described above for RT-PCR of human tissue samples.

### 2.2.3.3 Transduced murine cell lines

Immortalized ear fibroblasts from tapasin\(^{-/-}\) mice and C57BL/6 control mice generated by serial passaging were generously provided by Dr. Luc Van Kaer (Vanderbilt University, Nashville TN). Total RNA was extracted from tapasin\(^{-/-}\) fibroblasts and DC2.4 cells expressing each isoform or pMX-pie vector-alone, and 5 μg of RNA were converted to cDNA, as described above. RT-PCR was performed with the primer set that amplified all three isoforms simultaneously using 2.5 μl of template per 25μl PCR reaction. As controls, 2.5 μl of pMXpie plasmid containing each isoform at 10ng/ml were amplified with the same PCR mixture. To ensure equal loading of each PCR reaction with cDNA, 2.5 μl of template was also amplified 30 cycles with S15 primers. PCR products were separated on a 2% agarose gel as described above to confirm that the transduced population of tapasin\(^{-/-}\) fibroblasts and/or DC2.4 expressed the appropriate isoform.

### 2.2.3.4 TAP and tapasin expression in fibroblast cell lines

To assess mouse TAP1 and mouse TAP2 mRNA expression in tapasin\(^{-/-}\) fibroblasts (with TAP\(^{+/+}\) fibroblasts and DC2.4 cells as negative and positive controls, respectively), 1.5 μg of RNA was converted to cDNA as described above. PCR was
performed to detect mouse TAP1, mouse TAP2 (Table 2.2) or S15 loading controls with PCR conditions of 95°C 3min, 35 cycles of 95°C, 60°C, 72°C with a final elongation step of 72°C for 10 min using Taq polymerase (Invitrogen).

### 2.2.4 Real time PCR

Human B cells, monocytes and monocyte-derived dendritic cells were obtained from peripheral blood of healthy volunteers in the laboratory of Anne Hosmalin (Institut Cochin, France). B cells were isolated using CD19 MACS beads. Monocytes were obtained with a Monocyte Negative Isolation Kit (Invitrogen-Dynal) and checked for purity with CD14 and CD16 antibodies. Monocytes were differentiated into DCs in culture with GM-CSF (500 U/ml) and IL-4 (100 ng/ml) 6-7 days in RMPI with 10% FCS. DC cell-specific markers HLA-ABC, HLA-DR, CD1a, CD80, CD83, CD14 and CD16 were used to check DC phenotype and maturation status. DCs were matured by treatment with 100 ng/ml of LPS and 20 pg/ml IFNγ for indicated time periods, and verified for upregulation of DC activation markers (CD80, CD86, CD83) by flow cytometry. Cells were washed twice in PBS and frozen in RNAlater (Ambion), then total RNA was isolated with Trizol (Invitrogen) and DNase-I digested to remove genomic DNA contaminants. Equal amounts of RNA per sample (600-800 ng, depending on RNA yield for each repetition) were converted to cDNA with Superscript II and OligodT as per the manufacturer’s instructions, followed by RNase H digestion. Real-time PCR was used to evaluate expression of the different isoforms, normalized with the amount of S15 in each sample using standard curves for each primer set. S15 rRNA is often used as a “housekeeping” gene/loading control for RT-PCR because its expression level is
relatively constant across different cell lines and tissues, and is not often affected following drug treatments.

2.3 **Immunoprecipitation and western blots**

2.3.1 **Human cell lines**

Human tapasin protein expression was determined in various human cell lines by harvesting $8 \times 10^6$ cells of each type. Cells were washed once in PBS and lysed for 30 min on ice in 500 μl of lysis buffer (1% NP-40, 20mM Tris-HCl PH 8.0, 150mM NaCl, 5mM EDTA and 1 Complete Mini EDTA-free protease inhibitor cocktail (Roche, Mannheim, Germany) per 10mL). Nuclei were pelleted by centrifugation for 15 min at 4°C at 10,000Xg and samples were pre-cleared with 40 μl of pre-washed Protein G sepharose bead slurry (Protein G Sepharose 4 Fast Flow; Amersham Biosciences, Arlington Heights, IL) rotating overnight at 4°C. Human tapasin protein was immunoprecipitated with 1 μl of PaSta.1 mouse monoclonal antibody to human tapasin (105) (generously provided by Dr. Peter Cresswell, Yale University, New Haven, CT) rotating for four hrs at 4°C. Forty microlitres of Protein G sepharose bead slurry pre-washed with lysis buffer was added for 1 hr at 4°C, followed by 3 washes with lysis buffer before suspension in 80 μl of 2X protein sample buffer containing β-mercaptoethanol (Roche). Samples were heated to 95°C for 5 min and 20 μl were loaded onto a 10% SDS-polyacrylamide gel/5% stacking gel. Proteins were separated at 100V for 1.5hrs, followed by wet transfer to PVDF membrane (Biorad, Hercules, CA) at 100V for 1.5hrs. Membranes were blocked for 2 hrs at room temperature with blocking buffer (5% skim milk, 0.2% Tween-20 in PBS), followed by overnight incubation with a 1/2000 dilution of rabbit anti-human
MATERIALS AND METHODS

tapasin polyclonal (Rgp48N, generously provided by Dr. P. Cresswell, Yale University, (83)) in blocking buffer. The following day membranes were washed 4 times in blocking buffer for 10 min, incubated with 1/10,000 dilution of LI-COR secondary (AlexaFluor 680 goat anti-mouse or anti-rabbit IgG (H+L) (Molecular Probes, Eugene, OR)) for 1 hr room temperature, washed 3 times in blocking buffer for 15 min, followed by a final 15 min wash in PBS + 0.2% Tween-20 and visualization on a LI-COR Biosciences Odyssey Infrared Imager with Odyssey 2.0 software version 2.0.41. As a loading control, a mouse monoclonal to GAPDH (MAB374, Chemicon International) was used at 1/5000 with a secondary goat anti-mouse control (1/10,000) on 5 μl of lysate remaining followed immunoprecipitation, similarly separated by SDS-PAGE and blotted on PVDF membranes as described.

2.3.2 Transduced murine cell lines

To assess expression of the tapasin isoforms in transduced tapasin−/− fibroblasts and transduced DC2.4, 5x10^6 tapasin−/− fibroblasts or 1.4x10^7 DC2.4 cells expressing the tapasin isoforms or vector alone as a negative control were washed once in PBS and lysed in 500 μl of lysis buffer as described for the human cell lines above. A human B cell/T cell fusion cell line (T1) expressing high levels of human tapasin or the Raji B cell line expressing both isoform1/3 and isoform 2 proteins were used as a positive controls. Immunoprecipitation and SDS-PAGE were performed as described for the human cell lines, except incubation with the primary antibody for immunoprecipitation was overnight and a HRP-conjugated goat anti-rabbit IgG (H+L) (Jackson ImmunoResearch, West Grove, PA) was used as a secondary antibody for tapasin blotting and HRP-conjugated goat anti-mouse IgG (H+L) (Pierce, Rockford, IL) for GAPDH loading
control blotting, followed by standard ECL detection (Perkin Elmer Chemiluminescence Reagent, Boston, MA).

2.3.3 mTAP1 and mTAP2 expression in fibroblast cell lines

Four million tapasin−/− fibroblasts expressing the tapasin isoforms or vector alone as a negative control (C57BL/6 fibroblasts were the positive control) were lysed in 200 μl of 1% NP-40 lysis buffer as described above, and mixed with an equal volume of 2X protein sample buffer. Using SDS-PAGE, 25 μl of sample were separated on 10% separating, 4% stacking gels, transferred to PDVF and blocked as described. Membranes were incubated overnight with 1/2000 dilutions of rabbit polyclonal sera against either mTAP1 (TAP1 peptide sequence RGGCYRAMVEALAAPAD-C, (148)) or mTAP2 (made in the Jefferies lab by Xiao-Lin Li against the last 16 C-terminal amino acids of mouse TAP2 conjugated to KLH, (147)) or GAPDH as a loading control, followed by detection with HRP-conjugated secondary antibodies and ECL chemiluminescence as described in section 2.3.1.

Immunoprecipitation was performed on 1 mL of lysate made from 2x10⁷ cells, lysed as described in 1% NP-40 buffer, precipitated with 2 μl of rabbit antisera to mouse TAP1 or TAP2 and fifty microlitres of Protein G sepharose overnight. Samples were washed 3X in 1% NP-40, mixed with 60 μl of 2X protein sample buffer, separated by SDS-PAGE and probed by Western blot as described above for tapasin−/− and C57BL/6 fibroblasts.
2.3.4 ERp57 co-immunoprecipitation with tapasin isoforms

Immunoprecipitation, co-immunoprecipitation and Western blots were performed on 400 μl of lysate made from 2.5x10^6 tapasin^-/- fibroblasts expressing tapasin isoforms or vector alone, lysed as described in 1% NP-40 buffer. One hundred microlitres were removed for Western blot for ERp57 from whole cell lysates. To immunoprecipitate human tapasin, the remaining lysate was precleared for 2hrs rotating at 4°C with 50 μl of Protein G sepharose, and incubated overnight precipitated with 1 μl of PaSta.1 anti-human tapasin mouse monoclonal and fifty microlitres of Protein G sepharose. Samples were washed 3X in 1% NP-40, mixed with 80 μl of 2X protein sample buffer, separated by SDS-PAGE in duplicate and probed by Western blot on PDVF membranes with either the Rgp48N rabbit polyclonal to human tapasin or rabbit anti-ERp57 (Stressgen #SPA-585). Both primary antibodies were used at 1/2000 dilutions with HRP-conjugated anti-rabbit secondary antibody (Jackson ImmunoResearch) at 1/10,000.

2.3.5 Detection of APM protein expression in B16F10 transfectants and transductants by immunoblotting

Rat TAP1 and mouse TAP2 expression in B16/rTAP1 3-3 cells and B16/rTAP1 3-8 cells was examined by immunoblotting. Total extracts from 5x10^5 cells were separated on 10% SDS-PAGE gels and blotted onto nitrocellulose membranes. The blots were probed for mouse or rat TAP1 and TAP2 with relevant, specific, rabbit antiserum (described below) at a 1/2000 dilution. The blots were then incubated with HRP-labelled anti-rabbit IgG antibodies at a 1/20,000 dilution. The immune complexes were visualized by ECL according to the instructions of the manufacturer (GE Healthcare, Chalfont St.
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Giles, UK). The rabbit antiserum against mouse and rat TAP1 protein (148) was created by immunizing rabbits with a TAP1 peptide sequence, RGGCYRAMVEALAAPAD-C (common to both species), with a cysteine at the C-terminal, linked to keyhole limpet hemocyanin (Pierce Biotechnology Inc., Rockford, IL, USA). The specificity of the antiserum was confirmed by the detection of a band of approximately 70 kDa in size in lysates from TAP-expressing cells (RMA) that was absent in lysates of fibroblasts derived from TAP1−/− mice. The rabbit serum against mouse and rat TAP2 (116/4) was kindly provided by Dr. Geoff Butcher (University of Cambridge, Cambridge, UK). For mouse tapasin expression, 1.2x10^6 cells were lysed in 1% NP-40 lysis buffer for 30 min on ice, spun at 10,000Xg for 15 min at 4°C, and pre-cleared with 50 μl of Protein G-sepharose (GE Healthcare). Tapasin was immunoprecipitated with rabbit anti-tapasin antiserum number 2668 (163) (courtesy of Dr. Ted Hansen, Washington University School of Medicine, St. Louis, MO, USA) and Protein G sepharose, followed by separation by 12% SDS-PAGE, transferred to PDVF membranes (GE Healthcare), and probed with the same antiserum followed by ECL as described above.

B16F10 cells growing as a monolayer were infected with AdhTAP1 or Ψ5 at 50 PFU/cell. T1 cells were used as a positive control for human TAP1 expression. B16F10 cells treated with IFN-γ and untreated B16F10 cells were positive and negative controls, respectively, for mouse TAP1 (mTAP1), mouse TAP2 (mTAP2) and mouse tapasin (mTpn) expression. Two days after infection, the cells were washed with Tris saline (10 mM Tris HCl, pH 7.4, 120 mM NaCl) and extracted on ice for 50 min in RIPA buffer containing 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 1mM phenylmethylsulfonylfluoride, and aprotinin (1μg/ml) and a 1/100 dilution of protease
inhibitor cocktail (Sigma, Saint Louis, MO). Cell extracts were clarified by centrifugation 12,000Xg at 4°C for 15 min. The samples were subjected to SDS-PAGE, electrotransferred to nitrocellulose and probed with rabbit anti-hTAP1 antiserum with no cross-reactivity to mouse TAP1 (Stressgen Biotechnologies Corp, Victoria, BC, Canada), rabbit anti-mTpn antiserum (a gift from Dr. David Williams, University of Toronto), rabbit anti-mTAP1 and rabbit anti-mTAP2 antisera (made in the Jefferies lab as described above) and tested by Western blotting with fibroblasts from TAP1-expressing and TAP1-deficient mice), and mouse monoclonal anti-β-actin antibody (Sigma-Aldrich, Oakville, ON, Canada). The second antibodies were goat anti-rabbit IgG (H+L)-HRP and goat anti-mouse IgG (H+L)-HRP (Jackson ImmunoResearch Lab, West Grove, PA). Immunoreactive protein bands were visualized by exposure to Hyperfilm (Amersham Biosciences, Little Chalfont, Buckinghamshire, England) using an enhanced chemiluminescence (ECL) detection system (Amersham Biosciences).

2.3.6 APM expression after AdhTpn infection of CMT.64 cells

To examine Tpn and TAP expression in response to increasing doses of AdhTpn, CMT.64 cells were infected with AdhTpn at 1, 5, 25, 50, and 100 PFU /cell or Ψ5 (negative control) at 100 PFU/cell. T1 cells and 721.220 cells were, respectively, used as hTpn positive and negative controls. CMT.64 cells treated with IFN-γ were a positive control for mouse TAP1 (mTAP1), mouse TAP2 (mTAP2) and mouse Tpn (mTpn) expression. Two days after infection, cells were lysed and subjected to SDS-PAGE and electro-transferred to Hybond PVDF membrane (Amersham Biosciences, Buckinghamshire, England). The blot was treated with rabbit anti-hTpn antibodies (StressGen Biotechnologies Corp, Victoria, BC, Canada), rabbit anti- mTpn antibodies (a
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gift from Dr. David Williams, University of Toronto), rabbit anti-mTAP1 and rabbit anti-mTAP2 (described above), and mouse monoclonal antibody (mAb) against human β-actin (Sigma-Aldrich Oakville, ON, Canada). Goat anti-rabbit IgG (H+L)-HRP and goat anti-mouse IgG (H+L)-HRP (Jackson ImmunoResearch Lab, West Grove, PA) were used as secondary antibodies. The bands were visualized by enhanced chemiluminescence and exposure to Hyperfilm (Amersham Biosciences).

2.4 Generation of recombinant retroviruses and cell lines expressing the tapasin isoforms

2.4.1 Generation of retroviruses

Recombinant retroviruses encoding the human tapasin isoforms were generated by co-transfection of 2 μg of pMXpie plasmid encoding the tapasin isoform cDNA with 2 μg of pEco plasmid into BOSC23 cells plated in 10 cm plates at 80% confluence using FuGene6 (Roche) according to manufacturer’s instructions. Two days later, supernatant was removed from the transfected cells, fresh media was added, and supernatant was collected and pooled daily for four days and stored at 4°C. The viral supernatant was spun at 2000 rpm for 15 min at 4°C, filtered through 0.45 μm filters and flash frozen in dry ice/methanol in aliquots.

2.4.2 Infection of cell lines and selection

For retroviral infection, immortalized ear fibroblasts from tapasin−/− mice were plated in 10 cm dishes at 50% confluence, and viral supernatant was mixed at a 1:1 ratio with normal media with 4 μg/ml polybrene (hexadimethrine bromide) and incubated with
the cells at 37°C, 5% CO₂. After 48 hrs, cells were assessed for expression of GFP from the IRES-GFP cassette, and selected with 6 μg/ml puromycin for approximately two weeks until the majority of cells appeared GFP-positive. Cells were sorted on a FACSVantage cell sorter to select cells with equal GFP expression. Sorted cells were maintained in DMEM media with 5 μg/ml puromycin thereafter, with additional resorting if GFP levels diverged between populations. For transduction of the DC2.4 murine dendritic cell line, similar infections were performed but selection was with 2 μg/ml puromycin.

### 2.5 Construction of non-replicating adenovirus encoding human TAP1 (AdhTAP1) and human tapasin (AdhTpn)

Non-replicating adenovirus encoding hTAP1 under the control of human CMV immediate-early promoter (AdhTAP1) has been previously described in detail (146). See Appendix C for AdhTpn.

### 2.6 Flow cytometry

#### 2.6.1 Intracellular flow cytometry

To quantify the amount of human tapasin expressed by transduced tapasin⁻/⁻ fibroblasts and DC2.4, cells were harvested, washed once in PBS, and fixed with 3% paraformaldehyde in PBS for 30 min at room temperature. Cells were pelleted by centrifugation and resuspended in 0.1% saponin + 1% BSA in PBS for 30 min at room temperature to permeabilize cells and block non-specific antibody binding. Cells were then probed with a 1/500 dilution of the PaSta.1 monoclonal antibody to human tapasin
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for 30 min at 4°C, washed 3 times in 0.1% saponin + 1% BSA in PBS and incubated a further 30 min at 4°C with 1/500 dilution of AlexaFluor 647-conjugated goat anti-mouse secondary. Finally, the cells were washed 3 times in 0.1% saponin +1% BSA in PBS and assessed by flow cytometry with a FACSCalibur (Becton Dickinson, Oakville, ON) using CellQuest software. Results were analyzed with FlowJo version 4.5.9 (Tree Star Inc. & Stanford University, San Francisco CA).

2.6.2 Extracellular flow cytometry

To detect cell surface tapasin, a similar protocol was followed for intracellular flow cytometry described above, except the permeabilization step with saponin was omitted and cells were blocked and washed with 1% BSA in PBS alone at this step. For other experiments, cells were incubated at 4°C with saturating amounts of antibody for 30min, washed three times in PBS and, when unconjugated primary antibodies were used, incubated at further 30 min with fluorophore-conjugated secondary antibodies, washed three times in PBS and analyzed on a FACSCalibur or FACScan (Becton Dickenson) using CellQuest software. Results were analyzed with FlowJo version 4.5.9. Primary antibodies used were all used at 1/100 dilutions and were obtained from BD-Pharmingen (Mississauga, ON) except where indicated. These included PE anti-H-2Kb (clone AF.6-88.5) and PE anti-I-Ab (clone AF6-120.1). For H-2Db staining, undiluted supernatant from the 28.14.8S hybridoma (164) was used with a PE anti-mouse IgG + IgM F(ab’)2 (H+L) secondary (Jackson Immunoresearch) diluted 1/200. For H-2Kb/SIINFEKL complex detection, biotinylated antibody from the 25.D1.16 hybridoma (165) was used at 1/100 dilution with PE-Streptavidin secondary (Jackson Immunoresearch) at 1/200 dilution. For all flow cytometry involving DC2.4 cells, cells
were first incubated with Fc blocker (anti-mouse Fcγ III/II receptor clone 2.4G2, BD Pharmingen) diluted 1/100 for 30 min prior to staining with specific antibodies.

2.6.2.1 MHC Class I surface thermostability assay

Thermostability assays of surface H-2Kb were performed as described in (106). Briefly, tapasin<sup>−/−</sup> fibroblasts were incubated with 10 µg/ml of Brefeldin A (Sigma) and incubated for indicated time points, at which time cells were harvested, washed once in PBS, fixed in 3% paraformaldehyde, and stained with the Y3 (ATCC no. HB-176) anti-H-2Kb primary antibody followed by a PE anti-mouse IgG + IgM F(ab’<sub>2</sub>)<sub>(H+L)</sub> secondary (Jackson ImmunoResearch) antibody, and analyzed by flow cytometry as described above. The mean fluorescence intensity was compared at each time point to control cells that were not treated with BFA.

2.6.2.2 Exogenous SIINFEKL loading assay

Cells were harvested, counted, and approximately 5x10<sup>6</sup> were incubated in their respective supernatants with indicated amounts of SIINFEKL for 1 hr at 37°C, 5% CO₂, then washed three times in cold PBS, stained at 4°C for H-2Kb or H-2K<sup>b</sup>/SIINFEKL and analyzed, as described for extracellular flow cytometry. In experiments involving Brefeldin A (BFA), cells were first incubated with 10 µg/ml of BFA for 30 min prior to addition of SIINFEKL to the BFA-containing media, then assayed as per above. To detect an effect from soluble isoform 3, a 150 mm dish of tapasin<sup>−/−</sup> fibroblasts were incubated overnight with 0.45 µm-filtered supernatant from a confluent dish containing tapasin<sup>−/−</sup> fibroblasts expressing isoform 3, and assayed as described with vector alone and isoform 3-expressing tapasin<sup>−/−</sup> fibroblasts as controls.
2.6.2.3 Detection of surface H-2K\(^b\), H-2D\(^b\) antigen expression on B16F10 melanoma cells and CMT.64 lung carcinoma cells

B16F10 or B16/rTAP1 clone 3-8 cells were infected with VV-PJS-5 or VV-rTAP1 (multiplicity of infection (MOI) of 10) and incubated for 3 days (37\(^\circ\)C, 5% CO\(_2\)), followed by fixation and preparation for FACS analysis. Indirect immunofluorescence staining with conformational specific monoclonal antibodies (mAbs) for H-2K\(^b\) (Y-3) and H-2D\(^b\) (28.14.8.S) detected MHC Class I surface expression (166) (167). Aliquots of 10\(^6\) cells were incubated for 30 min at 4\(^\circ\)C with the primary Ab (50 µl undiluted hybridoma supernatant) for H-2K\(^b\) or H-2D\(^b\) antigens. After washing twice with PBS, the cells were re-suspended and incubated for 30 min at 4\(^\circ\)C in 1/100 FITC-conjugated rabbit anti-mouse IgG secondary Ab (Dakopatts, Glostrup, Denmark). A FACScan analyzer (Becton Dickinson) measured the mean logarithmic fluorescence intensity associated with the staining of surface antigens. Identical staining was performed for B16F10 following infection for 48 hrs with either AdhTAP1 or \(\Psi\)5 at 50 PFU/cell, and for CMT.64 cells infected with either AdhTpn or \(\Psi\)5 at 50 PFU/cell. Uninfected cells were used as negative controls, or treated with IFN-\(\gamma\) at 50 ng/ml for 48 hrs as positive controls.

2.7 Detection of secreted isoform 3

HEK293 cells were plated at 1x10\(^7\) cells/dish in 150 mm dishes in 20 mL of OptiPro Serum Free Media + 2mM glutamine (Invitrogen) and infected with recombinant adenovirus expressing isoform 1 or isoform 3 (Adiso1, Adiso3) at an MOI of 10 (described in Appendix C). Four hours later, 50 µl of protease inhibitor cocktail (Sigma
P1860) was added to the supernatant to prevent extracellular protein degradation. Forty-eight hours following infection, 20 mL of supernatant from the cells was collected and concentrated in Amicon Ultra 15 Centrifugal Filter Units 10 kDa cut-off (Millipore, Billerica, MA) for 30 min, reducing the volume to 500 μl (40X concentration). The infected cells were also collected and lysed in 300 μl of NP-40 lysis buffer for 30 min on ice, spun at 10,000Xg for 15 min, and the supernatant was mixed with an equal volume of 2X sample loading buffer. Protein levels in the samples were assessed by BCA assay (Pierce). Equal amounts of protein in each sample (whole cell lysate or concentrated supernatant) were separated by 10% separating/5% stacking SDS-PAGE, transferred to PVDF membranes and probed overnight with a 1/2000 dilution of the Rgp48N anti-human tapasin rabbit polyclonal antibody. Whole cell lysate samples were also probed for GAPDH protein loading controls. Western blots were visualized by probing either with 1/10,000 dilutions of HRP anti-rabbit or mouse secondary antibodies followed by ECL, or 1/10,000 dilutions with AlexaFluor 680 secondary antibodies (Molecular Probes) followed by laser LI-COR scanning as described.

### 2.8 Synthetic peptides

Vesicular stomatitis virus nucleoprotein (VSV)-NP$_{52-59}$ peptide (RGYVYQGL) and the B16F10 TAAs, tyrosinase-related protein-2 (TRP-2)$_{180-188}$ (VYDFFVWL) and gp100$_{25-33}$ (KVPRNQDWL), were made by the Peptide Synthesis Lab at the University of British Columbia. The purity of peptides was determined by HPLC to be >95% and the identities were confirmed by mass spectrometry. Lyophilized peptides were dissolved in DMSO at 10 mg/ml.
2.9 Cytotoxicity assays

2.9.1 Tapasin\(^{-}\) fibroblasts

Vesicular Stomatitis Virus, Indiana Strain (VSV), a gift from Frank Tufaro (University of British Columbia, Vancouver, Canada), was cultured on Vero cells (ATCC). CTLs were generated by intraperitoneal (i.p) inoculation of C57BL/6 mice with 3\(\times\)10\(^6\) tissue culture infectious particles (TCIP) of VSV. Splenocytes were harvested seven days later, separated into single cells, washed three times with PBS and incubated for 5 days at 37\(^{\circ}\)C, 5\% CO\(_2\) in 20 mL “CTL media” (RPMI-1640 containing 10\% heat-inactivated FBS (HyClone), 20 mM HEPES, 100 IU/ml penicillin, 100 \(\mu\)g/ml streptomycin, 0.1 mM non-essential amino acids, 1 mM Na-pyruvate, and 50 \(\mu\)M 2-ME) per spleen (approximately 10\(^6\) cells/ml) with 1\(\mu\)M of VSV-NP\(_{52-59}\) immunodominant peptide (RGYVYQGL). The day of the assay, CTLs were harvested, washed three times, counted and mixed with target cells at the indicated ratios. For target cells, cells were infected for 6 hrs with VSV at an MOI of 200, or left uninfected as negative controls or pulsed with VSV-NP\(_{52-59}\) exogenous peptide as positive controls. Cells were washed, resuspended in 200\(\mu\)l of CTL media per 2\(\times\)10\(^6\) cells and incubated with 10 \(\mu\)l (100 \(\mu\)Ci) of \(^{51}\)Cr (as sodium chromate; Amersham Biosciences) for 1 hr. Cells were washed 3X in PBS, counted and mixed with effector CTLs at the indicated ratios for 4 hrs at 37\(^{\circ}\)C, 5\% CO\(_2\). For maximal/minimal counts, target cells were either lysed with 5\% Triton X-100 or incubated with media lacking CTLs, respectively. Following the 4 hr incubation, cells were gently spun, 100 \(\mu\)l of supernatant was removed and quantified in a gamma counter (LKB Instruments, Gaithersburg, MD). To calculate percent specific killing, the
following formula was used: ((experimental - minimum control) / (maximum - minimum control)) x 100%.

### 2.9.2 B16F10 transfectants and transductants

All splenocytes were cultured in CTL media as described above. To generate H-2K^{b} antigen-restricted tyrosinase related protein-2 (TRP-2)-specific CTLs, TRP-2 peptide (VYDFFVWL) (100 μg) was mixed with 50 μl TiterMax adjuvant (Cedarlane Laboratories Ltd., Hornby, ON, Canada) and 50 μl PBS and injected subcutaneously (s.c.) into mice. This procedure was repeated after seven days. Fourteen days after the initial injection, mice received an additional injection (i.p.) with γ-irradiated RMA-S cells (5x10^6 cells in 300 μl). The irradiated RMA-S cells were prepared by incubating 5x10^6 cells with TRP-2 peptide (10 μg/ml peptide in 2 mL media) overnight at room temperature followed by γ-irradiation (10,000 rads). Cells were washed and re-suspended in PBS (300 μl). Seventeen days after the initial injection, the immunized spleen was removed and the splenocytes (10^8 cells) were cultured for five days with γ-irradiated naïve splenocytes (5x10^7 cells) pulsed with VYDFFVWL peptide (10 μg/ml).

To generate B16F10 tumor-specific CTLs, C57BL/6 mice were injected (i.p.) with γ-irradiated (10,000 rads) B16F10 cells, B16/PHβ 1-1 or B16/rTAP1 3-3 cells (3x10^6 cells/mouse). Five days after immunization, splenocytes were removed and cultured with stimulators at a 1:20 (stimulator/splenocyte) ratio for another 5 days at 37°C, 5% CO₂. The stimulators were prepared by incubating B16F10 cells, B16/rTAP1 3-3, or B16/PHβ 1-1 cells 1 hr at 37°C with mitomycin C (30 μg/ml) followed by γ-irradiated (10,000 rads) and three PBS washes before addition to the splenocyte culture.
The cytotoxic activities were measured in standard 4 hr $^{51}$Cr release assays. For TRP-2-specific and tumor-specific killing, B16F10 cells, B16/rTAP1 3-3, or B16/PHβ 1-1 target cells were untreated or infected with AdhTAP1 or Ψ5 at 50 PFU/cell or mock infected with PBS, labeled with Na$_2$$^{51}$CrO$_4$ (100 μCi/10$^6$ cells) (Amersham, Arlington Heights, IL) in 250 μl of complete RPMI medium for 1 hr at 37°C and washed extensively prior to incubation with indicated CTLs at indicated ratios for four hours. One hundred microlitres of supernatant were collected from each well, and the percentage of $^{51}$Cr release was calculated using the formula described above.

### 2.9.3 AdhTpn infected CMT.64/VSV-NP

Cytotoxicity was measured in a standard 4 hr $^{51}$Cr-release assay. In brief, stably-transfected CMT.64 cells (CMT/VSV-NP) expressing VSV-NP, which contains an immunodominant viral peptide consisting of amino acids 52-59, were infected with AdhTpn or Ψ5 at 50 PFU/cell for 1 day. These cells were labelled with Na$_2$$^{51}$CrO$_4$ (Amersham Biosciences) and used as targets for VSV-specific effector cells. VSV-specific CTL effectors were generated by i.p. injection of 5x10$^7$ PFU of VSV into mice. Splenocytes were collected five days after infection and cultured in RPMI-1640 complete medium plus 1 μM VSV-NP$_{52-59}$ peptide for five days.

### 2.10 ELISPOT assay

To generate B16F10 antigen specific splenocytes, 6x10$^6$ B16F10 cells were incubated with AdhTAP1 or Ψ5 at 50 PFU/cell or PBS at 37°C for 2 hrs. After incubation, the cells were irradiated (10,000 rad) for 30 min, then washed and re-suspended in PBS. On days 1, 4, and 8, mice were immunized by three separate
intraperitoneal (i.p.) injections of $2 \times 10^6$ irradiated cells (three mice per group). Nine days after the last immunization the spleens from each group were pooled and their splenocytes were isolated and cultured \textit{in vitro} in complete RPMI-1640 medium containing B16F10 tumor associated antigen peptide TRP-2 or gp100 (20 µg/ml) for 14 hrs. Controls contained no peptide. The frequency of B16F10 TAA-specific IFN-γ secreting cells was determined using an ELISPOT assay (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions. Briefly, dilutions of splenocytes ranging from $1 \times 10^6$ to $1 \times 10^4$ cells/well in 100 µl of complete RPMI-1640 medium were transferred to duplicate wells, with either TRP-2 or gp100 peptide (2 µg/ml) or without peptide. Following overnight incubation at 37°C in 5% CO$_2$ in air, the cells were removed, and the wells washed and incubated with biotinylated rabbit anti-IFN-γ antibody. After further washing, bound anti-IFN-γ antibody was detected with alkaline phosphatase-conjugated streptavidin. Spots were developed by incubating the plate with the chromogen BCIP/NBT. The color reaction was stopped by washing with deionized water. The plates were air-dried, and spots were visualized and counted using a dissecting microscope.

\textbf{2.11 Metabolic labeling, pulse-chase and endoglycosidase H experiments}

Tapasin$^{-/-}$ cells expressing each tapasin isoform were collected ($5 \times 10^6$ cells/sample), washed once in Cystine/Methionine-Free DMEM (CellGro Cat. No. 17-204-Cl, Mediatech, Herndon VA) + 5% FBS, once in PBS, resuspended in Cys/Met-Free DMEM + 5% FBS and plated in 6-well plates (one well per time point) for one hour of starvation and adherence to the plates. Ten microlitres (~20 µCi) of Pro-Mix L-$^{35}$S in
Cell Labeling Mix (Amersham Biosciences) were added per well for 15 min. Cells were then placed on ice, washed once with ice-cold PBS and normal DMEM + 10% FBS and cold Cys/Met for chase. At given time points, samples were removed and placed on ice, washed once in PBS and lysed in 1mL of TX-100 lysis buffer for 30 min, spun at 15 min at 4°C 10,000Xg, and pre-cleared overnight with pre-washed Protein G sepharose beads (Amersham Biosciences) and normal rabbit serum. The amount of labeled protein in each sample was quantified by TCA precipitation and counted in a scintillation counter. Normalized amounts of each sample were precipitated with antibodies to either H-2K\textsuperscript{b} (P8, recognizing all conformations of H-2K\textsuperscript{b}, courtesy of Jacques Neefjes, The Netherlands Cancer Institute) or human tapasin (PaSta.1, courtesy of Dr. Peter Cresswell, Yale University) for several hours or overnight, followed by binding to Protein G sepharose beads for one hour with rotation at 4°C. Samples were washed three times with 0.1% TX-100 in TBS and split into two separate samples: each was suspended in EndoH buffer but EndoH\textsubscript{f} enzyme (200 mIUB, New England Biolabs, P0703S) was added to only one sample. Both samples were incubated overnight at 37°C, then mixed with 2X protein sample buffer and separated by SDS-PAGE (12% separating/5% stacking). Gels were dried and exposed to a Phosphorimaging screen for 10 days, followed by visualization with a Phosphorimager SI (Molecular Dynamics), ImageQuant 5.2 software version 4.0, scanning at 100micron, PMT voltage 700.

2.12 Immunofluorescence and confocal microscopy

Cells were plated on sterile coverslips in 10 cm plates overnight. The following day, cells on coverslips were washed once in PBS, and fixed with 3% paraformaldehyde for 30 min at room temperature. Cells were washed again with PBS and permeabilized
and blocked for 1 hr with 0.1% saponin/1% BSA in PBS at room temperature. The cells were stained for one hour at room temperature with primary antibody listed in Table 2.3. Cells on slips were then washed four times in 0.1% saponin/1% BSA in PBS, and incubated with secondary antibody for one hour at room temperature with AlexaFluor 647 goat anti-mouse IgG (H+L) mixed with either AlexaFluor 568 goat anti-rabbit IgG (H+L), AlexaFluor 568 goat anti-rat IgG (H+L), or AlexaFluor 568 rabbit anti-goat IgG (H+L) - all from Molecular Probes, diluted 1/500. Cells were washed four times in 0.1% saponin/1% BSA in PBS and incubated 10 min in Slow Fade (Molecular Probes) equilibration buffer before mounting in Slow Fade glycerol solution. Slips were sealed to the slide with clear nail polish. Confocal microscopy was performed on a Nikon TE2000 inverted microscope with EZ-C1 software version 3.0, with 633 nm, 543 nm and 488 nm laser lines. Data analysis was performed with ImageJ.1 to select single slices and Adobe Photoshop CS version 8.0 for colour merging.

Table 2.3 Primary antibodies used for indirect immunofluorescence and confocal microscopy

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<th>Dilution</th>
<th>Organelle/ Target</th>
<th>Source/Reference</th>
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<td>Human tapasin</td>
<td>Dr. Peter Cresswell, Yale University, New Haven, CT, USA (105)</td>
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### MATERIALS AND METHODS

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### 2.12.1 Quantification of confocal results

The number of white (co-localized) pixels relative to total pixels were counted per cell with Adobe Photoshop CS version 8.0 in merged images. Bar graphs show the means +/- SEM.

### 2.13 Cross-presentation assays

Dendritic cells (DC2.4) were incubated overnight with indicated concentrations of soluble ovalbumin (Worthington Biochemical Corp., Lakewood, NJ) in PBS. The following morning, one well was pulsed with SIINFEKL peptide at 1 \(\mu\)g/ml for 1 hr at 37\(^\circ\)C, and cells from all samples were collected, washed 3 times with PBS and fixed for 10 min with 0.005% glutaraldehyde. Cells were resuspended in complete RPMI and either stained for flow cytometry with antibodies against total H-2K\(^b\) or with the 25.D1.16 antibody specific for H-2K\(^b\)/SIINFEKL complexes, or 1x10\(^5\) cells were mixed
1:1 with B3Z T cells overnight in 96 well plates. The following morning, cells were lysed in 100 μl of CPRG (Chlorophenol red-B-D-galactopyranoside, Roche) solution (91mg CPRG + 1.25ml NP-40, 9ml 1M MgCl₂ made up to 1 litre in PBS) and plates were read at 595 nm subtracting 655 nm background at 24 or 48 hrs to obtain a measure of the production of the β-galactosidase reporter under NF-AT elements by the B3Z TCR recognizing H-2Kᵇ/SIINFEKL complexes (157).

2.14 In vivo tumor growth assays

2.14.1 Animals

The mouse strain C57BL/6 (H-2ᵇ) was obtained from The Jackson Laboratory (Bar Harbor, ME, USA), and maintained at the Biotechnology Breeding Facility (University of British Columbia, Vancouver, BC, Canada). The mice were maintained according to the guidelines of the Canadian Council on Animal Care. Mice were kept on a standard diet with water ad libitum. The colony was routinely screened for *Mycoplasma pulmonis*, and *Mycoplasma arthritidis*, rodent coronaviruses (including hepatitis), and Sendai virus (SV) using the Murine ImmunoComb Test (Charles River Laboratories, Wilmington, MA, USA). The mice used in the experiments were between 6 and 12 weeks of age.

2.14.2 Viruses

Recombinant vaccinia virus (VV) either carrying rat-TAP1 cDNA (VV-rTAP1) or the empty plasmid PJS-5 (VV-PJS-5, vector negative control) has been described
previously (150). All VV strains were grown and titred in CV-1 cells (standard plaque forming units (PFU) assay).

The generation of recombinant adenoviruses expressing human TAP1 and human Tapasin are described in (146) and Appendix C, respectively. Viruses were grown and titred in HEK293 cells with standard plaque forming units (PFU) assays.

2.14.3 Tumor studies

2.14.3.1 Vaccinia virus

Mice (n = 49) were injected subcutaneously into the hindquarter with 1.5x10^5 cells of either B16F10 cells or B16/rTAP1 3-8 cells. One day and six days after introduction of tumor cells, mice were treated with injections localized to the tumor site of VV-rTAP1 (2x10^6 PFU/injection), VV-PJS-5 (2x10^6 PFU/injection), or PBS. Mice were sacrificed and the tumor masses measured 21 days after introduction of the tumor cells.

2.14.3.2 Adenovirus

For titration of the AdhTAP1 virus dose, tumors were established in six groups of 3 or 4 mice per group by i.p. injection of 4x10^5 CMT.64 cells in 500 µl PBS. On day 1, 3, 5, 8 days after the introduction of CMT.64 cells, the mice were further i.p. injected with either AdhTAP1 at 1.25, 2.5, 5.0, 10x10^7 PFU, ψ5 at 1x10^8 PFU in 500 µl PBS, or PBS and survival was followed for 90 days. For AdhTpn or AdhTpn plus AdhTAP1 treatment in CMT.64 tumor-bearing mice, tumors were established in five groups of 14 to 18 mice per group by i.p. injection of CMT.64 cells (4x10^5 cells in 500 µl PBS). On 1, 3, 5, and 8
days after the introduction of CMT.64 cells, the mice were further i.p. injected with AdhTpn, AdhTAP1, AdhTAP1 and AdhTpn, \( \Psi 5 \), (5x10^7 PFU/500 \( \mu l \) PBS.) or PBS and survival was followed for 90 days. To ensure all injection groups received the same number of Ad particles, mice treated with only one type of recombinant were complemented with enough \( \psi 5 \) vector to maintain a total Ad dose of 5x10^7 PFU. During the experiment four to eight mice of AdhTpn, \( \psi 5 \) or PBS groups were sacrificed from each group at selected times to observe tumor growth patterns and to measure the number of tumor-infiltrating CD4^+ and CD8^+ T lymphocytes and CD11c^+ DCs.

2.14.3.3 Treatment of B16F10 tumor-bearing mice with AdhTAP1

Twelve mice in each group were injected subcutaneously (s.c.) with 1.5 x 10^5 B16F10 cells in 100 \( \mu l \) PBS. On days 1, 4, and 8 after the introduction of B16F10 cells, mice were injected s.c. with either AdhTAP1, \( \Psi 5 \), or PBS (1 x 10^8 PFU/mouse/injection in 100 \( \mu l \) PBS). Fifteen days after the introduction of tumor cells, the mice were sacrificed and their tumor masses were measured. One tumor from each group was frozen for immunohistochemical (IHC) staining. The remaining tumors were pooled to measure the number of tumor infiltrating lymphocytes by flow cytometric analysis.

2.14.3.4 Tumor infiltrating lymphocytes (TILs) and DCs

TILs and tumor-infiltrating DCs were analyzed using both FACS and immunohistochemistry staining (IHC). Tumors were disaggregated into single cells and incubated with rat anti-mouse CD8 (Ly-2) mAb and R-PE-conjugated rat anti-mouse CD4 (L3T4) mAb, and the number of CD8^+ and CD4^+ TILs was quantified by FACS. Acetone fixed cryosections (8 \( \mu m \)) of frozen tumors were stained for tumor infiltrating
cells (CD8\(^+\), CD4\(^+\) T cells, and CD11c\(^+\) DCs) with rat anti-mouse CD4 mAb (RM4-5), rat anti-mouse CD8 mAb (53-6.7), or hamster anti-mouse CD11c (HL3). Rat IgG\(_{2a}\) was used as an isotope control for anti-CD8 and anti CD4 antibodies, whereas hamster IgG was the control for the antibody detecting CD11c\(^+\) cells. Antibody binding was detected with biotinylated polyclonal anti-rat IgGs and biotinylated anti-hamster IgG secondary antibodies and streptavidin-HRP and a DAB detection system (all the reagents were from BD Biosciences PharMingen).

### 2.15 Statistical analysis

Statistical analysis of real time PCR, flow cytometry and B3Z assays was performed with GraphPad Prism 4.00 software for Windows (GraphPad Software, San Diego CA, USA) using a one-way ANOVA with Tukey’s post test. Error bars represent the standard error of the mean.

The effect of VV-rTAP1 infection on surface MHC Class I antigen expression was analyzed using the Probability Binning Chi(T) Test (FlowJo software). Results were considered statistically different if the T(X) value was greater than 4, implying that the distributions are different with a p<0.01 (99% confidence).

The effect of rTAP transfection and treatment with vaccinia vectors on the growth of B16F10 tumors was analyzed by two-way ANOVA. The Tukey HSD Test was used for multiple comparisons to determine the differential effects of the treatments on tumor growth. The statistics for the \textit{in vivo} AdhTAP1 B16F10 tumor studies were performed using a one-way ANOVA and the binomial test. The data were considered statistically different if p<0.05. Error bars represent the standard error of the mean.
Survivorship data was analyzed with Kaplan-Meier plots and compared using the "Comparison of survival distributions" (http://biostat.hitchcock.org/BSR/Analytics/CompareTwoSurvivalDistributions.asp). The data were considered statistically different if $p<0.05$. 
Chapter 3: Expression and Subcellular Localization of the Tapasin Isoforms

3.1 Introduction

MHC Class I antigen presentation occurs in virtually all somatic nucleated cells of the body, allowing the immune system to survey the intracellular contents of the cells for pathogens or other abnormalities. As a result, components of the antigen processing machinery (APM) responsible for MHC Class I peptide generation and loading, including tapasin, are also widely expressed in nearly all cell types. However, should the tapasin isoforms be involved in specialized antigen processing and presentation pathways such as cross-presentation, then professional antigen presenting cells (pAPCs) such as dendritic cells should express high levels of the tapasin isoforms. Furthermore, tissues containing high proportions of these cells, such as lymph node and spleen where many immune responses are initiated, would also be expected to have similar high expression levels. To this end, the mRNA expression of the isoforms in an array of human tissues and cell lines was analyzed to see if any clues to their function could be gleaned from their expression patterns.

The subcellular localization of the isoforms should also reflect their specialized function in antigen presentation. Normally-spliced tapasin localizes principally in the ER, with some reports of small amounts in the cis-Golgi (170), similar to TAP (171). This form of tapasin contains a classical membrane protein ER-retention dilysine motif at its C-terminus, which is recognized by the COPI retrograde transport machinery in the Golgi and results in recycling to the ER of any tapasin molecules that exit (170). However, the
other two isoforms lack this dilysine motif. As a result, they might exit the ER more readily to other subcellular locations, such as the endolysosomal compartment where loading of cross-presented peptides is thought to occur. Immunofluorescence and confocal microscopy were used to assess co-localization of the tapasin isoforms with proteins found in specific organelles, including ER, Golgi, endosomes, and lysosomes as well as other APM components of interest such as MHC I molecules and the MHC II chaperones DM and DO, which define the MIIC/CIIV MHC II loading compartment. For the localization of tapasin, a monoclonal antibody specific for the common N-terminal region of human tapasin present in all isoforms was used. In the absence of antibodies specifically recognizing each individual isoform, it was necessary to subclone and express each isoform separately in cells in order to examine their subcellular localization. The isoforms were each expressed in two different murine cell lines: immortalized ear fibroblasts from tapasin-deficient mice (118) and a tapasin-expressing murine dendritic cell line, DC2.4 (161). The fibroblast cell line allowed the evaluation of each isoform in the absence of murine tapasin, and these cells were particularly well-suited for imaging studies due to their large size and clear subcellular structures. However, it was also important to assess the localization in dendritic cells, which express proteins essential for specific functions unique to this cell type. These in turn could influence the localization of the tapasin isoforms, particularly if the isoforms are themselves involved in these specialized processes (such as cross-presentation). Finally, this chapter also tests the hypothesis that isoform 3, predicted to be soluble, is secreted from cells.
3.2 Results

3.2.1 Identification of isoforms of human tapasin by PCR and development of isoform-specific PCR primer sets

The tapasin isoforms were identified in a human spleen cDNA library using RT-PCR with primers spanning exon 1 and exon 8 of the published human tapasin sequence (82;83) (GenBank accession number Y13582). The PCR products, corresponding to human tapasin cDNA, were subcloned into the pCR-Blunt II-TOPO cloning vector and several clones were sequenced. It was noted that while the majority of clones corresponded to the published, full-length tapasin primary sequence consisting of eight exons (“isoform 1”), some clones lacked exon 7 (“isoform 2”) or both exons 6 and 7 (“isoform 3”), suggesting alternative splicing of the tapasin RNA. PCR primers spanning exon 5 (forward) and exon 8 (reverse) of human tapasin were designed to more easily visualize the different expression patterns of the wild-type and two novel spliceforms of tapasin concurrently. However, since isoform 1 was more abundantly expressed in every tissue and cell type examined, visualization of the two other less abundant isoforms was often difficult with this primer set (see Figure 3.1b, upper left panel “Original primers”). Therefore, three more sets of primers with a common forward primer in exon 5 and reverse primers spanning exon-exon boundaries specific for each isoform were designed that specifically amplified only one isoform at a time (Figure 3.1a,b). These primer sets allowed for more precise quantification of the mRNA expression of each isoform from a mixed template.
Figure 3.1 Design of PCR primers for detection of tapasin isoforms.
(a) Original primers (orange) complementary to exon 5 (forward primer) and exon 8 (reverse primer). Isoform-specific primers still used a common forward primer in exon 5, but reverse primers spanned exon7-exon8 for isoform 1 (yellow), exon6-exon8 for isoform 2 (green) and exon5-exon8 for isoform 3 (purple). (b) PCR primers spanning exons 5 and 8 amplified all three transcripts from a mixed template but preferentially amplified isoform 1. Isoform-specific primers spanning exon-exon boundaries amplified each isoform specifically by PCR from mixed templates.
3.2.2 Expression of the tapasin isoforms in tissues and organs

To determine which tissues expressed the tapasin isoforms, RT-PCR was performed with each isoform-specific primer set and S15 primers as a loading control on oligo-dT-primed cDNA generated from a library of total RNA from twenty-one different human tissues. Each tissue sample contained RNA pooled from at least three different donors (Figure 3.2a). Results were analyzed by densitometry to obtain a semi-quantitative measure of the expression of each isoform in each tissue. Samples were normalized with the kidney sample (which showed the least variability between replicates) and results calculated relative to S15 loading controls. All tissues examined expressed the three forms of tapasin; however, there were tissue-specific differences in the expression of isoforms 2 and 3. Expression of isoform 1 was relatively consistent in all tissues, as expected since virtually all nucleated cells of the body present MHC I molecules at the cell surface (Figure 3.2b). Isoform 2 appeared to be more highly expressed in lymph node and spleen, with moderate levels in brain, liver, lung, testis and thymus (Figure 3.2c). This expression pattern is consistent with a role in immunity, including cross-presentation. DCs that have acquired antigen may require isoform 2 in lymph nodes and the spleen to initiate immune responses. It follows that isoform 2 will be expressed here and in many other tissues that DCs migrate through during different maturation stages. Isoform 3 expression was found to be highest in colon and trachea, with moderate expression in several other tissues such as lung, lymph node, placenta, small intestine, testis, and thymus (Figure 3.2d). Interestingly, the highest isoform 3 expression appeared to be localized to mucosal tissues (e.g. colon and trachea), which are in close and constant contact with commensal bacteria. It is possible that isoform 3 plays...
a role in mediating immune responses in these locations. All three isoforms displayed relatively low expression in some tissues: esophagus, kidney, ovary, prostate, and thyroid. Isoform 1 and 2 additionally showed lower expression levels in bladder and cervix that were not mirrored to the same extent by isoform 3 expression, which had average expression in these tissues relative to all the other tissues screened.

(a)

(b)

Isoform 1
Figure 3.2 Tapasin isoforms are widely expressed at the mRNA level in most tissues with high levels of isoform 2 in lymph node and spleen and high levels of isoform 3 in colon and trachea.

(a) Tissue-specific expression of the tapasin isoforms was determined by RT-PCR using isoform-specific primers and oligo-dT-primed cDNA generated from an RNA panel of normal human tissues. Densitometry of PCR bands was used to make semi-quantitative measurements of expression levels (b-d) relative to S15 loading controls and normalized with the kidney sample. All tissues expressed all three isoforms. Isoform 1 expression was quite high in many tissues, including brain, heart, liver, lung, lymph node, small intestine, spleen, testis and thymus (b). Isoform 2 appeared most highly expressed in lymph node and spleen (c), while isoform 3 was most highly expressed in colon and trachea (d). Relatively low expression of all three isoforms was observed in esophagus, kidney, ovary, prostate and thyroid. Data shown were compiled from three repetitions of the PCR experiment starting from the same cDNA sample. Each cDNA sample was generated two individual times.
3.2.3 Expression of the tapasin isoforms in human cell lines

Since tissues, by definition, contain many different cell types, isoform expression was also examined at the individual cell type level. The expression patterns of the tapasin isoforms were examined by RT-PCR using the isoform-specific primer sets (and S15 as a loading control) with oligo-dT-primed cDNA derived from RNA isolated from a series of human cell lines (Figure 3.3a). The cell lines available included T cells (Jurkat), B cells (Raji, BJA-B, Ramos, Daudi, 721.220, 721.221), monocyte-like (U937, KG-1), epithelial (HeLa) and embryonic kidney (HEK293). An African green monkey kidney cell line (COS-1) was also included to determine whether the tapasin RNA splicing extended to this species. Results were analyzed by densitometry as for the tissue panel, and were normalized with the HEK293 sample and to S15 loading controls. Similar to the human tissue samples, most cell lines examined expressed relatively consistent levels of isoform 1 (Figure 3.3b). Isoform 2 was also expressed in all cell lines tested; however, expression was highest in Raji and 721.221 B cells as well as KG-1 monocyte-like cells (Figure 3.3c). However, other B cell lines such as Ramos, BJA-B and Daudi showed very low isoform 2 expression. Jurkat T cells, U937 monocyte-like cells and HeLa epithelial cells also had very low isoform 2 expression. Raji and 721.221 B cells, as well as KG-1, similarly expressed isoform 3 at relatively high levels, as did Daudi and 721.220 B cells (Figure 3.3d). Low isoform 3 expression was observed in Ramos, Jurkat and HeLa, similar to isoform 2.

As demonstrated, differences do exist between the isoforms’ expression in various cell types and within the same class of cells (e.g. B cells), which may represent different stages of that cell type’s lineage and/or maturation stage. Interestingly, isoform 1
expression in Raji B cells was relatively low compared to isoform 1 expression in the other B cell lines examined, indicating that isoform 2 and isoform 3 expression may be upregulated in response to lower isoform 1 expression, or vice versa. The splicing of tapasin RNA to excise exons 6 and exons 6 and 7 also appears to extend beyond the human species as well: the monkey COS-1 cell line also expressed isoforms 1, 2 and 3, which were confirmed by DNA sequencing to be monkey tapasin.
Figure 3.3 Tapasin isoforms 1 and 3 are widely expressed at the mRNA level in most human cell lines, while isoform 2 expression is more variable.

(a) Cell line-specific expression of the tapasin isoforms was determined by RT-PCR using isoform-specific primers on oligo-dT-primed cDNA generated from RNA derived from a series of human cell lines. Densitometry of PCR bands was used to obtain a semi-quantitative measure of isoform 1 (b), isoform 2 (c) and isoform 3 (d) expression in each cell line relative to S15 loading controls and normalized to HEK293. All cell lines expressed all three isoforms, and isoform 1 expression was relatively high in all cell lines examined. Raji B cells expressed relatively low levels of isoform 1, but had amongst the highest levels of isoform 2 and 3 of the cell lines examined. 721.221 B cells and KG-1 monocytic-like cells also expressed high levels of isoforms 2 and 3 in addition to isoform 1. Ramos B cells, Jurkat, U937 and HEK293 expressed low levels of isoform 2 and 3. Data shown were compiled from three repetitions of the PCR experiment starting from the same cDNA sample, with each cDNA sample having been generated two individual times from independently-isolated RNA samples.
3.2.4 Expression of the tapasin isoforms in primary professional antigen presenting cells

The cell types most active in antigen presentation and cross-presentation include pAPCs such as B cells, monocytes and dendritic cells. Therefore, these cells were isolated for evaluation of tapasin isoform expression. Primary human cells were obtained from healthy donors, and immature monocyte-derived dendritic cells were generated by \textit{in vitro} culture for 6-7 days in GM-CSF and IL-4. Dendritic cells were matured over 24hrs with IFN\textgreek{g} and LPS, and cells were removed at indicated time points to follow tapasin isoform expression during maturation. Total RNA from each cell type was extracted, converted to cDNA with oligo-dT primers, and real time PCR using the isoform-specific and S15 primers was performed in triplicate to quantify the expression levels of the isoforms in each sample. The Ct values (the fractional PCR cycle number at which the reporter fluorescence is greater than the threshold baseline value) obtained were normalized with S15 values using standard curves for each primer set. B cells, monocytes and immature dendritic cells all expressed relatively low levels of the tapasin isoforms (Figure 3.4a). Upon maturation, however, DCs upregulated expression of the three isoforms to significantly higher levels. Maximal expression was reached after 24 hours (Figure 3.4b), but statistically significant differences were obvious even after two and four hours of stimulation in the case of isoforms 1 and 2.
Figure 3.4 Real time PCR analysis of isoform expression in primary human professional antigen presenting cells shows that mature DCs express the highest levels of all isoforms.

(a & b) Expression of tapasin isoforms was analyzed in triplicate by real time PCR in primary human antigen presenting cells (normalized to S15 expression using standard curves). Immature DCs were matured with LPS & IFNγ over 24hrs, with cells removed for analysis at indicated time points (b). Expression of all isoforms increased with DC maturation, and all were expressed at comparatively lower levels in immature DCs, B cells and monocytes. Data shown represents the results of one experiment, which was repeated three times independently.
3.2.5 Expression of the tapasin isoforms at the protein level in human cell lines

Expression of the tapasin isoforms at the protein level was examined in the human cell lines by immunoprecipitation with an anti-human tapasin mouse monoclonal antibody followed by Western blot with an anti-human tapasin rabbit polyclonal serum (Figure 3.5). Isoform 1 and 3 have nearly identical molecular weights and cannot be separated by SDS-PAGE; however, isoform 2 has slightly higher molecular weight and is distinguishable from the other two isoforms with this method. Raji B cells clearly expressed isoform 2 in addition to isoform 1/3 at nearly equal levels. 721.221 B cells also expressed isoform 2, but in lower amounts compared to isoform 1/3. The 721.220 B cells are deficient in human tapasin at the protein level (112;120) and were used as negative controls. Monkey tapasin appears to be poorly recognized by the human tapasin antibodies, and only a band of the isoform 1/3 size was detected in COS-1 cells. All other human cell lines displayed a single band corresponding to isoform 1/3, despite expression of isoform 2 at the mRNA level (Figure 3.3c). Notably, the two cells lines with the highest expression of isoform 2 at the mRNA level (Raji and 721.221) were also the cell lines with detectable isoform 2 protein expression. It is possible that the other cell lines may be expressing isoform 2 protein, but at levels below the detection limits of this method.
Figure 3.5 Two human B cell lines express detectable isoform 2 at the protein level. Expression of the tapasin isoforms was examined at the protein level by immunoprecipitation and Western blot in human cell lines. Because isoform 1 and 3 have very similar molecular weights, this experiment could only indicate whether isoform 2 was being expressed in addition to isoform 1 and/or isoform 3. Raji and 721.221 B cells were the only cell lines found to express isoform 2 protein in addition to isoform 1 and/or 3: all other cell lines expressed only isoform 1 and/or 3 at the protein level. 721.220 B cells are a negative control for human tapasin expression at the protein level. Data shown represents the results of one experiment, which was repeated at least twice for each cell line.

3.2.6 Predicted subcellular localization of the tapasin isoforms

For preliminary determination of the subcellular localization of the tapasin isoforms, the primary amino acid sequence of each was analyzed with the PSORT II program (172) to search for any known trafficking motifs present within the novel cytoplasmic tails of the isoforms. Consistent with the known localization of isoform 1, PSORT II predicted that it was most likely (44.4%) to be found in ER. However, isoform 2 was predicted to be equally likely to be found in ER, Golgi or plasma membrane.
Isoform 3 was strongly predicted (77.8%) to be extracellular or secreted from the cell (see Table 3.1).

### Table 3.1 Predicted subcellular localizations of the tapasin isoforms using the PSORTII prediction program.

Isoform 1 is most likely to localize in the ER and isoform 3 is strongly predicted to be extracellular or secreted from the cell. Isoform 2 was equally likely to be localized to ER, Golgi or the cell membrane according to the program algorithm.

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<th>Isoform 2</th>
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### 3.2.7 Cloning and construction of recombinant retroviruses encoding the tapasin isoforms

To determine the subcellular localization of the isoforms experimentally, the full-length isoforms were subcloned into the pMXpie retroviral vector, and recombinant retroviruses encoding each isoform or vector alone were generated. These were used to stably transduce immortalized ear fibroblasts derived from tapasin<sup>−/−</sup> mice (118) or the mouse DC cell line (with normal mouse tapasin expression), DC2.4 (161). Transduced cells were selected in puromycin and normalized for transgene expression by sorting for equal GFP expression driven by IRES-GFP cassette encoded by the pMXpie retroviruses (Figure 3.6a, Figure 3.7a). Expression of the isoforms in these cells was confirmed at the RNA level by RT-PCR using primers capable of amplifying all three isoforms (Figure 3.6b, Figure 3.7b), as well as by Western blot (tapasin<sup>−/−</sup> fibroblasts, Figure 3.6c) or immunoprecipitation followed by Western blot (DC2.4, Figure 3.7c) for detection of
expression of the isoforms at the protein level. Both RT-PCR and Western blot bands were of the expected size for each isoform. However, it was noted that despite equal GFP expression and protein loading of the SDS-PAGE gels, isoform 3 was always present at lower levels in cell lysates compared to isoform 1 and 2 (Figure 3.4c). In DC2.4 cells, expression of isoform 3 in protein lysates was not detectable by Western blot (Figure 3.7c). This is consistent with secretion of isoform 3 into the extracellular media, leading to lower protein levels within the cells and cell lysates.
Figure 3.6 Generation of tapasin $^-$ fibroblasts expressing the tapasin isoforms. Immortalized ear fibroblasts derived from tapasin $^-$ mice were transduced with non-replicating recombinant pMX-pie retroviral vectors encoding each isoform or vector alone as a negative control. Equal expression of each isoform was selected for by sorting cells expressing the same levels of GFP from the IRES-GFP cassette encoded within the vector (a), with uninfected cells as a negative control for GFP expression. Isoform expression was assessed by RT-PCR for RNA expression (b) using plasmids encoding the isoforms as controls, and Western blot for protein expression using T1 cells as a positive control for human isoform 1 and/or isoform 3 expression (c). It was noted that isoform 3 protein levels were lower in lysates from tapasin $^-$ fibroblasts expressing this isoform despite equal GFP levels, perhaps due to secretion from the cells. Data shown represents the results of one experiment, which was repeated at least twice.
Figure 3.7 Generation of DC2.4 mouse dendritic cells expressing the tapasin isoforms.

DC2.4 cells, a mouse DC-like cell line that expresses mouse tapasin, were transduced using the non-replicating recombinant pMXpie retroviral vectors expressing the tapasin isoforms or vector alone negative control and sorted for equal GFP levels with uninfected DC2.4 as negative controls for GFP expression (a). Transduced cells were tested for RNA expression of the isoforms by RT-PCR using plasmids encoding the isoforms as controls (b) and for protein expression by immunoprecipitation followed by Western blot using Raji B cells as a control for human isoform 1 and/or 3 and isoform 2 expression (c). Isoform 3 expression was very low in the DC2.4 cell line, consistent with secretion of this isoform from the cell. Data shown represents the results of one experiment, which was repeated at least twice.
To better quantify protein levels of the isoforms within cells, intracellular flow cytometry was performed with the anti-human tapasin monoclonal antibody PaSta.1. Results were in agreement with Western blot data, and showed that isoform 3 was found in lower abundance within both tapasin$^{-/-}$ fibroblasts and DC2.4, despite equal GFP levels and equal background staining in negative controls (Figure 3.8, right panels). Isoform 3 levels in DC2.4 were significantly lower than in tapasin$^{-/-}$ fibroblasts as observed by Western blot. Isoform 2 protein levels were also slightly lower than isoform 1 in both cell lines. To determine whether any isoform 3 (or either of the other two isoforms) was associated with the exterior of the cells, cells were fixed and stained similarly to the intracellular experiment, except the permeabilization with saponin was omitted so only extracellular epitopes could be detected. No tapasin was detected on the outside of either tapasin$^{-/-}$ or DC2.4 expressing any of the isoforms, suggesting no form of tapasin is associated with the outside of the plasma membrane of these cells (Figure 3.8, left panels).
Figure 3.8 Detection of tapasin isoform expression in transduced tapasin<sup>−/−</sup> fibroblasts and DC2.4 or on the cell surface using intra- and extra-cellular flow cytometry, respectively. Cells were stained with the PaSta.1 anti-human tapasin monoclonal antibody and AlexaFluor 647-conjugated anti-mouse secondary following saponin permeabilization to quantify the amount of tapasin protein expressed within tapasin<sup>−/−</sup> (top right panel) and DC2.4 cells (bottom right panel) expressing each isoform. Vector alone-expressing cells were used as negative controls for human tapasin expression. Intracellular isoform 3 levels were found to be consistently lower than other isoforms despite equal GFP levels, consistent with secretion of this isoform. Isoform 2 levels were also slightly reduced in both cell lines relative to isoform 1. Cells were also stained with the PaSta.1 anti-tapasin antibody without saponin permeabilization of the cells to determine if any tapasin protein was associated with the cell surface or plasma membrane in cells expressing isoform 3 (left panels). No differences were noted between cells expressing vector alone controls and any of the isoforms, suggesting no tapasin is localized to the cell surface. Data shown represents the results of one experiment, which was repeated three times.
3.2.8 Lack of acquisition of endoglycosidase H resistance of isoforms suggests mainly ER localization

In an effort to follow the secretion of isoform 3 biochemically, a pulse-chase assay with S\(^{35}\)-labelled methionine and cysteine was performed in tapasin\(^{-/-}\) fibroblasts expressing the isoforms or pMX-pie vector alone followed by immunoprecipitation with the anti-tapasin monoclonal antibody PaSta.1 and Endoglycosidase H (EndoH) digestion. Many proteins are sensitive to EndoH digestion until passage through the medial Golgi and acquisition of carbohydrate moieties by mannosidase II that confer resistance to digestion by the enzyme. Therefore, acquisition of EndoH resistance is frequently used as an indicator of ER exit and passage through the medial Golgi (86;87;101;173;174). Results indicated that isoform 1 and 2 did not acquire resistance to EndoH even after two hours of chase, suggesting they remain in the ER and do not passage through the medial Golgi. However, no isoform 3 was detected even at the beginning chase period, suggesting the isoform 3 protein synthesized within the initial 15 minute pulse rapidly exits the cell. Attempts to shorten pulse times to detect intracellular isoform 3 before secretion failed to detect the protein. Background proteins of unknown identity were detected in all four samples, including the vector alone negative control, likely due to nonspecific interactions with either the antibody or the beads used in the immunoprecipitation.
Figure 3.9 Tapasin isoforms 1 and 2 do not acquire EndoH resistance.

Acquisition of EndoH resistance by the tapasin isoforms was assessed by pulsing tapasin−/− fibroblasts expressing each isoform or vector alone with 35S-labelled methionine and cysteine for 15 minutes, followed by a two-hour chase. Cells were removed at indicated time points, lysed and immunoprecipitated with the anti-human tapasin monoclonal antibody PaSta.1. EndoH resistance, indicative of passage through the medial Golgi and exit from the ER, was assessed. Results show that isoform 1 and 2 remain EndoH sensitive even after two hours of chase, suggesting the majority of these proteins remain in the ER/pre-median Golgi. Isoform 3 appears to rapidly leave the cell during the 15 minute pulse, and was not detectable in this assay. Data shown represents the results of one experiment, which was repeated three times.

3.2.9 Detection of isoform 3 in the supernatant of isoform 3-expressing cells

Since attempts to track the exit of isoform 3 from the ER after synthesis were not successful, we sought to detect secreted isoform 3 in the supernatant of cells expressing this protein. In order to achieve high enough isoform 3 expression to detect secreted isoform 3 protein by Western blot, recombinant adenoviruses were generated that expressed isoform 1 or isoform 3 from a CMV promoter (see Appendix C). These viruses
were used to infect HEK293 cells for 48 hours, after which time both the supernatant and the cells were collected. Whole cell lysates were made from the infected cells, the supernatant was concentrated 40X, and samples were assessed for the presence of human tapasin levels by Western blot. While both isoform 1 and 3 were detected in whole cell lysates from infected 293 cells, only supernatant from isoform 3-infected cells showed a band of the correct size for tapasin (Figure 3.10). These results suggest that isoform 3 is secreted into the supernatant.

![Figure 3.10 Soluble isoform 3 is detectable in concentrated supernatant from isoform 3-expressing cells.](image)

Western blot of human tapasin from whole cell lysates or concentrated supernatants of HEK293 cells 48hrs after infection with a non-replicating recombinant adenoviruses encoding isoform 3 or isoform 1. A band of the correct size was consistently detected in supernatant from isoform 3-expressing cells, suggesting isoform 3 is secreted into the media. Data shown represents the results of one experiment, which was repeated three times.
**3.2.10 Determination of subcellular localization of tapasin isoform by immunofluorescence and confocal microscopy**

To further examine the subcellular localization of the tapasin isoforms within tapasin⁻/⁻ fibroblasts and DC2.4 cells, indirect immunofluorescence staining for tapasin and several organelle marker proteins followed by confocal microscopy was performed. The tapasin isoforms were stained with the PaSta.1 anti-human tapasin monoclonal antibody, which showed very little background staining of vector-alone expressing cells. This was followed by an AlexaFluor 647-conjugated anti-mouse secondary antibody and was visualized with the 633nm laser and coloured green in all figures. All organelle co-stains used either rat monoclonal or rabbit polyclonal antibodies, which were visualized with respective AlexaFluor 568-conjugated secondary antibodies using the 547nm laser line and coloured magenta in all figures. Co-localization is visible as white colour, and white pixels were quantified as a percentage of the total number of pixels per cell in at least six individual cells for each cell type and co-stain that showed quantifiable co-localization.

In both tapasin⁻/⁻ fibroblasts and DC2.4 expressing the tapasin isoforms, extensive co-localization of isoform 1 and 2 - as well as isoform 3 in tapasin⁻/⁻ fibroblasts - was evident with the ER-resident protein cytochrome P450 reductase (Figure 3.11a,b). Isoform 3 levels in DC2.4 were too low to detect with this technique. Similar co-localization of the isoforms with other ER proteins (PDI, calnexin) was also observed (data not shown), suggesting that high amounts of all forms of tapasin localized primarily in the ER, consistent with the pulse-chase/EndoH experiments. Isoform 3, while likely secreted, would be expected to be found at least transiently in the ER immediately after
co-translational insertion through the Sec61 complex, so it is not surprising that co-localization with the ER marker protein was detected.

**Figure 3.11 The tapasin isoforms co-localize with the cytochrome P450 reductase ER marker.**

All isoforms showed equal amounts of co-localization with this marker, indicating all three isoforms are found in the ER in tapasin−/− fibroblasts (a) in similar quantities, and isoform 1 and isoform 2 are also found in the ER of the DC2.4 dendritic cell line (b). Data shown were compiled from at least 18 (DC2.4) or 4 (tapasin−/− fibroblasts) individual cells from two to three separate individual preparations. See following pages.
TAPASIN ISOFORM EXPRESSION & SUBCELLULAR LOCALIZATION

(b) Free GFP | Tapasin | Organelle Marker | Merged

Isoform 1

Isoform 2

Isoform 3

Vector Alone

DC2.4 - Cytochrome P450 Reductase

Percent colocalization

Isoform 1 | Isoform 2
In addition, all isoforms co-localized with H-2K\textsuperscript{\textbeta} in tapasin\textsuperscript{+/−} fibroblasts (Figure 3.12a), the majority of which appears to be ER-localized in this cell type. Very little co-localization of the isoforms with H-2K\textsuperscript{\textbeta} was noted in DC2.4 (data not measurable, Figure 3.12b), presumably because more MHC I molecules in these cells are found in non-ER compartments and the cell surface compared to fibroblasts.

Figure 3.12 The tapasin isoforms co-localize with H-2K\textsuperscript{\textbeta} in tapasin\textsuperscript{+/−} fibroblasts but not in DC2.4.
(a) All isoforms showed equal amounts of co-localization with this MHC Class I molecule, indicating all three isoforms may be capable of interactions with MHC I molecules. Data shown were compiled from at least 3 individual cells from two separate individual preparations. (b) No co-localization of the tapasin isoforms and H-2K\textsuperscript{\textbeta} was observed in DC2.4 cells (data not quantifiable). See following pages.
(a) TAPASIN ISOFORM EXPRESSION & SUBCELLULAR LOCALIZATION

Free GFP  Tapasin  Organelle marker  Merged

Isoform 1

Isoform 2

Isoform 3

Vector Alone

Tapasin^+ - H-2K^b

percent co-localization

isoform 1  isoform 2  isoform 3

95
(b) | Free GFP | Tapasin | Organelle Marker | Merged
| ISOFORM EXPRESSION & SUBCELLULAR LOCALIZATION | 96

Isoform 1

Isoform 2

Isoform 3

Vector Alone
Differences in subcellular localization between the isoforms were observed with the trans Golgi network (TGN) marker, furin convertase. Isoform 2 showed higher levels of co-localization with this marker than isoform 1 in both tapasin\textsuperscript{−/−} fibroblasts and DC2.4 cells (Figure 3.13a,b). In addition, levels of isoform 3 co-localization with furin convertase were between isoform 1 and isoform 2 levels in tapasin\textsuperscript{−/−} fibroblasts, though differences were not statistically significant (Figure 3.13a). These results suggest that very little isoform 1 is present in the TGN of these cell lines, but that isoform 2 does localize to this compartment and possibly some isoform 3 as well. In the case of isoform 3 it would pass through the TGN as it transits to the cell surface. Co-localization was also assessed with the cis/medial Golgi compartment using Giantin as a marker; however, no co-localization of any of the isoforms was observed in either cell type with this marker (data not measurable, see Figure 3.13c,d).

**Figure 3.13 Co-localization of the tapasin isoforms with Golgi markers.**
Isoform 2 showed higher levels of co-localization with this trans Golgi network (TGN) marker in both tapasin\textsuperscript{−/−} fibroblasts (a) and the DC2.4 dendritic cell line (b) compared to isoform 1. In tapasin\textsuperscript{−/−} fibroblasts, isoform 3 levels in the TGN were also slightly elevated, though to a lesser extent than isoform 2. However, this increase was not statistically significant. Data shown were compiled from at least 16 (DC2.4) or 11 (tapasin\textsuperscript{−/−} fibroblasts) individual cells from two to three separate individual preparations. None of the isoforms was observed to co-localize with the cis/medial Golgi marker Giantin in either tapasin\textsuperscript{−/−} fibroblasts (c) or DC2.4 (d). See following pages.
(b) TAPASIN ISOFORM EXPRESSION & SUBCELLULAR LOCALIZATION

<table>
<thead>
<tr>
<th>Free GFP</th>
<th>Tapasin</th>
<th>Organelle Marker</th>
<th>Merged</th>
</tr>
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<tbody>
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<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
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<tr>
<td>Isoform 3</td>
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<tr>
<td>Vector Alone</td>
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<td><img src="image11.png" alt="Image" /></td>
<td><img src="image12.png" alt="Image" /></td>
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</tbody>
</table>

**DC2.4 - Furin Convertase**

- **p < 0.0001**

Percentage colocalization:
- Isoform 1: 0.2
- Isoform 2: 1.8

Significance levels:
- three asterisks indicate p < 0.001.
TAPASIN ISOFORM EXPRESSION & SUBCELLULAR LOCALIZATION

(c) Free GFP Tapasin Organelle marker Merged

Isoform 1

Isoform 2

Isoform 3

Vector Alone
<table>
<thead>
<tr>
<th>Isoform 1</th>
<th>Free GFP</th>
<th>Tapasin</th>
<th>Organelle Marker</th>
<th>Merged</th>
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<tr>
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<td><img src="image3.jpg" alt="Image" /></td>
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<tr>
<td>Isoform 3</td>
<td><img src="image1.jpg" alt="Image" /></td>
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<td>Vector Alone</td>
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</table>
There were also measurable differences in the co-localization of isoforms 1 and 2 with different markers of the endosomal/lysosomal pathway. Isoform 2 showed high levels of co-localization with the endosomal marker Rab5. Interestingly, isoform 2 showed higher levels of co-localization with this marker only in the tapasin<sup>−/−</sup> fibroblast cell line (Figure 3.14a): in the DC2.4 cells both isoforms co-localized equally with this marker (Figure 3.14b), suggesting that the localization of isoform 2 to endosomes is regulated differently in these different cell lines. In DC2.4, both isoforms 1 and 2 showed co-localization with another early endosome marker EEA1, and isoform 1 levels appeared higher than isoform 2 levels in this compartment (Figure 3.14d). In tapasin<sup>−/−</sup> fibroblasts, however, none of the isoforms were observed to co-localize with EEA1 (data not measurable, Figure 3.14c).

Figure 3.14 Co-localization of the tapasin isoforms with endosomal markers.
Isoform 2 showed higher levels of co-localization with the endosomal marker Rab5 in tapasin<sup>−/−</sup> fibroblasts (a). In the DC2.4 dendritic cell line, both isoform 1 and 2 co-localized equally with Rab 5 (b). Both isoforms also co-localized with a second endosomal marker, EEA1, in DC2.4, but isoform 1 co-localized with this marker to a larger extent than isoform 2 (d). None of the isoforms co-localized with EEA1 in tapasin<sup>−/−</sup> fibroblasts (c). Data shown were compiled from at least 10 (DC2.4) or 4 (tapasin<sup>−/−</sup> fibroblasts) individual cells from two to three separate individual preparations. See following pages.
(a) TAPASIN ISOFORM EXPRESSION & SUBCELLULAR LOCALIZATION

<table>
<thead>
<tr>
<th>Free GFP</th>
<th>Tapasin</th>
<th>Organelle marker</th>
<th>Merged</th>
</tr>
</thead>
</table>
| Isoform 1 | ![Image]
| Isoform 2 | ![Image]
| Isoform 3 | ![Image]
| Vector Alone | ![Image]

**Tapasin**^+^ - Rab5

- \( p < 0.01 \)

<table>
<thead>
<tr>
<th>Isoform 1</th>
<th>Isoform 2</th>
<th>Isoform 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>0.6</td>
<td>0.1</td>
</tr>
</tbody>
</table>
(b) Free GFP  Tapasin  Organelle Marker  Merged

Isoform 1

Isoform 2

Isoform 3

Vector Alone

DC2.4 - Rab5

percent colocalization

Isoform 1  Isoform 2

0.00  0.25  0.50  0.75  1.00
(d) TAPASIN ISOFORM EXPRESSION & SUBCELLULAR LOCALIZATION

<table>
<thead>
<tr>
<th>Free GFP</th>
<th>Tapasin</th>
<th>Organelle Marker</th>
<th>Merged</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoform 1</td>
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<td>Vector Alone</td>
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</table>

**DC2.4 - EEA1**

![Graph showing data comparison between Isoform 1 and Isoform 2]
Isoform 2 also showed high levels of co-localization with the late endosome/lysosome marker Rab7 (Figure 3.14a), but again only in tapasin^{-/-} fibroblasts. There was no observable co-localization of any of the isoforms with Rab7 in the DC2.4 cell line (data not measurable, Figure 3.15b), nor was there any co-localization of any of the isoforms with another lysosomal marker, LAMP2, in either cell line (data not measurable, Figure 3.15c,d). Together, these data suggest that isoform 1 and 2 can localize to endosomal or lysosomal compartments, but that the degree of localization varies between different cell types, presumably due to cell-specific regulation of trafficking of these molecules. In addition, the endo/lysosomal compartments to which they co-localize seem to be defined by certain endo/lysosomal markers (e.g. Rab5 and Rab7) but not others (e.g. EEA1 and LAMP2). The latter observation could also be due to the quality of the antibody being used for indirect immunofluorescence, and the protein expression level in endo/lysosomal compartments of each marker.

Figure 3.15 Co-localization of the tapasin isoforms with lysosomal markers. Isoform 2 co-localized with the late endosome/lysosome marker Rab7 in tapasin^{-/-} fibroblasts only (a) and not in DC2.4 (b). None of the isoforms co-localized with the LAMP2 lysosomal marker in tapasin^{-/-} fibroblasts (c) or DC2.4 (d). Data shown were compiled from at least 10 (DC2.4) or 4 (tapasin^{-/-} fibroblasts) individual cells from two to three separate individual preparations. See following pages.
<table>
<thead>
<tr>
<th>Isoform</th>
<th>Free GFP</th>
<th>Tapasin</th>
<th>Organelle Marker</th>
<th>Merged</th>
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<td>Isoform 1</td>
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<td>Isoform 2</td>
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<td>Isoform 3</td>
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<tr>
<td>Vector Alone</td>
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(c)  

<table>
<thead>
<tr>
<th></th>
<th>Free GFP</th>
<th>Tapasin</th>
<th>Organelle marker</th>
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<td>Isoform 2</td>
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<tr>
<td>Isoform 3</td>
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<tr>
<td>Vector Alone</td>
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</table>
(d) TAPASIN ISOFORM EXPRESSION & SUBCELLULAR LOCALIZATION

Isoform 1

Isoform 2

Isoform 3

Vector Alone
In DC2.4, further co-stains were performed with the pAPC-specific proteins H-2M and H-2O (169), which are residents of the MHC Class II peptide loading compartment/Class II-containing vesicles (MIIC/CIIV) (175). However, no co-localization of any of the tapasin isoforms was observed with either of these proteins, suggesting that none of the tapasin isoforms localizes to the Class II loading compartment (Figure 3.16). MHC Class I antigen cross-presentation is believed to occur in an endolysosomal compartment; therefore, the data indicating tapasin isoform localizing with endo/lysosomal markers would be consistent with a role for the isoforms in cross-presentation. However, the lack of co-localization with the MIIC compartment suggests that MHC I molecules are loaded with exogenous antigens in a compartment distinct from that in which loading of MHC II with exogenous antigens occurs.

**Figure 3.16 Co-localization of the tapasin isoforms with MIIC/CIIV markers.**
In DC2.4, none of the isoforms was found to co-localize with MIIC/CIIV resident proteins H-2M (a) or H-2O (b). See following pages.
(b) Free GFP  Tapasin  Organelle Marker  Merged

Isoform 1

Isoform 2

Isoform 3

Vector Alone
3.3 Discussion

To function in MHC I cross-presentation of exogenous antigens, the novel tapasin isoforms must be expressed in the tissues and cell types where this process occurs, and must also be localized at the subcellular level to the compartments in which loading of exogenously-derived peptides onto MHC I takes place. In so doing, the tapasin isoforms could assemble functional peptide-loading complexes in these novel subcellular locations and cell types to mediate the loading of cross-presented peptides.

3.3.1 Tapasin isoform expression in tissues and cells

While classical MHC I antigen presentation occurs in almost every cell of the body, cross-presentation is thought to be performed most efficiently by dendritic cells matured by “danger” signals that trigger the Toll-like receptors (TLRs) and related pattern-recognition receptors (22;37;80). The hypothesis is that if the tapasin isoforms were involved in cross-presentation, then mature dendritic cells should express high levels of the isoforms. Furthermore, tissues with high levels of mature DCs that are involved in the initiation of immune responses, such as lymph node and spleen, should also express the isoforms at high levels.

The results showed that, indeed, isoform 1 was expressed in all tissues and cell types examined with relatively small differences between the different samples tested (Figure 3.2b). On the other hand, lymph node and spleen expressed the highest levels of isoform 2 relative to nineteen other human tissue samples (Figure 3.2c), supporting a novel role in antigen presentation in these locations. Interestingly, isoform 3 seemed most highly expressed in certain mucosal tissues such as colon and trachea (Figure 3.2d). It can
therefore be conjectured that this isoform might play a slightly different role in immunity, perhaps in balancing appropriate immune responses against pathogens while avoiding or inhibiting such responses against normal gut flora and/or food antigens. How the immune system achieves this balance in the gut is still poorly understood.

Tissues, by definition, consist of mixes of different cell types, therefore the isoforms would either have to be expressed by many of the cell types making up the tissue or contain one cell type that very highly over- (or under-) expresses the isoforms to be able to observe differences in expression levels. To distinguish between these two possibilities, individual cell types were examined. Since it is difficult to obtain RNA from all the primary human cells making up these tissues, only pAPCs (human B cells, monocytes and dendritic cells) were assessed. For other cell types, human cells lines were used as substitutes for primary cells. Primary human DCs matured by LPS and IFN-γ did express the highest levels of all three tapasin isoforms relative to immature DCs, B cells and monocytes, supporting the hypothesis that these isoforms play a role in a DC-specific processes such as cross-presentation (Figure 3.4).

B cells are also thought to be capable of cross-presentation under certain circumstances, though they may only present the antigen bound and internalized by their B cell receptor (BCR) (54-56). Interestingly, amongst the human cell lines examined for tapasin isoform expression, those that had high mRNA expression of isoforms 2 and 3 and had high enough levels of isoform 2 protein to be detectable were both B cell lines: Raji and 721.221 (Figure 3.5). However, other B cell lines examined did not express isoform 2 at the protein level. B cells undergo a complicated maturation process that can be followed based on the expression of various cell surface markers (see Figure 3.17a).
Many B cell lines represent cells arrested at some stage of this development (176-178), as shown in Figure 3.17b.

(a) Normal B cells progress from a common lymphoid progenitor cell to pro B, pre B and finally immature and mature B cells, expressing different cell surface markers as they develop. Cells in the latter two stages express surface IgM, but only mature B cells express surface IgD.

(b) Figure 3.17 Stages of B cell development.
express surface IgD. After antigen stimulation, B cells evolve into antibody-secreting plasma cells. Reproduced from (179) with permission. (b) B cell malignancies often correspond to distinct stages of normal B cell development. From www.lymphomainfo.net/images/b-cancer-chart.gif.

Different maturation stages could account for the varied expression between the B cell lines. B cells are generally thought to present antigen at later, more mature stages of development after they express functional BCRs (180). If the tapasin isoforms are important in B cell antigen presentation, they would be expected to be predominantly expressed in cell lines representative of these late stages of B cell development. Another factor to consider when examining B cell lines is the presence or absence of Epstein-Barr Virus (EBV), which encodes proteins that alter the antigen presentation abilities of the cell line (181). The maturation state and other properties of the cell lines used in this study are summarized in Table 3.2 and discussed below.

Table 3.2 Summary of B cells and their maturation markers used in this study.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Type</th>
<th>EBV status</th>
<th>Surface markers</th>
<th>Maturation stage</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raji</td>
<td>African Burkitt’s lymphoma</td>
<td>+</td>
<td>Little/no secreted IgM Surface IgD unknown</td>
<td>Mature B</td>
<td>(176-178;182-185)</td>
</tr>
<tr>
<td>Daudi</td>
<td>African Burkitt’s lymphoma</td>
<td>+</td>
<td>High surface IgM Little/no secreted IgM Surface IgD unknown</td>
<td>Mature B</td>
<td>(176-178;182-184;186;187)</td>
</tr>
<tr>
<td>Ramos</td>
<td>American Burkitt’s lymphoma</td>
<td>-</td>
<td>Surface and secreted IgM No surface IgD</td>
<td>Immature B</td>
<td>(176-178;182-184)</td>
</tr>
<tr>
<td>BJA-B</td>
<td>American Burkitt’s lymphoma</td>
<td>-</td>
<td>High levels of secreted IgM No surface IgD</td>
<td>Immature B</td>
<td>(158;176-178;182-184)</td>
</tr>
<tr>
<td>721.221</td>
<td>Lymphoblastoid cell line</td>
<td>+</td>
<td>Unknown, but likely to secrete IgM</td>
<td>Mature B</td>
<td>(156;176-178;182-184)</td>
</tr>
</tbody>
</table>
Many of the cell lines examined (Raji, Daudi, LCL 721.220/721.221) are positive for EBV, which is commonly used to generate lymphoblastoid cell lines (LCLs) from normal B cells *in vitro*, or is present in patients with infectious mononucleosis from whom B cell lines can be naturally-derived *ex vivo* (177;178). Raji and Daudi cells were both derived from African Burkitt’s lymphoma patients (185;187). The LCL 721.220 and 721.221 cell lines are derivatives of normal B lymphocytes that were infected with EBV *in vitro* (156). Ramos and BJA-B are EBV negative, but are considered to be Burkitt’s-like lymphoma cell lines because they have chromosomal translocations typical of this disease (158;177). Generally, it is felt that the EBV+ “African” Burkitt’s lymphoma cell lines, of which Raji is considered a prototype, represent somewhat more mature B cell lineages than the “American” EBV- Burkitt’s-like lymphoma lines such as Ramos (177;182).

B cell maturation stage is often graded by assessing Ig production and secretion. Ramos cells express both surface and secreted IgM (182), and BJA-B secrete high levels of IgM associated with J chain (158;177;182): neither expresses surface IgD. Some feel that Ramos and BJA-B represent earlier stages of B cell development (immature B) when antigen-independent secretion transiently occurs (182;183). However, this hypothesis is not universally accepted as others feel that Ig secretion is a characteristic of more mature B cells only (176;182). Both Raji and Daudi cells secrete little to no IgM, and Daudi cells have unusually high surface levels of IgM (182). They are thought to represent a later stage of development where synthesis and secretion of IgM are more controlled as the B cells become sensitive to antigen (182). No information could be found on surface IgD expression on Raji and Daudi cells. Ig expression in the LCL 721 derivative cell lines has
not been measured to our knowledge, but other groups who have generated similar cell lines reported that these cells represent late, mature stages of B cell differentiation capable of secretion of large amounts of IgM (178;182).

Alternatively, the stage of differentiation of the B cell lines alone might not fully explain the differences observed between the different cell lines. Another possibility is that the presence of EBV influences tapasin isoform expression. TAP levels in EBV-expressing cells are known to be much higher compared to EBV-negative cells, where TAP is more limiting (174;184); it is likely that tapasin expression is also altered by the virus as well.

In summary then, the two cell lines that expressed isoform 2 at the protein level (Raji and 721.221) are EBV⁺ cell lines that may represent more mature B cell lineages, which would be more likely to engage in antigen presentation and might utilize isoform 2. The less mature, EBV⁻ B cell lineages (represented by Ramos and BJA-B) might not yet express this isoform because they do not present antigen. The exception to this trend is the Daudi line, which is EBV⁺ and considered to be very similar to Raji, yet it did not express isoform 2 at the protein level. However, Daudi cells are deficient in β2m expression (186), and could potentially have other underlying defects in antigen presentation that might preclude tapasin isoform 2 expression.

In general, however, transformed cell lines are somewhat problematic for this type of study due to their inherent genomic instability: many have chromosomal translocations and are somewhat heterogeneous, especially after long term culture, making them imperfect, but nonetheless useful, study subjects. For these reasons, it
would be advantageous to look at primary human B cells at various stages of development and stimulated with different cytokines or TLR ligands.

### 3.3.2 Subcellular localization

Isoform 2 and 3 expression at the RNA and protein levels did correlate with tissues and cell types involved in MHC I cross-presentation; therefore, the effect of the novel C-termini encoded by isoforms 2 and 3 in directing subcellular trafficking of the isoforms to distinct locations in the cell was examined. No known addressin sequences were encoded in the novel 26 amino acids encoded at the C-terminus of isoform 2 and 3, but isoform 1 was expected to be retained in the ER due, at least in part, to the C-terminal dilysine motif lacking in the other two isoforms. Isoform 2 was hypothesized to still be membrane-bound due to its transmembrane domain; however, isoform 3 was predicted to be soluble and perhaps secreted from the cell. Nevertheless, secretion would not exclude the possibility that isoform 3 could subsequently be internalized (perhaps along with exogenous antigen and/or recycling MHC I molecules) and participate in peptide loading onto MHC I.

Loading of exogenously-derived peptides onto MHC I molecules during cross-presentation has been proposed to occur in several locations within the cell, as described in the introduction. In one model, loading occurs in the ER similarly to classical MHC Class I antigen presentation after endocytosed antigens have escaped into the cytosol through as-yet undefined mechanisms (62-64). Another model proposes that during phagocytosis of exogenous particulate antigens, the ER membrane fuses with the plasma membrane undergoing invagination to form the phagosome. This results in all the antigen presentation machinery components being present within this membrane and creating an
independent vesicle capable of exporting antigens into the ER for proteasomal degradation. The peptides thus generated are proposed to be imported back into the phagosome through TAP and loaded onto MHC I within the same compartment (Figure 1.9) (69;70). Neither of these two models would necessarily require novel forms of tapasin, as both would employ normally-spliced ER tapasin and other PLC components for MHC I peptide loading, although the phago-ER model could explain how isoform 1 with its dilysine motif came to co-localize with Rab5 and EEA1 in DC2.4 cells. However, a third mechanism of cross-presentation is thought to occur in endo-lysosomal compartments, in which normally-spliced tapasin has not been reported to be present (65-68). Co-localization of the novel tapasin isoforms with markers for a variety of subcellular organelles, including endosomes and lysosomes, was assessed for evidence of tapasin localization in compartments where cross-presentation might be occurring.

The localization of the isoforms was examined in two transduced cell lines lacking human tapasin to allow for expression and detection of each isoform in isolation since no antibody exists to distinguish the isoforms at the protein level. The only human cell line deficient in human tapasin expression (721.220) does not express HLA-A or -B molecules due to a chromosomal deletion (112;120), making it a poor model for both functional studies and also localization studies in which interactions with MHC I might play a key role in directing subcellular localization. Previous studies have demonstrated that human and mouse APM components are generally similar enough to allow them to function together (89;116;188); therefore, a murine tapasin-deficient cell system (tapasin\(^{-}\) fibroblasts) was chosen for initial studies and later the DC2.4 system as a dendritic cell model. A dendritic cell line was also examined because of the high isoform expression
observed in mature human DCs, and because DCs might express co-factors needed for isoform localization to cross-presentation compartments that are not found in the non-cross-presenting fibroblasts.

Both cell lines were used to assess the subcellular localization of the isoforms by intracellular flow cytometry and immunofluorescence followed by confocal microscopy. Isoform 3 levels were found to be lower relative to the other two isoforms in protein lysates and in intracellular flow cytometry in both cell types, suggesting it is being secreted from the cell (Figure 3.6, 3.8). This is consistent with its ER signal peptide and lack of a transmembrane domain and/or ER-retention motif. However, isoform 3 levels were much lower in DC2.4 cells than in tapasin\textsuperscript{-/-} fibroblasts, which may be indicative of faster exit from the cell in DCs (Figure 3.7, 3.8). Isoform 3 levels were so low in DC2.4 (despite equal GFP expression from the IRES-GFP cassette encoded in the retroviral construct) that this isoform was not detectable by immunofluorescence in the confocal studies. However, soluble isoform 3 was detected in supernatant of cells infected with adenovirus encoding isoform 3 and not from cells infected with adenovirus encoding isoform 1, also supporting its secretion from cells (Figure 3.10). Because isoform 3 retains the N-terminal domain needed for interaction with MHC I, it was speculated that it might associate with MHC I trafficking to the cell surface and subsequently be released. Since DC2.4 cells appear to have much higher levels of MHC I reaching the cell surface, this is one possible explanation for the lower isoform 3 levels observed in these cells relative to tapasin\textsuperscript{-/-} fibroblasts.

Immunofluorescence and confocal microscopy studies suggested that the majority of isoform 1 and 2 localizes to the ER, which is supported by the EndoH pulse-chase
assays that indicate neither isoform acquired any significant resistance to EndoH digestion over a two-hour chase period (Figure 3.9, 3.11). This ER localization was observed in both tapasin-/- fibroblasts and DC2.4, and in the former case also correlated with extensive co-localization with MHC Class I, which appears to be mainly ER-localized in fibroblasts but not in the DC2.4 (Figure 3.12), as mentioned above. Interestingly, in tapasin-/- fibroblasts in which isoform 3 was detectable (isoform 3 did not appear to be uniformly expressed within the cell population), it was also found to co-localize with ER, perhaps in a transient state following co-translational insertion into the ER via the Sec61 complex and prior to secretion.

The localization of isoform 2 to the ER is somewhat surprising given its lack of an ER-retention motif. However, other studies have mutated the tapasin dilysine and found that it still does not leave the ER in large amounts, leading to speculation that tapasin is also retained in the ER by other motifs, interactions with other molecules, or clustering exclusion from ER exit sites (189). The tapasin-related protein (discussed further in Chapter 6), which is structurally very similar to tapasin but lacks an ER-retention motif, was also found to be mainly ER-localized (190). These results may indicate that that isoform 2 is present in non-ER compartments in small amounts insufficient for detection by pulse-chase and EndoH digestion. It is also possible that tapasin might not be capable of acquiring EndoH resistance despite passing through the medial Golgi, a phenomenon that is known for a small number of proteins such as the IP30 glycoprotein (174). On the other hand, one study following a mutant tapasin molecule lacking the dilysine motif that was tagged at the C-terminus with GFP did observe some exit of tapasin from the ER and acquisition of EndoH resistance,
suggesting that tapasin is subject to modification by this enzyme if it escapes the ER (101).

Despite the extensive ER co-localization, small amounts of non-ER localization were observed for both isoform 1 and 2, though there were differences between the two cell lines. Both isoforms were found to co-localize with the endosomal marker Rab5 in DC2.4 in approximately equal amounts, while in tapasin⁻/⁻ fibroblasts only isoform 2 colocalized extensively with Rab5 (Figure 3.14). This indicates cell type-specific mechanisms (such as the phago-ER) may be responsible for isoform 1 localization to Rab5⁺ compartments in DCs. Interestingly, both isoforms were also found to co-localize with a second endosomal marker, EEA1, in DC2.4, but isoform 1 co-localized in higher amounts with this marker than isoform 2. It therefore appears that both isoform 1 and 2 can localize to endosomal compartments in DC2.4, and that the specific compartments defined by the Rab5 and EEA1 markers, respectively, might not wholly overlap, resulting in the differential amounts of isoform 1 and 2 observed to colocalize with each marker. This phenomenon was also observed for the two lysosomal markers employed in this study: Rab7 and LAMP2 (Figure 3.15). Whereas extensive colocalization of isoform 2 was observed in tapasin⁻/⁻ fibroblasts with Rab7, no colocalization was found with LAMP2. This could be due to the quality of the staining obtained with the two different antibodies, the expression levels of the proteins detected by the antibodies, or might indicate different subsets of lysosomes, with isoform 2 only localizing to those expressing Rab7. It is interesting that co-localization of isoform 2 with Rab7 was not observed in DC2.4 cells, which again might be indicative of cell type-specific trafficking mechanisms. Clearly, the more marker antibodies used to co-stains a given organelle, the
higher the resolution of the localization. This is especially true for the endosomal/lysosomal system, which consists of many vesicles at different stages of maturation (191) in a dynamic equilibrium, with the marker proteins themselves constantly cycling between compartments.

Co-localization of the isoforms was not observed with cis-Golgi (Figure 3.13), unlike other studies that have reported small amounts of tapasin transiently localized to that compartment (170). TAP1 and 2 have been reported to localize to cis-Golgi in small amounts as well (192), which together might indicate occasional escape of the whole TAP-Tapasin complex from the ER or a small degree of cycling between the two compartments. However, isoform 2 was found to co-localize extensively with the TGN marker furin convertase in both DC2.4 and tapasin⁻/⁻ fibroblasts (Figure 3.13). MHC I molecules have been proposed to pass through the TGN when being recycled from the cell surface for MHC Class I cross-presentation, a pathway thought to be controlled by a tyrosine residue in the cytoplasmic tail of MHC I (26). However, it is not known whether or not MHC I molecules actually load peptides in the TGN. Though the results were not statistically significant, it also appeared that isoform 3 was present in the TGN in tapasin⁻/⁻ fibroblasts, which could be transient as isoform 3 is secreted from the cell, but could also be due to internalization from the extracellular milieu in conjunction with recycling MHC I molecules. The subcellular localizations of the tapasin isoforms as determined by this study are summarized in Figure 3.18.

In conclusion, the tissue and cellular expression of the tapasin isoforms, as well as their localization in some cases to the TGN and endosomal compartments, supports a potential role for the novel isoforms in DC-specific antigen presentation processes such
as cross-presentation. The localization of the tapasin isoforms to these compartments may be indicative of assembly of the whole PLC in these novel locations, since the isoforms retain the N terminal domain needed for scaffolding of other components, such as ERp57 and calreticulin.

(a) In tapasin−/− fibroblasts, all three isoforms co-localized with ER markers (pink) and MHC I molecules, which are found at high levels in the ER of this cell type and at low levels on the cell surface. Isoforms 2 and 3 showed partial co-localization with a TGN (orange) marker, furin convertase. Isoform 2 also co-localized with the endosomal marker Rab5 (blue) and occasionally with the lysosomal marker Rab7 (purple). Although not detectable, isoform 3 is likely to be secreted at low levels from the tapasin−/− fibroblasts (b) In DC2.4 cells, levels of isoform 3 were too low for detection, presumably due to high levels of secretion from the cell. Isoforms 1 and 2 co-localized with ER markers, but less with MHC I as most MHC I in DCs is cell surface-localized. Isoform 1 and 2 were also found co-localized with endosomal markers Rab5 and EEA1 (green). Only isoform 2 co-localized with the trans-Golgi network marker in DC2.4 cells.

Figure 3.18 Summary of findings regarding the tapasin isoforms’ subcellular localization in tapasin−/− fibroblasts (a) and DC2.4 cells (b).
Chapter 4: The Functions of Tapasin and Its Isoforms in MHC Class I Antigen Presentation

4.1 Introduction

The very existence of novel isoforms of tapasin suggests that they have a unique function in biology. Given the only known function for tapasin is in immunity - specifically as a “chaperone” in MHC I antigen presentation - it therefore seemed likely that the two novel isoforms might play slightly different roles in this same process. All the isoforms have an N-terminal domain needed for interaction with MHC I and other peptide loading complex proteins, such as ERp57, and therefore may possess the ability to influence the functions of these proteins in antigen processing and presentation. To investigate this possibility, the functions of the isoforms were examined in the context of the two known forms of MHC I antigen presentation: classical endogenous MHC I antigen presentation and MHC I cross-presentation of exogenous antigens.

The isoforms were expressed in tapasin−/− fibroblasts and DC2.4 dendritic cells, as described in Chapter 3. The tapasin−/− fibroblasts allowed us to evaluate the role of each individual isoform, as well as combinations of different isoforms, on a tapasin−/− background. However, these studies were limited to an examination of endogenous MHC I antigen presentation and related processes since fibroblasts are not thought to be capable of cross-presenting exogenous antigens. The isoforms were therefore also expressed in a murine dendritic cell line DC2.4 to evaluate their potential contribution to cross-presentation of the model antigen ovalbumin. In addition, expression of the isoforms in this cell line allowed testing of the hypothesis that the isoforms might play a
role MHC Class II antigen presentation, which is normally restricted to DCs, macrophages and B cells. The expression of combinations of the isoforms in tapasin−/− fibroblasts, and individually in DC2.4 (but with mouse tapasin present), also allowed testing of the hypothesis that the isoforms might exert a dominant negative or competitive effect on MHC I peptide loading by another isoform, particularly isoform 3, which in lacking the transmembrane domain might serve to block the MHC I peptide-binding groove in a dominant-negative or competitive fashion. The blocking effect of isoform 3 was also tested using MHC I-binding peptides added exogenously to the media, mimicking a potential in vivo pathway of exogenous peptide presentation.

4.2 Results

4.2.1 Effect of the tapasin isoforms on MHC Class I surface expression in fibroblasts from tapasin−/− mice

The effect of the tapasin isoforms on MHC I surface expression levels was examined in tapasin−/− fibroblasts expressing each isoform or pMX-pie vector alone (described in Chapter 3) by flow cytometry with conformation-dependent antibodies against the two MHC I alleles expressed by these cells: H-2Kb and H-2Db. Tapasin−/− fibroblasts naturally express very low surface MHC Class I (117;118), similar to TAP1−/− fibroblasts (38). Expression of isoform 1 and 2 resulted in restoration of surface H-2Kb and H-2Db expression to levels similar to C57BL/6 fibroblasts (Figure 4.1). However, isoform 3 expression was not able to restore the surface MHC I expression of either allele, and the low levels were statistically no different from those of tapasin−/− fibroblasts expressing vector alone or TAP1−/− fibroblasts.
Figure 4.1 Surface MHC Class I levels in tapasin−/− fibroblasts expressing the tapasin isoforms.
Isoform 1 and 2 restored both H-2Kb (a) and H-2Db (b) surface expression to wild-type levels as assessed by flow cytometry. Surface MHC I expression was very low, comparable to TAP1−/− fibroblasts, in tapasin−/− fibroblasts expressing vector alone or isoform 3. Data were compiled from at least three independent experiments.
4.2.2 Effect of the tapasin isoforms on susceptibility to specific CTL killing of fibroblasts from tapasin^{-/-} mice

Classical $^{51}$Cr release CTL assays were performed to determine whether the increase in surface MHC I expression in tapasin^{-/-} fibroblasts mediated by isoform 1 and 2 resulted in restoration of the ability of the cells to be recognized and killed by specific CTLs. Tapasin^{-/-} fibroblasts expressing each isoform or vector alone were infected with Vesicular Stomatitis Virus (VSV), loaded with $^{51}$Cr, and incubated with splenic CTLs specific for a VSV immunodominant peptide (VSV-NP_{52-59}) in the context of H-2K^{b}, which were isolated from C57BL/6 mice infected with VSV. As positive and negative controls, C57BL/6 fibroblasts were infected with VSV or left uninfected, respectively. Tapasin^{-/-} fibroblasts expressing each isoform or vector alone that were not infected with VSV were also included as additional negative controls and showed virtually no susceptibility to killing (data not shown). Consistent with the flow cytometry results, tapasin^{-/-} fibroblasts expressing isoform 1 and 2 were recognized and killed as efficiently as C57BL/6 fibroblasts, while tapasin^{-/-} fibroblasts expressing vector alone or isoform 3 were not killed, similar to the uninfected C57BL/6 negative control. Killing efficiency decreased when decreasing numbers of CTLs were mixed with the target cells in a titration curve, indicating that the specific CTLs were mediating the cell lysis (Figure 4.2).
Figure 4.2 51Cr-release assay using VSV-infected tapasin−/− fibroblasts expressing the tapasin isoforms as targets for VSV-specific CTLs. Tapasin−/− fibroblasts expressing isoforms 1 and 2 and infected with VSV were efficiently killed by VSV-specific CTLs, while cells expressing vector alone, isoform 3 and uninfected control cells were not killed. Data shown represents the results of one experiment, which was repeated three times.

4.2.3 Ability of tapasin isoforms to stabilize TAP

The underlying mechanism governing the ability of isoforms 1 and 2 to restore MHC I surface expression and present specific peptides to CTLs was revealed by Western blotting for mouse TAP1 and TAP2 protein levels in tapasin−/− fibroblasts expressing each isoform. Although mouse TAP1 and TAP2 mRNA was expressed normally in tapasin−/− fibroblasts (Figure 4.3a), TAP1 and TAP2 protein levels in these cells were undetectable (see “vector alone” lane, Figure 4.3b). Tapasin is known to stabilize TAP1 and TAP2 proteins, and it is the transmembrane domain of tapasin that mediates its interactions with the TAP heterodimer (87;99;100). Isoforms 1 and 2 retain
the tapasin transmembrane domain and were therefore able to stabilize mTAP1 and TAP2 protein to levels similar to C57BL/6 cells (Figure 4.3b). However, vector alone- and isoform 3-expressing tapasin−/− fibroblasts had no detectable TAP protein, presumably because isoform 3 lacks the transmembrane domain necessary for TAP stabilization.

(a)       (b)

Figure 4.3 TAP expression in tapasin−/− fibroblasts expressing the tapasin isoforms. (a) RT-PCR indicates that tapasin−/− fibroblasts express mouse TAP1 and TAP2 mRNA (DC2.4 DC cell line = positive control for mTAP1 and mTAP2 mRNA, TAP1−/− = negative control for mTAP1 mRNA, S15 = mRNA loading control for RT-PCR). (b) TAP1 and TAP2 protein levels are undetectable by Western blot of tapasin−/− fibroblasts expressing vector alone or isoform 3, but are stabilized at levels similar to C57BL/6 wild-type fibroblasts upon expression of isoforms 1 and 2. GAPDH = protein loading control (~35kDa). Both TAP1 and TAP2 proteins are ~70kDa in size. Data shown represents the results of one experiment, which was repeated twice.
4.2.4 ERp57 interacts with the tapasin isoforms

Since all three isoforms of tapasin retained the N-terminal domain responsible for interactions with ERp57 and other members of the PLC, it was determined whether any physical interaction could be detected between tapasin and ERp57. Tapasin−/− fibroblasts expressing the tapasin isoforms or vector alone were lysed, and Western blots or immunoprecipitations were performed to detect ERp57 in association with tapasin. ERp57 was co-immunoprecipitated and detected by ERp57 antibodies in human tapasin-immunoprecipitated samples (Figure 4.4, middle panel), indicating the mouse ERp57 interacts with the tapasin isoforms.

![Figure 4.4 ERp57 co-immunoprecipitates with the tapasin isoforms.](image)

**Figure 4.4 ERp57 co-immunoprecipitates with the tapasin isoforms.** Tapasin−/− fibroblasts expressing the tapasin isoforms or vector alone were immunoprecipitated with an anti-human tapasin monoclonal antibody and probed with a rabbit anti-human tapasin polyclonal (top panel) or rabbit anti-mouse ERp57 polyclonal (middle panel). ERp57 co-immunoprecipitated with all three tapasin isoforms. GAPDH = protein loading control from lysates used for IP.
4.2.5 Rate of ER exit of MHC Class I molecules loaded by the tapasin isoforms

To ascertain any novel functions of isoform 2 compared to isoform 1, a series of assays were employed to assess the quality of the peptides being loaded onto MHC I in tapasin−/− fibroblasts expressing either isoform. Previous studies have demonstrated that loading of peptides that suboptimally stabilize MHC I can result in different kinetics of ER exit of MHC I molecules, or more rapid turnover of MHC I at the cell surface (87;117;118;173;174). To address the former point, pulse-chase analysis followed by immunoprecipitation with an antibody (P8) recognizing all forms (both folded and unfolded) of H-2Kb was performed. This was followed by Endoglycosidase H (EndoH) digestion to assess the rate of H-2Kb exit from the ER of tapasin−/− fibroblasts expressing each of the tapasin isoforms or pMX-pie vector alone. H-2Kb was immunoprecipitated from cell lysates at various time points up to four hours following a fifteen minute pulse with S35-labeled cysteine and methionine. The rate of acquisition of resistance to Endo H digestion, as described in Chapter 3, is indicative of passage through the medial Golgi. In tapasin−/− fibroblasts expressing isoform 3 and vector alone, very little H-2Kb acquired EndoH resistance after four hours of chase. At two hours, it appeared that much of the H-2Kb was being degraded. This indicates that very little H-2Kb exited the ER and was likely degraded after a few hours, consistent with flow cytometry experiments which showed almost no surface expression of MHC I in these cells. In contrast, some H-2Kb from tapasin−/− fibroblasts expressing isoform 1 and 2 started acquiring resistance to EndoH digestion after ten minutes of chase. By thirty minutes, approximately half of the labeled H-2Kb molecules had acquired EndoH resistance, and by sixty minutes virtually
all of the labeled H-2K\textsuperscript{b} had acquired EndoH resistance. The rate of acquisition of EndoH resistance was similar between tapasin\textsuperscript{−/−} fibroblasts expressing isoform 1 and 2, suggesting that both isoforms mediate similar kinetics of peptide loading and ER exit of this MHC I allele in these cells (Figure 4.5, left panel). Furthermore, these effects were apparent even without the EndoH digestion step. H-2K\textsuperscript{b} is glycosylated and acquires higher carbohydrate structures after leaving the ER, leading to an increase in molecular weight that is evident by decreased mobility in SDS-PAGE gels. This up-shift in molecular weight started to occur by ten minutes of chase in tapasin\textsuperscript{−/−} fibroblasts expressing isoform 1 and 2, although there was still some lower molecular weight ER-form visible as well, leading to the detection of two bands. The higher molecular weight band is equivalent to the EndoH-resistant band in EndoH-digested samples. This higher molecular weight band constituted the majority of H-2K\textsuperscript{b} in the cells by thirty minutes of chase in cells expressing isoform 1 and 2, and was the only form present thereafter (Figure 4.5, right panel). In contrast, in tapasin\textsuperscript{−/−} fibroblasts expressing isoform 3 or vector alone, the lower molecular weight form predominated over the four hour chase. A small amount of a higher molecular weight form did appear in both samples after ten minutes, but did not increase in intensity beyond thirty minutes. This is consistent with EndoH-digested samples, in which the majority of H-2K\textsuperscript{b} remains EndoH sensitive in these cells.
Figure 4.5 Pulse-chase and EndoH digestion assay of H-2K<sup>b</sup> maturation rate in tapasin<sup>-/-</sup> fibroblasts.
Isoforms 1 and 2 allow H-2K<sup>b</sup> molecules to traffic through the Golgi and acquire EndoH resistance and higher molecular weight glycosylation in tapasin<sup>-/-</sup> fibroblasts at similar rates, with acquisition of EndoH resistance and a concurrent increase in molecular weight in undigested controls due to higher glycosylation beginning at ten minutes and completed by sixty minutes. In contrast, very few H-2K<sup>b</sup> molecules acquire EndoH resistance or higher glycosylation in cells expressing vector alone or isoform 3, indicating that the majority of H-2K<sup>b</sup> in these cells remains in the ER. H-2K<sup>b</sup> was immunoprecipitated with the P8 polyclonal antibody recognizing the C terminal region of H-2K<sup>b</sup> in both folded and unfolded molecules. * Non-specific, unidentified band found in all samples. EndoH<sup>R</sup> = Endoglycosidase H resistant, EndoH<sup>S</sup> = Endoglycosidase H sensitive. Data shown represents the results of one experiment, which was repeated three times.
4.2.6 Thermostability of MHC Class I molecules loaded by tapasin isoforms

The decay of H-2K\textsuperscript{b} on the cell surface is another indirect measure of the quality of peptides loaded onto MHC I in the ER. Loading of suboptimal peptides in the ER, which has been observed in tapasin-deficient cells (87;117), leads to more rapid turnover and disassembly of the MHC I complex once it reaches the cell surface. In order to assess the stability of surface MHC I, de novo MHC I being synthesized and assembled in the ER must be blocked from reaching the cell surface and replenishing the MHC I being turned over. This is achieved by blocking ER to Golgi transport with Brefeldin A (BFA), followed by measurement of surface H-2K\textsuperscript{b} by flow cytometry with a conformation-dependent antibody at various time points after BFA addition at 37°C compared to untreated cells. As shown in Figure 4.6, tapasin\textsuperscript{−/−} fibroblasts expressing isoform 1 and 2 had very similar decay curves, suggesting that the peptides loaded by each tapasin isoform onto H-2K\textsuperscript{b} in the ER of these cells were very similar in their binding affinity and ability to stabilize the H-2K\textsuperscript{b} complex. Surface H-2K\textsuperscript{b} levels on tapasin\textsuperscript{−/−} fibroblasts expressing isoform 3 and vector alone were too low to assay their stability with this technique.
Figure 4.6 The thermal stability at 37°C of surface H-2K\textsuperscript{b} molecules in tapasin\textsuperscript{-/-} fibroblasts expressing tapasin isoforms 1 or 2 is very similar. Surface H-2K\textsuperscript{b} molecules, measured by flow cytometry, decay at similar rates upon blockage of \textit{de novo} MHC I ER exit, suggesting the peptides loaded onto H-2K\textsuperscript{b} in these cells by the two isoforms are similar in their binding affinity and ability to stabilize this MHC I molecule. Data shown were compiled from three independent experiments.

4.2.7 Loading of exogenous peptides onto MHC Class I by isoform 3

Isoform 3 is unable to mediate MHC I surface expression in tapasin\textsuperscript{-/-} fibroblasts, likely because of its inability to stabilize the TAP complex due to its lack of the tapasin transmembrane domain. It was also found that tapasin\textsuperscript{-/-} fibroblasts expressing this isoform demonstrated a decreased ability to mediate the loading of an exogenously-added H-2K\textsuperscript{b}-binding peptide when compared to tapasin\textsuperscript{-/-} fibroblasts expressing vector alone. Tapasin\textsuperscript{-/-} fibroblasts expressing isoform 1 and 2 did not show any reproducible differences between them (data not shown).
When SIINFEKL, a peptide derived from ovalbumin that binds to H-2K\textsuperscript{b}, is added to the media in which the cells are growing, it associates with H-2K\textsuperscript{b} through a peptide exchange mechanism. This association can be detected with the 25.D1.16 antibody, which recognizes H-2K\textsuperscript{b} loaded specifically with the SIINFEKL peptide; the association may also be detected with the B3Z hybridoma T cell line, whose TCR specifically recognizes SIINFEKL peptide-loaded H-2K\textsuperscript{b} and results in the cells producing β-galactosidase under the control of the NF-AT promoter in response to TCR engagement (157). When the amount of H-2K\textsuperscript{b}/SIINFEKL staining on tapasin\textsuperscript{-/-} fibroblasts expressing isoform 3 was compared with tapasin\textsuperscript{-/-} fibroblasts expressing vector alone by flow cytometry, the isoform 3-expressing cells consistently showed a small but reproducible decrease in fluorescence intensity. This indicates that isoform 3-expressing cells form fewer H-2K\textsuperscript{b}/SIINFEKL complexes compared to vector alone-expressing cells (Figure 4.8a, left panel). This effect was apparent at all three doses of exogenously-added SIINFEKL used in the assay, and is presumably due to an inhibitory function of isoform 3 on SIINFEKL loading/exchange onto H-2K\textsuperscript{b} that is absent in vector alone-expressing cells.

In addition, the number of total H-2K\textsuperscript{b} complexes on the cell surface of SIINFEKL-incubated cells was detected with a conformation-dependent antibody recognizing H-2K\textsuperscript{b} regardless of the specific peptide loaded. Total surface H-2K\textsuperscript{b} levels were virtually undetectable without the addition of peptide, as seen in Figure 4.1. However, addition of exogenous SIINFEKL peptide at higher doses did lead to an increase in total H-2K\textsuperscript{b} levels. This effect is presumably due to exchange of low affinity self-peptides for the high affinity SIINFEKL peptide, allowing cell-surface expression of
H-2K\textsuperscript{b} through stabilization of the trimeric heavy chain/β2m/peptide complex. When a low affinity peptide is loaded onto MHC-I in the ER, the complex may reach the cell surface but is unstable and rapidly dissociates; however, a high affinity peptide prolongs the half-life of the complex and allows detection by flow cytometry with the conformation-specific antibody. Isoform 3-expressing cells also had lower levels of total H-2K\textsuperscript{b} on the cell surface compared with vector-alone expressing cells following incubation with any given dose of exogenous SIINFEKL (Figure 4.8b, left panel). This also supports the hypothesis that isoform 3 has an inhibitory effect on peptide exchange.

Furthermore, the H-2K\textsuperscript{b}/SIINFEKL complexes generated by the addition of exogenous peptide were able to stimulate B3Z T cells. Consistent with the flow cytometry experiments, isoform 3-expressing cells demonstrated a reduced ability to activate B3Z T cells at a given dose of exogenous SIINFEKL compared to vector alone-expressing cells (Figure 4.7). These experiments also demonstrate that the H-2K\textsuperscript{b}/SIINFEKL complexes generated by addition of exogenous SIINFEKL peptide to the media are functional in their ability to stimulate NF-AT activation in T cells.
Figure 4.7 Loading of exogenous SIINFEKL peptide onto H-2K\textsuperscript{b} is decreased in tapasin\textsuperscript{−/−} fibroblasts expressing isoform 3 compared to vector alone-expressing cells, resulting in decreased activation of B3Z T cells.

Tapasin\textsuperscript{−/−} fibroblasts expressing isoform 3 or vector alone were incubated with SIINFEKL at indicated doses for 1 hr at 37°C, washed extensively, fixed and incubated overnight at a 1:1 ratio with B3Z T cells, whose TCR recognizing SIINFEKL in the context of H-2K\textsuperscript{b} leads to the production of β-galactosidase under the control of an NF-AT element. Levels of β-galactosidase produced were measured by absorbance at 595 nm with the 605 nm wavelength subtracted with CPRG substrate.

It was further investigated whether the observed loading of exogenous SIINFEKL was mainly due to peptide exchange at the cell surface, or required trafficking of nascent H-2K\textsuperscript{b} molecules from the ER. ER to Golgi transport was blocked by pre-treating tapasin\textsuperscript{−/−} fibroblasts expressing isoform 3 or vector alone with Brefeldin A (BFA) prior to adding exogenous SIINFEKL. Only very small amounts of H-2K\textsuperscript{b}/SIINFEKL complexes formed on the cell surface of both isoform 3 and vector alone-expressing BFA-treated fibroblasts, though the amount formed was again slightly higher in vector alone-expressing cells compared to isoform 3-expressing cells (Figure 4.8a, right panel). BFA
treatment also completely abrogated the increase in total H-2K\textsuperscript{b} observed previously in both isoform 3 and vector alone-expressing cells to an equal degree (Figure 4.8b, right panel). Increasing doses of SIINFEKL resulted in only very small increases of H-2K\textsuperscript{b}/SIINFEKL complexes on BFA-treated cells and did not appreciably affect total H-2K\textsuperscript{b} levels. This suggests that a small number of H-2K\textsuperscript{b} already present at the cell surface of these cells may have exchanged intracellularly-loaded, low affinity peptides for SIINFEKL at the cell surface. However, due to the inability for further H-2K\textsuperscript{b} molecules to reach the cell surface from the ER, the number of H-2K\textsuperscript{b}/SIINFEKL complexes remained static and were close to saturation with SIINFEKL even at the lower doses of SIINFEKL added. Therefore, it appears that ER to Golgi transport of nascent MHC I molecules is important for the larger amounts of exogenous SIINFEKL loading observed in the absence of BFA.
Figure 4.8 Loading of exogenous peptide onto H-2K^b and total H-2K^b surface levels are decreased in tapasin−/− fibroblasts expressing isoform 3, and this process is abrogated by Brefeldin A.

Addition of exogenous SIINFEKL peptide to the media of tapasin−/− fibroblasts expressing isoform 3 or vector alone results in formation of SIINFEKL/H-2K^b peptide complexes (a) and total H-2K^b levels (b) with increasing amounts formed with increasing doses of SIINFEKL. However, Tapasin−/− fibroblasts expressing isoform 3 form fewer SIINFEKL/H-2K^b peptide complexes at the cell surface compared to cells expressing vector alone (a- left panel) at any given SIINFEKL dose (statistically significant for 100
and 1000 ng/ml doses), as detected by flow cytometry with a SIINFEKL/H-2K\textsuperscript{b}-specific antibody 25.D1.16. The formation of these complexes is abrogated by blocking nascent H-2K\textsuperscript{b} trafficking to the cell surface with BFA, although isoform 3-expressing cell still have slightly reduced SIINFEKL/H-2K\textsuperscript{b} compared to vector alone under these conditions (a - right panel). (b) Tapasin\textsuperscript{-/-} fibroblasts expressing isoform 3 also have lower total H-2K\textsuperscript{b} surface levels compared to cells expressing vector alone upon addition of exogenous SIINFEKL peptide to the media (b, left panel), but this effect is totally abrogated by addition of BFA and no difference is observed between vector alone and isoform 3-expressing cells (b -right panel). Data shown were compiled from at least two independent experiments, and was performed at least three independent times.

To assess whether intracellular or extracellular isoform 3 is responsible for reducing the formation of SIINFEKL/H-2K\textsuperscript{b} complexes, supernatant from isoform 3-expressing tapasin\textsuperscript{-/-} fibroblasts was transferred to vector alone-expressing fibroblasts and incubated overnight prior to addition of increasing doses of SIINFEKL. Both SIINFEKL/H-2K\textsuperscript{b} complex formation (Figure 4.9a) and total H-2K\textsuperscript{b} surface expression (Figure 4.9b) appeared to be intermediate between isoform 3 and vector alone-expressing controls. This suggests that part of the inhibitory effect of isoform 3 on SIINFEKL loading may be from the extracellular form but that full effects require either intracellular expression of isoform 3 or more consistent synthesis from within the cell, as the half-life of isoform 3 in the media is unknown.

Together these results suggest that isoform 3 may actively suppress the exchange and loading of exogenously-added peptides onto the H-2K\textsuperscript{b} MHC I allele. This could form part of a mechanism that evolved to avoid potential killing of bystander cells located close to pathogen-infected cells that might bind pathogenic peptides released from infected cells and thereby be targeted for CTL-mediated killing.
Figure 4.9 Soluble isoform 3 is at least partially responsible for decreasing the loading of exogenous peptide onto H-2K\(^b\) in tapasin\(^{-/-}\) fibroblasts.

Loading of exogenous SIINFEKL by vector alone-expressing cells pre-incubated overnight with isoform 3 supernatant shows intermediate levels of SIINFEKL/H-2K\(^b\) complexes (a) and total surface K\(^b\) levels (b), which are in between vector alone and isoform 3-expressing controls, indicating extracellular isoform 3 may be partially responsible for the decreased loading of SIINFEKL observed. Data shown represents the results of one flow cytometry experiment, which was repeated three times.
4.2.8 Expression of combinations of isoforms in tapasin<sup>−/−</sup> fibroblasts

Since all cells, cell lines and tissues examined in Chapter 3 naturally express isoform 1, isoform 2 and in some cases isoform 3, the effect of expressing the different isoforms in combinations was evaluated in tapasin<sup>−/−</sup> fibroblasts to observe any synergistic effects on MHC I expression, including potential dominant negative effects (particularly for isoform 3). Tapasin<sup>−/−</sup> fibroblasts were infected with a 1:1 mixture of recombinant retroviruses encoding isoform 1 + isoform 2, isoform 1 + isoform 3, isoform 2 + isoform 3, or all three isoforms, which were then selected in puromycin, and individual clones were sorted by FACS based on GFP expression and expanded in culture. Clones were then screened for expression of more than one isoform. The majority of cells had only been infected with one of the retroviruses and expressed only one isoform. No clones were identified that expressed all three isoforms. However, two clones each were identified that expressed each combination of two isoforms (Figure 4.10).

![Figure 4.10 Tapasin<sup>−/−</sup> fibroblasts clones expressing combinations of tapasin isoforms were generated by co-infection with mixtures of recombinant retrovirus and screening individual clones for expression of both tapasin isoform mRNAs by RT-PCR.](image-url)
The H-2K\textsuperscript{b} and H-2D\textsuperscript{b} surface expression of the clones expressing combinations of isoforms were evaluated by flow cytometry (Figure 4.11a,b). Although expression levels varied between each pair of clones, none were statistically different from one another. Overall, it appeared that in general expression of more than one isoform resulted in slightly lower MHC I surface expression compared to cells expressing either isoform 1 or 2 alone. Only clone 2 of the isoform 2 + 3 combination had statistically lower H-2K\textsuperscript{b} surface expression compared to cells expressing either isoform 1 or isoform 2 alone, or wild-type C57BL/6 fibroblasts (*p<0.01). H-2D\textsuperscript{b} expression on isoform 1+3 clone 2 was statistically lower than cells expressing isoform 1 alone (p<0.01), but was not statistically different than cells expressing isoform 2 alone (p>0.05). However, since both these differences were seen in only one clone from each pair, and only in one of the two MHC I alleles, it was hypothesized that the differences might be due to positional effects in the site of retroviral gene insertion rather than an effect from the genes themselves. A true gene-specific effect would have been mirrored by both clones of each group. Therefore, combinations of isoforms did not appear to exhibit any dominant negative or other synergistic effects in this cell line.
Figure 4.11 Surface MHC Class I expression levels on tapasin^{-/-} fibroblasts expressing combinations of isoforms.

Resting surface levels of both H-2K^b (a) and H-2D^b (b) alleles were examined by flow cytometry. Expression of more than one tapasin isoform decreased surface MHC I expression compared to cell expressing a single isoform. Only clone 2 of isoform 2 + 3 was found to express statistically lower H-2K^b levels compared to cells expressing either isoform 1 or 2 alone (a, *p<0.01). Data were compiled from each least three individual experiments.
4.2.9 Isoform expression in the DC2.4 cell line

Another way to address potential synergistic effects between isoforms was to express the isoforms in cells already expressing mouse tapasin, which is very similar to human isoform 1 in structure and function. The transformed dendritic cell line DC2.4 was selected because it also allowed for the evaluation of the isoforms’ effect on MHC II surface expression and on cross-presentation of exogenous antigens on MHC I, as these are thought to occur only in pAPCs such as DCs and not in fibroblasts (193). Recombinant retroviruses were used to express each isoform or vector alone in the DC2.4 cell line, followed by selection in puromycin and sorting for cells expressing equal levels of GFP as described in Chapter 3. The MHC Class I surface expression of the transduced DC2.4 cells was evaluated by FACS for both H-2K<sup>b</sup> (Figure 4.12a) and H-2D<sup>b</sup> alleles (Figure 4.12b). Interestingly, the effect of tapasin isoform expression on H-2K<sup>b</sup> in DC2.4 cells seemed to be the opposite of the effect of the isoforms in tapasin<sup>-/-</sup> fibroblasts: DC2.4 expressing isoform 1 and 2 appeared to have lower surface H-2K<sup>b</sup> compared to cells expressing isoform 3 and vector alone; however, these differences were not statistically significant (Figure 4.12a). H-2D<sup>b</sup> levels were unaffected by any expression of the isoforms compared to vector alone (Figure 4.12b). Interestingly, all transduced cells had statistically significantly lower expression of H-2K<sup>b</sup> and H-2D<sup>b</sup> compared to uninfected DC2.4, suggesting the retroviral transduction may have caused an overall decrease in MHC I surface expression. This effect did not appear to extend to MHC II expression, however. MHC II expression varied considerably between assays, but statistically there was no significant effect from any isoform or vector alone expression on overall levels of MHC II surface expression (Figure 4.12c).
FUNCTIONS OF TAPASIN ISOFORMS

(a) 

(b) 

(c) 

Fold difference relative to negative control

Vector alone  Isoform 1  Isoform 2  Isoform 3  Uninfected

Fold difference relative to negative control

Vector alone  Isoform 1  Isoform 2  Isoform 3  Uninfected

Fold difference relative to negative control

Vector alone  Isoform 1  Isoform 2  Isoform 3  Uninfected
**Figure 4.12** Cell surface MHC Class I and Class II expression on DC2.4 mouse dendritic cell lines stably infected with recombinant retroviruses expressing the tapasin isoforms, vector alone or uninfected cells.

Flow cytometry shows MHC Class II and H-2D\(^b\) levels were unaffected by expression of the tapasin isoforms versus vector alone (b,c). H-2K\(^b\) levels were slightly reduced in DC2.4 expressing isoform 1 and 2 compared to vector alone- and isoform 3-expressing cell lines, but these differences were not statistically significant (a). Surface expression of both MHC I alleles was statistically higher in uninfected cells compared to cells infected with any of the recombinant retroviruses (a,b), suggesting that transduction itself reduced surface MHC I levels. Data were compiled from each least three individual experiments.

### 4.2.10 Cross-presentation of soluble ovalbumin by DC2.4 expressing tapasin isoforms

To evaluate the potential contribution of the tapasin isoforms to exogenous antigen cross-presentation, soluble ovalbumin at various concentrations was added overnight to DC2.4 expressing the tapasin isoforms. The following day, excess ovalbumin not internalized by the cells was washed off, cells were gently fixed to halt MHC Class I turnover and nascent MHC I loading, and the cells were mixed with B3Z to assess the amount of H-2K\(^b\)/SIINFEKL generated. All cells presented increased amounts of H-2K\(^b\)/SIINFEKL with increasing amounts of ovalbumin added, as expected; however, expression of the isoforms did not have an appreciable effect on the ability of DC2.4 to cross-present exogenous ovalbumin-derived peptides on H-2K\(^b\) (Figure 4.13). At each given dose of ovalbumin, there was no statistically significant difference between the cells expressing the different isoforms (p>0.05) though the increases observed with the highest dose of ovalbumin were statistically significant (p<0.01 or lower). At higher ovalbumin doses, it appeared that isoform 1 and 2-expressing cells expressed fewer H-2K\(^b\)-SIINFEKL complexes compared to isoform 3 and pMX-pie vector-alone expressing cells; however, this is also consistent with the slightly lower overall H-2K\(^b\) surface
expression levels on these cells seen earlier in flow cytometry experiments (Figure 4.12a) and is further supported by the fact that pulsing cells with exogenous SIINFEKL (as a positive control) resulted in lower B3Z activation by isoform 1 and 2-expressing cells compared to pMX-pie vector alone and isoform 3.

![Graph showing absorbance at 595nm-650nm for different ovalbumin doses and tapasin isoforms](image)

**Figure 4.13 Cross presentation of soluble ovalbumin by DC2.4 expressing the tapasin isoforms or vector alone increases with increasing doses of ovalbumin but is unaffected by isoform expression.**

DC2.4 cells expressing tapasin isoforms were incubated overnight with soluble ovalbumin, excess ovalbumin was washed away and cells were incubated at a 1:1 ratio with B3Z T cells to assess the ability of the DCs to present SIINFEKL peptides. No statistically significant differences were observed between DC2.4 cells expressing the tapasin isoforms or vector alone. Data shown represents the results of one experiment, which was repeated three times.
4.3 Discussion

4.3.1 Protein expression of TAP depends on the presence of the tapasin transmembrane domain

The ability of tapasin isoforms 1 and 2 to mediate classical endogenous MHC Class I surface expression and presentation of viral peptides to CTLs in tapasin⁻/⁻ fibroblasts appears to be directly linked to their ability to stabilize TAP1 and TAP2 at the protein level (Figure 4.1, 4.2, 4.3). The regions of TAP that interact with tapasin are the N-terminal extensions of the core multiple membrane spanning domains common to all ABC transporters (79;97;98). The regions of tapasin responsible for mediating interactions with TAP have also been well characterized and consist mainly of residues within the tapasin transmembrane domain (99;100). These detailed studies have contributed a great deal to our understanding of how tapasin stabilizes the TAP heterodimer, and since both isoform 1 and 2 encode the transmembrane domain responsible for this function, it is expected that these isoforms stabilize TAP and isoform 3, which does not contain any of these critical TAP-stabilizing regions, does not. However, this does make it very difficult to ascertain and/or separate additional novel functions of the tapasin isoforms for isoform 3, since the function of tapasin is inextricably linked to the presence of TAP, and any potential subtle differences might be undetectable in its absence. Thus, we must rely more heavily on tissue distribution and expression data of the tapasin isoforms to understand their function.
4.3.2 Tapasin isoforms may assemble peptide loading complexes

The finding that ERp57 co-immunoprecipitates with all three tapasin isoforms (Figure 4.4) suggests that the tapasin isoforms may mediate assembly of a complete and functional peptide loading complex, including other members such as calreticulin and MHC I. Indeed, tapasin has been proposed to require covalent linkage to ERp57 for proper function (104). This could indicate that PLC assembly occurs in the non-ER compartments where tapasin isoforms have been found to localize in small amounts, such as TGN and lysosomes, as described in Chapter 3. While isoform 3 is not likely to associate with TAP, its association with ERp57 could mean that it too acts as a scaffold for PLC assembly, though it remains unknown whether the secreted isoform also interacts with ERp57 and other PLC components. It is also unknown if a soluble PLC complex in the absence of TAP association would function in a different manner than TAP-associated PLCs.

4.3.3 Isoform 1 versus Isoform 2

It was important to ascertain whether isoform 1 and 2 mediated different effects on MHC I antigen presentation beyond their common ability to stabilize TAP. Tapasin is known to influence the repertoire of peptides presented by MHC I molecules, but eluting and sequencing peptides bound by surface MHC I molecules loaded by the different isoforms is a costly experiment. Instead, we began by employing more indirect assays to assess the quality of peptides being loaded by the different isoforms onto H-2Kb. It was hypothesized that the nature of the peptides loaded might differ if the isoforms altered the peptide-binding preferences of the MHC I molecules in distinct ways. Other studies have shown that suboptimally-loaded MHC I molecules, such as those from cells of transgenic
animals deficient in various APM components like ERp57 (106) and tapasin itself (117;118), exit the ER at faster rates and have decreased cell surface thermostability at 37°C. Suboptimal peptide loading leading to thermally unstable surface MHC I can have serious consequences in terms of generating CD8 T cell-mediated immune responses, as the MHC I molecules might disassemble and turn over prior to interacting with CD8 T cells for periods long enough to generate and/or sustain an effective immune response. However, both ER exit rates and cell surface thermostability of H-2Kb molecules appeared very similar in tapasin−/− fibroblasts expressing isoform 1 and isoform 2 (Figure 4.5, 4.6), suggesting that the loaded peptides possessed similar abilities to stabilize this MHC I allele. Nevertheless, it is known that different MHC I alleles, particularly in humans, possess greatly varying dependencies on tapasin for optimal peptide loading, with some alleles such as HLA-B8 and A1 being highly dependent on tapasin, and others such as HLA-B7, A2 and A3 being virtually unaffected by the presence or absence of tapasin (110-112). Since the assay used in this thesis examines human tapasin isoforms in conjunction with a single mouse MHC I allele, H-2Kb, it is possible that this allele may be among those that are not very dependent on tapasin for optimal peptide loading. The dependencies of mouse MHC I alleles on tapasin have been less well studied than human MHC I alleles, and the degree of influence tapasin exerts on H-2Kb specifically has not been studied extensively. However, the low surface expression of H-2Kb in tapasin−/− mice on a C57BL/6 background does suggest it has a tapasin-dependent phenotype. Nevertheless, it remains possible that differences might be observed between isoform 1 and 2 if loading of a different MHC I allele were tested.
4.3.4 Isoform 3

It was initially somewhat surprising that no effect on MHC I from isoform 3 was observed in the tapasin−/− fibroblasts, since another group reported a very similar soluble form of tapasin that did enhance MHC I loading in cells lacking tapasin (174). Lehner et al. (174) demonstrated that a soluble form of human tapasin created by deleting the transmembrane and cytoplasmic regions could still reconstitute MHC I surface expression in 721.220 cells stably expressing various HLA molecules, even though the soluble tapasin no longer associated with TAP (174). More recently, a study by Gao et al. (173) identified a novel isoform of human tapasin that retains introns 5, 6 and 7, resulting in the expression of a truncated tapasin protein due to the introduction of a new stop codon that terminates translation immediately prior to the transmembrane domain. This truncated tapasin protein was predicted to be soluble, as it retained the ER signal peptide and tapasin N-terminal domain but contained 8 novel amino acids at its C terminus and lacked an ER retention motif. When this isoform was expressed in 721.220 cells stably expressing HLA-B8, it was also capable of restoring MHC I antigen presentation despite a lack of binding to TAP. However, the peptides loaded were found to have suboptimal affinity for MHC I compared to those loaded by wild-type tapasin (173). Both these studies were done in the human tapasin-deficient 721.220 cell line transfected with HLA-B8 (or other HLA alleles) rather than the mouse tapasin−/− fibroblast line used in this thesis. Unlike the murine tapasin−/− cells, 721.220 cells express large amounts of TAP since they have been transformed by EBV; TAP levels in EBV-expressing cells are known to be much higher compared to EBV-negative cells, in which TAP is more limiting (174;184). It is likely true that tapasin expression in EBV-transformed cells is
higher than in uninfected cells as well. Furthermore, the 721.220 cells do still produce a
N-terminally truncated form of tapasin that localizes in small amounts to the ER and
contains the TAP-stabilizing transmembrane domain, which is likely sufficient to
stabilize TAP protein expression (120). Therefore, it is difficult to compare our studies to
the studies done in 721.220, as clearly these cells are not completely tapasin (and TAP)
deficient. It would be very interesting to express isoform 3 in these cells; however, the
721.220 cells stably transfected with the otherwise-deleted HLA genes were not available
for this study, and many cloned HLA molecules (which could have been used to generate
our own stable transfectants) appear to be proprietary and were similarly inaccessible.

The existence of isoform 3 suggests that it must have a function distinct from
stabilizing TAP, and certainly there are many other different functions ascribed to tapasin
(83;86;87;89;90). Two approaches were tried to ascertain the function of isoform 3: 1) addition of a known H-2K\textsuperscript{b}-binding peptide, SIINFEKL, to the extracellular media,
thereby bypassing the need for peptide import into the ER by TAP, and 2) expression of
isoform 3 in combination with the other two TAP-stabilizing tapasin isoforms to see if
there was any synergistic or dominant–negative function for isoform 3 that might be
revealed upon restoration of TAP protein expression by the other isoforms. For the latter
experiments the tapasin isoforms were expressed in combinations (isoform 1+2, isoform
2+3 and isoform 1+3) in tapasin\textsuperscript{−/−} fibroblasts, and also each isoform was expressed
individually in DC2.4 cells that have endogenous mouse tapasin capable of stabilizing
TAP, to look for any combinatorial effects (Figure 4.11, 4.12). In both cell lines, no
obvious dominant negative effects from isoform 3 were observed to influence surface
levels of either H-2K\textsuperscript{b} or H-2D\textsuperscript{b}; indeed if anything it appeared that expression of more
than one tapasin isoform decreased overall surface MHC I expression relative to cells transduced with only a single isoform, regardless of which isoform combinations were expressed. This may indicate that higher-than-normal tapasin expression results in more stringent editing functions in the ER, leading to an overall decrease in surface expression as more MHC I molecules remain in the ER. Only one clone (isoform 2+3, clone 2) was statistically different from cells expressing either isoform 1 or 2 alone. However, a second clone (clone 1) expressing the same two isoforms did not have any significant decrease in surface MHC I. Therefore, the decreased surface MHC I observed could be due to positional effects from the site of integration of the retroviral constructs rather than an effect from the genes themselves, which should have been apparent in all clones examined. It would be useful to study larger numbers of clones to draw further conclusions as to any combinatorial effects, two clones being a small sample size. However, having two different retroviruses infect the same cell is a rare occurrence, and would require the screening of many more clones. No effects on MHC II surface levels were observed in DC2.4 transductants (Figure 4.12), suggesting the novel isoforms do not influence basal MHC II presentation in these cells. It has been recently proposed that the MHC II chaperone invariant chain can bind and direct MHC I molecules (194;195), but from the data presented here it appears that the reverse (i.e. tapasin chaperoning MHC II) does not occur.

An isoform 3-mediated effect on MHC I loading of exogenously-added SIINFEKL peptides was observed in tapasin\textsuperscript{−/−} fibroblasts relative to vector-alone expressing cells that may be indicative of a novel function of isoform 3. Tapasin\textsuperscript{−/−} cells expressing isoform 3 formed fewer H-2K\textsuperscript{b}/SIINFEKL complexes at the cell surface (as
detected by an antibody or via activation of T cells with TCRs specific for this complex) compared to vector-alone expressing fibroblasts (Figure 4.7, 4.8). This occurred despite equally low overall surface H-2K^b levels prior to addition of peptide (Figure 4.2.1a). Levels of total surface H-2K^b were also lower following peptide incubation in isoform 3-expressing tapasin^c^ fibroblasts relative to vector alone, though this antibody does not discriminate between H-2K^b^ loaded with SIINFEKL versus any other peptide. However, since the antibody used in the latter experiment recognizes only properly folded and assembled H-2K^b^ (196), this likely indicates that isoform 3 decreases loading of SIINFEKL onto H-2K^b^ associated with endogenous mouse β2-microglobulin, rather than exchange of the endogenous peptide and re-assembly of the complex with bovine β2-microglobulin from the media FBS (151;197), which would not be recognized by this antibody. Furthermore, almost all loading of SIINFEKL onto H-2K^b^ was blocked by Brefeldin A (Figure 4.8), which arrests the ER to Golgi transport of nascent MHC I molecules (198;199). Therefore, the loading of exogenous peptides appears to a large extent to require new MHC I molecules trafficking from the ER. Two non-mutually exclusive scenarios can be envisioned that could explain this observation, summarized in Figure 4.14: 1) MHC I loaded with low-stability self-peptides, such as excised signal peptides, traffic from the ER and then bind and are stabilized by SIINFEKL at the cell surface, leading to an accumulation of MHC I bound to SIINFEKL at the plasma membrane: this process is decreased in the presence of isoform 3 and potentially other members of the PLC, or 2) studies have shown that exogenous proteins and peptides can access the ER through unknown mechanisms (200-203), so the exogenous SIINFEKL might reach the ER and bind MHC I molecules within the ER, leading to their
stabilization and escape from the ER. This latter process could also be abrogated by BFA and could be inhibited by isoform 3 either in the ER or exogenous isoform 3 having trafficked back into the ER from the cell surface similarly to the peptide. Isoform 3’s effects did seem to be at least partially due to the secreted form, because transfer of supernatant from isoform 3-expressing fibroblasts to vector alone-expressing fibroblasts did decrease the amount of SIINFEKL loaded onto H-2K\(^b\), but not to the same degree as observed in cells actually synthesizing isoform 3 \textit{de novo} (Figure 4.9). In the present studies, however, it is not possible to distinguish between these two possible scenarios of peptide loading, nor the specific mode of action of isoform 3. It is also possible that isoform 3 influences exogenous peptide loading in other compartments as well, such as Golgi or lysosomes, providing the peptides, soluble tapasin and PLC components, and MHC I molecules co-localize in these compartments.
Figure 4.14 Two potential models of isoform 3’s effect on inhibiting loading of exogenous peptides.
(a) Exogenous isoform 3 (potentially in conjunction with other PLC components) inhibits loading of exogenous peptides onto MHC I molecules present at the cell surface that have escaped the ER either empty or loaded with low affinity peptides due to the absence of endogenous ER peptides from low to no TAP expression. (b) Exogenous peptides gain
access to the ER through unknown mechanisms where they bind newly-synthesized MHC I molecules, a reaction inhibited by ER-localized (or internalized) soluble tapasin and potentially other PLC components such as ERp57. It is also possible that both models operate simultaneously, or that exogenous peptide loading might also occur in other compartments as well (such as Golgi or endo/lysosomes).

Whatever the exact molecular mechanism, it does seem apparent that isoform 3 plays a role in blocking at least some exogenous peptides from binding to MHC I molecules. This could assist in avoiding detrimental effects in vivo such as uninfected bystander cells being targeted for CTL killing during viral infections by binding viral peptides derived from nearby infected cells, for example. Isoform 3 may act in a manner similar to invariant chain, which prevents MHC II molecules from binding peptides until reaching lysosomal compartments. By preventing MHC I molecules, perhaps some of which are recycling from the cell surface or may be newly synthesized ER forms, from binding peptides until the appropriate cross-presenting compartment is reached, isoform 3 could assist in cross-presentation by ensuring loading of peptides only at the correct time and place. This might also avoid potentially autoreactive immune responses caused by MHC I binding self peptides on the cell surface of activated DCs in the lymph node. Similarly to invariant chain, other groups have identified tapasin-derived peptides eluted from HLA binding grooves by mass spectrometry (see Table 4.1), suggesting that tapasin does occupy the peptide binding groove at certain times or under certain circumstances. This is discussed further in Chapter 6.
Table 4.1 Tapasin-derived peptides eluted from HLA molecules and sequenced by mass spectrometry.

<table>
<thead>
<tr>
<th>Tapasin-derived peptide</th>
<th>HLA molecule</th>
<th>Source of data</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tapasin 354-362</td>
<td>HLA-B*1509</td>
<td>(204;205)</td>
</tr>
<tr>
<td></td>
<td>HLA-B*1510</td>
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</tr>
<tr>
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<tr>
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<td>HLA-A*1101</td>
<td><a href="http://www.SYFPEITHI.de">www.SYFPEITHI.de</a></td>
</tr>
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4.3.5 Cross-presentation

The isoforms did not appear to influence cross-presentation of the ovalbumin-derived peptide SIINFEKL onto H-2K<sup>b</sup> molecules in the DC2.4 cell line (Figure 4.13). There are several possible explanations for this observation. The first is that the isoforms, despite their high expression in mature DCs and localization to endo/lysosomal compartments, do not participate in antigen cross-presentation and have another function altogether. However, the many proposed models of cross-presentation testify to the complexity of this process, and the molecular mechanisms involved have not yet been clearly delineated and are still a matter of considerable debate within the field. It is therefore possible that the isoforms might exert an effect on cross-presentation if tested with another antigen taken up by another route or in a different DC subset.

Soluble ovalbumin is cross-presented after uptake via the mannose receptor (206). Other routes of uptake, such as phagocytosis of particulate antigen, apoptotic cells, or endocytosis of antigen bound by antibodies (as immune complexes), may lead to distinct mechanisms of cross-presentation (207). It has been shown that the same antigen (ovalbumin) is processed through different pathways depending on whether it is
presented in a soluble or particulate form (208). Other antigens may be processed
differently still, but unfortunately very few reagents such as the 25.D1.16 antibody or the
B3Z T cell line exist for other antigens, making detection of cross-presentation of these
antigens difficult.

Another explanation for the lack of cross-presentation by the isoforms is that the
form of cross-presentation mediated by the isoforms may operate only in specific subsets
of DCs not represented by the DC2.4 cell line. Many different DC subsets have been
discovered, each with slightly different characteristics, locations and functions (209;210).
While all, to some extent, are capable of antigen capture and presentation to naïve T cells,
some are more efficient at this than others. For example, conventional mouse myeloid
DCs expressing CD8 (for which no equivalent in human has yet been identified) are
thought to be the most efficient at cross-presentation (211), but skin Langerhans cells
(212;213) and debatably plasmacytoid DCs (214-217) are also thought to cross-prime
naïve CD8+ T cells. However, not all DC subsets can be easily cultured, transfected or
transduced in vitro, limiting our ability to test many of their functions at the molecular
level.

The effect of the isoforms could also be hidden by endogenous mouse tapasin in
the DC2.4 cell line; indeed, isoform 3’s effect in decreasing the loading of exogenous
SIINFEKL peptides on tapasin−/− fibroblasts was not readily observable in DC2.4 cells
(data not shown), in which the mouse tapasin expression could have masked this effect.
However, attempts to express the isoforms in DCs from tapasin−/− mice were hampered by
technical difficulties in expressing the isoforms in these primary cells while not
interfering with their ability to cross-present. In addition, it was not really possible to
assess the function of isoform 3 in tapasin−/− DCs, since these cells, like their fibroblasts counterparts, do not express any TAP protein in the absence of tapasin’s transmembrane domain (data not shown). This effectively makes it impossible to assess the contribution of this isoform in the absence of another tapasin isoform containing the domain needed to stabilize TAP. While TAP is not specifically required for exogenously-derived peptide loading in the lysosomal model of cross-presentation, TAP is still likely required in this model to initially get MHC I molecules to the cell surface for subsequent internalization into the lysosomal compartments for intersection with the exogenous antigens. In the absence of any TAP in the ER, the sequestration of peptide-deficient MHC I in the ER likely decreases the efficiency of this pathway significantly.

It is also possible that the isoforms might have more influence on other MHC I alleles that could be more sensitive to their effects than H-2Kb (as discussed above) or that the human isoforms might mediate an effect specific to some or all human MHC alleles only. However, due to the unavailability of a human tapasin-deficient dendritic cell line and appropriate detection reagents, this cannot be assessed at present.

In vitro assays of cross-presentation are inherently inefficient compared to in vivo assays. The levels of SIINFEKL complexed with MHC I generated by cross-presentation of soluble OVA are quite low in the tissue culture setting. Therefore, the best assay for the role of the isoforms in cross-presentation would be the creation of transgenic animals expressing the isoforms systemically on an otherwise tapasin−/− background, allowing for cross-presentation assays without the need to virally-infect the cells to achieve isoform expression, which can have undesirable side effects such as premature DC maturation or viral inhibition of cross-presentation. These models would also allow the assessment of
cross-presentation in the context of a complete, interacting immune system with the possibility to test different antigens and routes of inoculation. Proposed experiments of this nature are discussed in Chapter 6.
Chapter 5: Reconstitution of TAP and Tapasin Expression in MHC Class I-deficient Tumors

5.1 Introduction

The discovery of tumor-associated antigens has led to research and the development of prototypic therapeutic vaccines designed to generate anti-tumor responses with the aim of controlling tumor growth and metastasis (218). A wide variety of vaccination approaches and protocols have been tried or are currently being tested (219). In general, the results show that even though specific cellular immune responses can be generated against tumor-associated antigens (TAAs), the response of the disease to the treatment is low (220;221). The reasons for the low response rates are thought to be the low immunogenicity of the antigens, which are usually self-antigens, and the natural selection of tumor variants that have immune-suppressive phenotypes arising through a process called immuno-editing (140;222).

A version of this chapter has been published.

The nature of the immune suppression may include secretion of immunosuppressive cytokines (223), the expression of ligands (FAS-L) that initiate apoptosis in cytotoxic T cells (224), and the selection of tumor variants that are deficient in antigen processing and presentation (225).

Tumors that are deficient in the MHC I antigen processing pathway do not express MHC I molecules on the cell surface. As a consequence, specific cytotoxic T cells (CTLs) generated by the vaccine protocol are unable to recognize and kill these tumor variants due to defective presentation of TAA-derived peptides recognized by the CTLs. Any alterations, mutations, deletions or deficiencies in the antigen presentation pathway can lead to such non-immunogenic tumor variants. In most cases, these tumors are characterized by the down-regulation of components of the MHC I antigen presentation pathway, including TAP1, TAP2 and tapasin (134;225). Down-regulation of TAP1 has been shown to be a critical factor in MHC I antigen deficiencies in tumors and has been associated with disease progression and death (131;132;139). Conversely, TAP1 expression has been associated with increased numbers of tumor infiltrating lymphocytes, a characteristic of good clinical outcome (131;226;227) and spontaneous tumor regression (228). Loss of tapasin expression is also common in many human carcinomas such as breast cancer (229;230), melanoma (231), colorectal carcinoma (232), and both small cell and non-small cell lung carcinoma (146), as well as mouse cancers such as mouse fibrosarcoma (233) and mouse melanoma (234). Tapasin loss is particularly common in metastases (128;130). Remarkably, in human colorectal cancers, tapasin is more frequently lost than TAP1, LMP2 and LMP7 (235), suggesting that the loss of tapasin could be a key event in overcoming immune-surveillance in these tumors.
Since the downregulation of APM components in carcinomas has been hypothesized to play a key role in their escape from CD8+ T cell immune recognition and destruction, the effect both in vitro and in vivo of expressing the missing components ectopically was determined. Previous studies in our laboratory demonstrated that restoration of rat or human TAP1 expression alone seemed to be capable of restoring MHC I surface expression and recognition by CTLs in a murine lung carcinoma model, CMT.64 (146;150). TAP1 restoration of antigen processing and presentation has also been observed by other groups in human carcinoma models (152;153). CMT.64 is derived from a spontaneous lung carcinoma in a C57BL/6 mouse (236) that is characterized by the down-regulation of many components of the antigen presentation pathway, including MHC Class I heavy chain, β2-microglobulin, LMP2 and LMP7, TAP1 and TAP2 and Tpn (237;238). In this chapter, the effect of rat and human TAP1 expression in another model was investigated. B16F10 is a subclone of the mouse B16 melanoma cell line that was established after 10 successive selections for lung metastases after intravenous injection, and it is highly metastatic and weakly immunogenic (155;239). B16F10 has been widely used as a tumor model for tumor-host immune interactions and the evaluation of tumor therapies. The B16F10 tumor cells, like most metastatic carcinomas (including our previously-studied model CMT.64) have a deficiency in MHC I surface expression due to downregulation of several components of MHC I antigen-processing pathway, including TAP1, TAP2, the proteasome subunits LMP2, LMP7, and LMP10, PA28α and β, and tapasin (155;240). This downregulation of the antigen presentation pathway can be reversed by IFN-γ treatment. The hypothesis tested was that restoration of TAP1 expression in B16F10 cells increases MHC I surface
expression and immunogenicity, making these cells visible to immune surveillance mechanisms. The efficacy of recombinant vaccinia or adenovirus vectors expressing TAP1 was also tested in an \textit{in vivo} setting to obtain further proof-of-principle of the efficacy of the TAP1 viral construct for use in a cancer vaccine setting.

A significant stabilizing effect on tapasin protein levels was observed in B16F10 transduced with either rat or human TAP1, suggesting the observed effects could be due to concurrent upregulation or stabilization of endogenous tapasin protein expression in addition to the ectopic TAP1 expression. To further investigate the role of tapasin in antigen processing and presentation in tumor cells, a recombinant adenovirus expressing human tapasin (isoform 1) was created, and its effects on tumor antigen processing, presentation, and immunogenicity were evaluated in the CMT.64 carcinoma model. Results indicate that tapasin also can restore a functional degree of antigen processing and presentation in tumor cells, and leads to even more potent anti-tumor immunity when combined with TAP1.

\section*{5.2 Results}

\subsection*{5.2.1 Expression of APM components in MHC Class I-deficient B16F10 after rat or human TAP1 expression}

Previous studies have shown that expression of rat or human TAP1 through stable transfection or infection with recombinant viruses expressing TAP1 resulted in small but significant increases in MHC I surface expression in the murine lung carcinoma, CMT.64 (146;150). The goal of this study was to recapitulate these results in another model, B16F10, and to further investigate the mechanism by which restoration of TAP1
mediates MHC I surface expression. To this end, B16F10 cells were stably transfected with a rat TAP1 cDNA construct under the control of a β-actin promoter, and two clones (B16/rTAP1 3-3 and B16/rTAP1 3-8) were selected for further examination. Not surprisingly, the clones expressing rTAP1 had detectable TAP1 protein by Western blot, while untransfected or an empty vector-alone transfected clone (B16/PHβ1-1) had no detectable TAP1 expression (Figure 5.1, top panel). However, the clones expressing rat TAP1 had detectable levels of TAP2 and tapasin protein as well (Figure 5.1). This suggested that TAP1 expression was somehow stabilizing low levels of the other two APM proteins, allowing their accumulation to detectable levels in the cells.

Figure 5.1 Stable transfection of rat TAP1 restores TAP2 and tapasin protein expression in B16F10 melanoma cells.
Stable transfectants expressing rat TAP1 were generated in B16F10 murine melanoma cells and specific clones (B16/rTAP1 3-3 and 3-8) were analyzed for their expression of antigen processing and presentation components, including TAP1, TAP2 and Tapasin, compared with stable transfectants expressing the empty vector alone (B16/pHβ1-1) or untransfected B16F10 cells. RMA cells were used as a positive control, and CMT.64 cells were used as a negative control for these proteins. Tapasin−/− fibroblasts were an additional negative control for mTpn. Expression of rat TAP1 led to increased expression
of mTAP2 and mTpn at the protein level, suggesting that reconstituting TAP1 expression resurrected many components of the antigen processing pathway. Data shown represents the results of one experiment, which was repeated at least twice.

Immunoblot analysis showed that infection of B16F10 cells with a recombinant adenovirus expressing human TAP1 (AdhTAP1) resulted in high levels of expression of the transgene hTAP1. In addition, hTAP1 expression in AdhTAP1-infected B16F10 cells increased endogenous mTpn expression, but not mTAP1 and TAP2 expression (Figure 5.2).

Figure 5.2 Human TAP1 expression after infection by AdhTAP1 leads to increased endogenous mTpn expression.
Murine B16F10 melanoma cells were infected with AdhTAP1 or Ψ5 50 PFU/cell and harvested 48 hrs later. The infected cells were analyzed for hTAP1, mTAP1, mTAP2, and mTpn expression by immunoblotting. β-actin was used as a control for protein loading. T1 and T2 cells, respectively, were used as positive and negative controls for hTAP1 expression. IFN-γ-treated B16F10 cells and untreated B16F10 cells, respectively, were used as positive and negative controls for mTAP1, mTAP2 and mTpn expression.

5.2.2 TAP1 expression increases MHC Class I surface expression on B16F10.

Cell surface MHC I expression on B16F10 cells stably transfected with rat TAP1 was examined by flow cytometry with antibodies recognizing the two MHC I alleles expressed by these cells: H-2K^b and H-2D^b. Untransfected and vector-alone transfected B16F10 expressed almost no H-2K^b and very low levels of H-2D^b. However, the two clones stably expressing rat TAP1 had significantly higher levels of both MHC I molecules, indicating that TAP expression had resurrected surface expression of these proteins (Figure 5.3).

![Figure 5.3 Stable transfection of B16F10 cells with rat TAP1 induces MHC I surface expression.](image)

Surface H-2K^b and H-2D^b expression was detected by flow cytometry. Purple filled areas represent negative controls, and green lines represent H-2K^b or H-2D^b stained cells. Data shown represents the results of one experiment, which was repeated at least twice.

The effect of increased hTAP1 expression on MHC Class I surface expression in AdhTAP1-infected B16F10 cells was investigated by flow cytometry. The results showed that the cell surface expression of both H2-K^b and H2-D^b was significantly increased in B16F10 cells infected with AdhTAP1 when compared with uninfected cells or cells infected with the vector \( \Psi 5 \) alone. The expression of TAP1 alone at least partially restored MHC Class I expression on the surface of B16F10 cells when compared to MHC Class I expression on the surface of IFN-\( \gamma \) treated cells (Figure 5.4).

**Figure 5.4 AdhTAP1 infection increases surface MHC Class I expression.**
(a) H-2K^b and (a) H-2D^b surface expression in B16F10 cells was assessed by flow cytometric analysis. B16F10 cells were infected with AdhTAP1 or \( \Psi 5 \) at 50 PFU/cell. \( \Psi 5 \) - adenovirus vector alone (negative control) and IFN-\( \gamma \) (positive control).
5.2.3 TAP1 expression restores B16F10 susceptibility to killing by specific CTLs

To determine if the increase in MHC I surface expression had a functional effect in terms of increased tumor associated antigen presentation and recognition by tumor-specific CTLs, CTL assays were performed using TRP-2-specific CTLs as effectors and stably transfected B16F10 cells as targets in a chromium-release assay. TRP-2 and gp100 are known differentiation antigens expressed in B16F10 cells and other melanoma cells from human patients. B16F10 cells express the H-2K\textsuperscript{b}-restricted TRP-2 tumor associated antigen (TAA); however, due to their lack of APM components, they cannot display the TRP-2 peptide on the cell surface unless expression of these components is restored, either by transfection or IFN\textgamma treatment. B16F10 cells stably transfected with rat TAP1 were efficiently recognized and killed by TRP-2-specific CTLs, whereas untransfected or vector alone-transfected control cells were not, indicating that rat TAP1 transfection restored presentation of the TRP-2 peptide (Figure 5.5).
Figure 5.5 TAP1-transfected B16F10 cells present tumor-associated antigenic peptides to specific CTLs, rendering them susceptible to killing.

A standard 4-hour cytotoxicity assay was performed to detect the tumor-associated antigen presentation capacity of TAP1 transfectants of B16F10 cells. Effectors were splenocytes from mice immunized with the tumor-associated antigen peptide, TRP-2, followed by irradiated RMA cells pulsed with TRP-2 peptide. B16F10 cells, B16/PHβ 1-1 cells, B16/rTAP1 3-3 cells and B16/rTAP1 3-8 cells were used as targets. Data shown represents the results of one experiment, which was repeated at least twice.

Cytotoxicity assays were performed in order to test whether the AdhTAP1-induced MHC Class I expression enhanced the ability of B16F10 cells to present TRP-2. The results showed that the AdhTAP1-infected B16F10 cells were sensitive to the cytolytic activity of the TRP-2-specific effectors, while the uninfected B16F10 cells (not shown) or the Ψ5-infected cells (negative controls) were resistant to killing (Figure 5.6). This indicates that hTAP1 expression and activity by AdhTAP1 infection can restore sufficient MHC Class I-restricted antigen presentation of a TAA, TRP-2, to render these cells susceptible to killing by specific cytotoxic lymphocytes.
Figure 5.6 Infection of B16F10 cells with AdhTAP1 (50 PFU/cell) restores MHC Class I antigen presentation of the TRP-2 epitope and increases susceptibility to lysis by TRP-2 specific effector cells. Splenocytes from mice immunized with TRP-2 peptide followed by irradiated RMA-S cells pulsed with TRP-2 were used as effectors. Targets: B16F10, B16F10 infected with Ψ5 (adenovirus vector control) or B16F10 infected with AdhTAP1.

5.2.4 TAP1 expression increases B16F10 tumor immunogenicity

To establish the immunogenicity of the tumor cells themselves, stably-transfected B16F10 clones were irradiated and injected into to mice to assess their ability to initiate anti-tumor immune responses. Spleens from mice immunized with the tumor cells were then removed and further stimulated in vitro for five days with irradiated tumor cells of the same type used in the immunization protocol. CTLs from these cultures were then tested for their ability to kill both the rat TAP1 stable transfectants of B16F10, or the untransfected or vector-alone transfected B16F10. Surprisingly, mice immunized with the
B16/rTAP1 3-3 stable transfectant generated CTLs capable of recognizing not only the same B16/rTAP1 3-3 cells against which they were immunized, but they also killed untransfected and vector alone transfected B16F10 cells with only slightly lower efficiency (Figure 5.7). This indicated that the control cell lines, despite their very low levels of surface MHC I, did express enough surface MHC I to render them susceptible to killing when the CTLs were generated with a TAP-expressing immunogen. When untransfected or vector-alone transfected B16F10 were used as immunogens, the CTLs generated were not able to recognize or kill any of the target cell lines, even those expressing TAP.

**Figure 5.7 Rat TAP1-transfected B16F10 cells are more immunogenic.**
A standard cytotoxicity assay was performed to test the capacity of TAP1-transfected B16F10 cells to generate tumor-specific T cells. Splenocytes from mice immunized with irradiated B16F10 cells, B16/PHβ1-1 cells and B16/rTAP1 3-3 cells were used as effectors against the indicated targets. Data shown represents the results of one experiment, which was repeated at least twice.
TRP-2- and gp100-specific cellular immune responses elicited by the AdhTAP1-infected B16F10 cells were measured by IFN-γ ELISPOT assay. Mice that were vaccinated with irradiated, AdhTAP1-infected B16F10 cells showed a significant increase in the number of both TRP-2- and gp100–specific, IFN-γ-secreting splenocytes when compared to those vaccinated with either irradiated uninfected cells or irradiated Ψ5-infected B16F10 cells. These results indicate that AdhTAP1 infection of B16F10 cells induced a Th1-type tumor specific immune response (Figure 5.8).

**Figure 5.8 TAP1 expression in B16F10 cells increases the numbers of tumor-specific, IFN-γ-secreting splenocytes.**

Bars represent the mean number of IFN-γ secreting splenocytes isolated from mice immunized with γ-irradiated B16F10 cells infected *ex vivo* with AdhTAP1, Ψ5 (Adenovirus vector control) or no treatment (PBS). Splenocytes from immunized mice were stimulated with the tumor associated antigens TRP-2 or gp100 or incubated without peptide. The numbers of tumor antigen-specific, IFN-γ-secreting precursors were determined by ELISPOT assay. Precursor frequency is reported as IFN-γ-secreting cells per 10⁶ splenocytes (IFN-γ SC/10⁶ splenocytes).
5.2.5 Expression of TAP1 from recombinant vaccinia virus results in increased surface MHC Class I levels and suppresses tumor growth in vivo

To extend our findings to an in vivo setting, a more efficient delivery vector was needed for the rat TAP1 gene to tumor cells in a whole animal mouse model. Recombinant vaccinia viruses were generated expressing rat TAP1 (VV-rTAP1) or empty virus alone (VV-PJS-5) under the control of two synthetic VV promoters (241). The viruses were first tested in vitro. Infection of B16F10 cells with the recombinant VV-rTAP1 virus resulted in increased H-2K\textsuperscript{b} and H-2D\textsuperscript{b} surface expression, similar to results seen when the rat TAP1 cDNA was introduced by stable transfection (Table 5.1, first row). When B16/rTAP1 3-8 cells already stably transfected with rat TAP1 were infected with VV-rTAP1, surface MHC I was further increased, but the magnitude of the increase was less than when untransfected cells were infected (Table 5.1, second row). This is likely due to TAP levels (due to prior stable transfection) already being able to provide sufficient peptides to the ER for near maximal surface MHC I expression, which was not enhanced greatly by additional TAP1 expression from the virus.
Table 5.1 VV-rTAP1 infection of B16F10 and B16/rTAP1 3-8 cells results in increased surface MHC Class I expression compared to VV-PJS-5.
Data represent the fold increase in mean fluorescence intensity of VV-rTAP1-infected cells with the fold increase in VV-PJS-5-infected cells subtracted, as detected by FACS using antibodies to H-2K^b and H-2D^b.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Fold increase H-2K^b</th>
<th>T(X) value, p value</th>
<th>Fold increase H-2D^b</th>
<th>T(X) value, p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>B16F10</td>
<td>0.52</td>
<td>4.4711, p&lt;0.01</td>
<td>1.8</td>
<td>24.052, p&lt;0.01</td>
</tr>
<tr>
<td>B16/r TAP1 3-8</td>
<td>0.16</td>
<td>10.462, p&lt;0.01</td>
<td>0.81</td>
<td>2.1617, 0.01&lt;p&lt;0.17</td>
</tr>
</tbody>
</table>

Tumors were established in C57BL/6 mice by subcutaneous injection of either untransfected B16F10 or the stably transfected B16/rTAP1 3-8 clone. The mice were then treated with intratumoral injections of the VV-rTAP1 virus, empty VV-PJS-5 virus, or PBS as controls. Tumor mass was measured after 21 days. The tumors that grew from the B16/rTAP1 transfectant were significantly smaller than those that grew from untransfected B16F10 (Figure 5.9, black bars). Untransfected B16F10 tumors treated with VV-rTAP1 were also significantly smaller than those treated with PBS or empty virus alone (Figure 5.9). This was also true in tumors derived from B16/rTAP1, but in this case treatment with either VV-rTAP1 or VV-PJS-5 resulted in smaller tumor size, indicating that when TAP is already present (from stable transfection), the virus itself likely acts as an adjuvant regardless of the addition of further virally-generated TAP, perhaps by providing further immunogenic viral epitopes that increase the immune response against the virally-infected tumor.
Figure 5.9 Rat TAP1 expression decreases the growth of subcutaneous B16F10 and B16/rTAP1 3-8 tumors.
Mice were injected subcutaneously with 1.5x10^5 cells in the hindquarter. One day and seven days later, mice were injected at the site of the tumor with 2x10^6 PFU of VV-rTAP1, VV-PJS-5 (vector control) or PBS. Tumor mass (mean ± SE) was measured after 21 days. Two-way ANOVA showed that both TAP1 transfection and infection with VV-TAP1 retarded tumor growth.
(*) Significant reduction in B16F10 cells tumor growth when treated with VV-rTAP1
(**) Significant reduction in B16/rTAP1 3-8 tumor growth when treated with VV-rTAP1 or VV-PJS-5

5.2.6 AdhTAP1 inhibits tumor growth in B16F10 tumor-bearing mice

B16F10 tumor formation and growth in syngeneic mice was examined following AdhTAP1 treatment. The mean tumor weight in tumor-bearing mice treated with AdhTAP1 was 28 mg, in sharp contrast to the 232 mg and 172 mg means of the Ψ5- and PBS- treated groups, respectively. AdhTAP1 treatment of tumor-bearing mice therefore significantly reduced the tumor mass in comparison to those treated with and Ψ5 and PBS (p<0.01, Figure 5.10a). The data also showed that five (42%) of the B16F10 tumor-bearing mice treated with AdhTAP1 were tumor-free, in comparison with one (8%) and
two (17%) tumor-free mice respectively in the Ψ5- and PBS-treated groups (Figure 5.10b).

Figure 5.10 AdhTAP1 treatment retards tumor growth in mice bearing B16F10 tumors.
C57BL/6 mice (12 mice per group) were injected s.c. with 1.5 x 10^5 B16F10 cells/mouse and 1, 4, and 8 days after B16F10 cells were introduced, mice were treated s.c. with 10^8 PFU/mouse of AdhTAP1 or Ψ5 or PBS only. (a) AdhTAP1 significantly retarded tumor growth of B16F10-bearing mice (p<0.01) and (b) increased the proportion of tumor-free mice compared to the Ψ5 and PBS-treated mice.
5.2.7 AdhTpn Increases MHC Class I Surface Expression and Immunogenicity in CMT.64 Cells

Previously, the Jeffries laboratory demonstrated that AdhTAP1 treatment of tumors elicited enhanced antigen processing, presentation and tumor immunity in both CMT.64 cells and here in B16F10. In B16F10, increases in levels of tapasin protein in treated tumor cells were also observed. To further examine the role of tapasin in this process, CMT.64 cells were infected with recombinant, nonreplicating adenovirus encoding human tapasin (AdhTpn). Human tapasin was expressed in these cells in a dose dependent manner (Figure 5.11a). However, no increase in endogenous mTpn, mTAP1 and mTAP2 protein expression was detected by Western blot in AdhTpn-infected CMT.64 cells. Nevertheless, flow cytometry analysis showed that cell surface expression of H-2K^b and H-2D^b was increased in CMT.64 cells infected with AdhTpn (Figure 5.11b), whereas cells infected with empty adenovirus vector alone Ψ5 showed no such increase. CMT.64 cells treated with IFN-γ were used as a positive control and showed much larger increases in H-2K^b and H-2D^b surface expression (Figure 5.11b), as well as increases in endogenous mTpn, mTAP1 and mTAP2 protein levels in Western blot analysis (Figure 5.11a). AdhTpn also enhanced the ability of CMT.64 stably transfected with the VSV nucleoprotein minigene (CMT/VSV-NP) to present the immunodominant VSV-NP_{52-59} peptide to CTLs. CMT/VSV-NP cells infected with AdhTpn were sensitive to the cytolytic activity of VSV-specific effector T lymphocytes, while CMT/VSV-NP cells alone or infected with Ψ5 were resistant to killing (Figure 5.11c), presumably due to the lack of H-2K^b/VSV peptide on the cell surface of the latter cells. These results show that tapasin expression and activity following AdhTpn infection can restore sufficient
MHC Class I-restricted antigen presentation of a specific epitope (VSV-NP$_{52-59}$) to make these cells susceptible to specific CTL activity.

Figure 5.11 Tapasin expression in CMT.64 cells after infection with recombinant adenovirus expressing human tapasin isoform 1 (AdhTpn) is dose dependent and leads to increased surface MHC Class I levels and presentation of a viral epitope. (a) CMT.64 cells were infected with AdhTpn at MOI of 1, 5, 25, 50, and 100 PFU/cell of AdhTpn or $\Psi_5$ at 100 PFU/cell and harvested 48 hr later. Western blotting was carried out with anti-hTpn, mTAP1, and mTAP1 polyclonal antibodies and $\beta$-actin mAb. $\beta$-actin was used as a control for protein loading. T1 = positive control for hTpn, .220 = negative control for hTpn. No bands were observed for any of the endogenous mouse APM components. (b) AdhTpn infection increases both H-2K$^b$ and H-2D$^b$ surface expression in CMT.64 cells. $\Psi_5$ - adenovirus vector control, IFN-\(\gamma\) – positive control. (c) Infection of CMT.64 cells with AdhTpn restores MHC Class I antigen presentation of VSV-NP epitope and increases susceptibility to lysis by VSV-NP-specific effector cells. Targets:
CMT/VSV-NP – CMT.64 transfected with VSV-NP52-59 minigene, CMT/VSV-NP infected with Ψ5 (adenovirus vector control), or CMT/VSV-NP infected with AdhTpn. Effectors: splenocytes from VSV-infected mice.

5.2.8 AdhTpn treatment increases survival of tumor-bearing mice: maximal protection is achieved by combining both AdhTpn and AdhTAP1

Previously, we demonstrated that treatment of CMT.64 tumor-bearing mice with recombinant adenovirus expressing human TAP1 (AdhTAP1) resulted in increased survival compared to mice treated with Ψ5 or PBS alone (146). Since AdhTpn increases MHC I antigen surface expression and restores susceptibility to CTL killing in a manner similar to AdhTAP1 treatment, we examined if AdhTpn in combination with AdhTAP1 could enhance the inhibition of CMT.64 tumor formation. In order to avoid cytotoxicity associated with high adenoviral loads, a suboptimal dose of 2.5x10^7 PFU of AdhTAP1 determined by titration (data not shown) that was demonstrated to have a protective effect, was used in combination with an equal dose of AdhTpn. To balance the viral load for the single dose treatments with that of the double dose treatment, AdhTAP1 and AdhTpn alone treatments were mixed with an equal number of Ψ5 viruses. Dual treatment with AdhTpn and AdhTAP1 resulted in even greater mouse survival than either virus with Ψ5 alone, with 50% long-term survival without visible tumors (greater than 100 days) compared to 30% with AdhTpn and 10% with AdhTAP1 (Figure 5.12). The median survival was also maximal at 75 days for AdhTpn + AdhTAP1 compared to 48.5 days for AdhTpn alone, 22 days for AdhTAP1 alone, 23 days for Ψ5 and 21 days for the PBS control. The dual treatment was statistically more effective than Ψ5 or AdhTAP1.
treatment alone at the same viral dose (p<0.01), but not statistically different from AdhTpn treatment alone at the same dose.

Figure 5.12 AdhTpn and AdhTAP1 prolong the survival of tumor-bearing mice.
C57BL/6 mice were injected i.p. with CMT.64 cells (4x10^5 cells/mouse) and were treated on days 1, 3, 5, and 8 with AdhTpn, AdhTAP1, AdhTAP1 and AdhTpn, Ψ5, (5x10^7 PFU/500 μl PBS) or PBS and survival was followed for 90 days (n = 10 mice per group). To ensure all groups received the same number of Ad particles, mice treated with AdhTAP1 alone or AdhTpn alone were complemented with an equal amount of Ψ5 vector to maintain a total Ad dose of 5x10^7 PFU. At the same dose, AdhTAP1 and AdhTpn together resulted in maximal protection that was statistically greater than AdhTAP1 alone and Ψ5 and PBS controls, but not AdhTpn alone (p = 0.0061 for AdhTAP1 + AdhTpn vs. AdhTAP1 alone).
5.2.9 AdhTpn treatment increases TILs and tumor-infiltrating DCs in tumors

Between four to eight mice from the AdhTpn treatment group, as well as Ψ5 and PBS control groups, were examined for patterns in tumor growth 20 days after the last treatment injection. The peritoneal cavities of mice treated with AdhTpn were tumor-free or had only a few small tumors less than 1 or 2 millimeters in diameter. Both the liver and intestine appeared normal upon visual inspection. This was in sharp contrast to mice treated with PBS or Ψ5. These mice had large volumes of bloody ascites fluid (2-5 mL) and many tumors distributed throughout the peritoneal cavity. Tumors were observed growing on the liver and intestine and were associated with large fibrotic adhesions. Tumors harvested from the mice were examined for TILs and DCs infiltrates by FACS and immunohistochemistry (IHC) staining. IHC staining showed that mice treated with AdhTpn had significantly greater numbers of CD8⁺ and CD4⁺ T cells and CD11c⁺ DCs in the tumor mass than in tumors taken from mice treated with Ψ5 or PBS (Figure 5.13a). FACS analysis also confirmed that tumors from mice treated with AdhTpn had significantly greater CD8⁺ and CD4⁺ TILs (p = 0.011 and p = 0.042, respectively) than tumors taken from mice treated with Ψ5 and PBS (Figure 5.13b). These results are consistent with our previous findings treating CMT.64 tumor-bearing mice with AdhTAP1 (146), and they suggest that AdhTpn treatment may function in a similar manner by increasing tumor antigen-specific immune responses.
TAPASIN AND TAP EXPRESSION IN TUMORS

Figure 5.13 Tumor infiltrating lymphocytes and DCs were increased in CMT.64 tumors treated with AdhTpn *in vivo*.
(a) IHC staining for CD4\(^+\) (a, b, c), CD8\(^+\) (d, e, f) or CD11c\(^+\) (g, h, i) cells in CMT.64 tumors treated with AdhTpn (a, d, g) or \(\Psi 5\) (Ad vector control) (b, e, h), or PBS (c, f, i). Tumors were analyzed 19 days after CMT.64 cells were introduced into mice. C57BL/6 mice were injected i.p. with CMT.64 cells (4\(\times\)10\(^5\) cells/mouse) and were treated on days 1, 3, 5, and 8 with either 2.5 \(\times\) 10\(^7\) PFU/mouse of AdhTpn or \(\Psi 5\) or PBS only. A positive stain is indicated by the intense brown labelling of cell surface membranes (200X magnification). (b) Tumor infiltrating lymphocytes were increased in CMT.64 tumors treated with AdhTpn *in vivo* by FACS analysis (**p= 0.011 for CD8 in treated vs. PBS control. *p = 0.042 for CD4 in treated vs. PBS control after a square root transformation to satisfy homogeneity of variance). Tumor infiltrating CD4\(^+\) and CD8\(^+\) lymphocytes are presented as a percentage of total cells in tumors.

5.3 Discussion

The restoration of antigen processing, MHC I surface expression and tumor cell immunogenicity by transfection or infection of rat or human TAP1 alone in B16F10 melanoma cells or infection with AdhTpn in CMT.64 lung carcinoma cells occurs despite other deficiencies in the antigen presentation pathway. This has also been demonstrated in other cell lines with similar antigen presentation-deficient phenotypes, such as human
melanoma, small cell lung carcinoma, squamous cell carcinoma of the head and neck (SCCHN), and renal cell carcinomas (133;151-155;240). In B16F10 cells, rat TAP1 expression stabilized the expression of TAP2 and increased the expression of endogenous tapasin (Figure 5.1). Human TAP1 expression also increased endogenous tapasin levels, but no TAP2 was detectable (Figure 5.2). These results indicate that the re-expression of TAP1 may lead to a general reconstitution of several other components of the MHC I antigen-processing pathway, and may therefore increase the amount of antigenic peptides available for assembly onto MHC I molecules in the ER, leading to enhanced surface MHC I expression. Other studies have shown that pre-existing TAP1 is needed for TAP2 expression, but not the other way around (242). This could explain why this effect was not observed when TAP2 alone was re-introduced into tumor cells in our lab’s previous studies (150;151). Restoration of TAP expression in TAP-deficient cancer cells should make a wide variety of peptides derived from tumor-specific and tumor-associated antigens available, and this may compensate for any other unpredictable deficiencies in a given tumor’s MHC I allele repertoire. Interestingly, although AdhTpn expression also resulted in increased MHC I surface expression similarly to rat and human TAP1 transfection, no stabilization of other APM components to detectable levels was detected (Figure 5.11a). Residual transport of the peptides into the ER may be due to low levels of TAP expression (undetectable by Western blot) providing sufficient MHC Class I peptide complexes in the presence of tapasin-mediated chaperone activity for a significant increase in susceptibility to killing by specific T cell effectors. Steady state levels of other components of the antigen presentation pathway including TAP have been shown to be stabilized by Tpn (243-245). Therefore, tapasin expression in tumor cells may stabilize
the low level of TAP present in these cells, and therefore significantly increase the H-2K\textsuperscript{b} and H-2D\textsuperscript{b} surface expression and immunogenicity of CMT.64 cells in this manner. Expression of Tpn in the Tpn-deficient mouse hepatoma cell line H6 and the human HepG2 cell line has been similarly shown to increase surface MHC Class I (246;247) expression, suggesting this approach could be effective in many carcinomas.

In the case of B16F10 cells, rat or human TAP1 gene transfer was able to resurrect the presentation of the appropriate TRP-2 peptide on H-2K\textsuperscript{b} to allow for TRP-2-specific CTL killing (Figure 5.5 & 5.6). B16/rTAP1 cells have also been shown to present H-2D\textsuperscript{b} antigen-specific peptides derived from gp100, making B16/rTAP1 cells susceptible to lysis by CTLs specific for this epitope both \textit{in vitro} and \textit{in vivo} as well (248). CMT.64 cells stably transfected with the VSV-NP protein, which encodes an H-2K\textsuperscript{b} binding epitope, were also susceptible to killing by specific CTLs following treatment with AdhTpn but not vector alone (Figure 5.11c). This is consistent with previous studies with AdhTAP1, in which similar increases in susceptibility to killing was observed (146).

Vaccination by irradiated cells expressing TAP1 greatly enhanced the CTL activity not only towards B16/rTAP1 target cells but also to untransfected B16F10 target cells (Figure 5.7). This indicates that it is likely not necessary for every cell to re-express TAP1 in order for immune tolerance to the tumor to be broken, allowing CD8\textsuperscript{+} cytotoxic T cell responses to occur. This is further supported by a previous \textit{in vivo} study in our lab with CMT.64 lung carcinoma, in which mice initially immunized with CMT.64 cells expressing TAP1 were better able to reject a challenge with untransfected CMT.64, unlike mice initially immunized with untransfected CMT.64 (150). Perhaps encouraging
for applications to metastatic disease, the lysis of B16F10 cells by splenocytes generated by vaccination with irradiated B16/rTAP1 cells demonstrates that there is sufficient H-2 antigen on the surface of B16F10 cells to facilitate cytolytic activity. In addition, TAA-specific (including gp100 and TRP-2) IFN-γ-secreting splenocytes were observed after vaccination with irradiated B16F10 cells infected with AdhTAP1, indicating that TAP activity in tumor cells can promote Th1 responses (Figure 5.8). TAP1 activity or the products of TAP1 activity in B16/rTAP1 cells must be transferred in some way to the dendritic cells involved in the cross-presentation of MHC I tumor antigens, a crucial step in generating specific CD8⁺ cytotoxic T cell responses. TAP1 expression in B16F10 cells results in a source of antigen that may be bound to MHC I on the surface of these cells, and these MHC I-restricted antigens may be transferred to the dendritic cell MHC I. Alternatively, dendritic cells may access processed tumor antigens from the ER compartment of TAP1-expressing cells during internalization and antigen cross-presentation, perhaps by heat shock proteins (HSPs). Further studies will be necessary to answer these questions.

The enhanced immunogenicity of TAP-expressing B16F10 cells has a significant effect on tumor growth in mice. It was shown previously that mice vaccinated with melanoma antigens restricted to H-2Dᵇ were protected from B16/rTAP1 tumor challenge by CD8⁺ T cells but not by challenge with B16F10 cells (248). In this study, B16/rTAP1 tumor growth was retarded without prior vaccination with tumor associated antigens (Figure 5.9), though the protection was likely not as complete as it would have been with prior vaccination. The effect also appears when TAP1 is expressed in vivo by vaccinia or adenoviral vectors (Figure 5.9, Figure 5.10). In this case, not only does TAP1 expression
have an effect on tumor growth, but the vector also appears to act as an adjuvant. It is likely that upon infection \textit{in vivo} the tumor cells also present viral antigens in the presence of TAP (whether from infection or transfection) that provide further epitopes for CTL recognition in addition to tumor antigens. The presence of the delivery virus during processing of tumor antigens by DCs may also assist in breaking tumor cell tolerance by the immune system by acting as a “danger signal” that contributes to the priming of tumor antigen-specific immune responses through TLR signaling by the DCs (249-251). TAP1 expression, in conjunction with viral gene transfer vectors, appears to then promote a Th1 type response that can function to retard tumor growth.

Similarly, in CMT.64 it appears that the enhanced MHC Class I surface expression and immunogenicity due to AdhTpn infection \textit{in vivo} significantly retards tumor growth and enhances mouse survival (Figure 5.12). AdhTpn injections at the site of the tumor infect the tumor cells and increase the activity of the endogenous antigen presentation pathway, leading to surface expression of MHC Class I-restricted tumor antigens that can then be recognized by the increased numbers of tumor-infiltrating CD8$^+$ T cells, assisted by CD4$^+$ T cells and CD11c$^+$ DCs. A recent paper (252) showed that CD8$^+$ T cell infiltration into colon cancers was the best prognostic marker for patient survival, more so than classical histochemical staging approaches. This supports our view that CD8$^+$ T cell infiltration is indicative of eliciting an active anti-tumor immune response.

Combining AdhTAP1 and AdhTpn in treating CMT.64 carcinomas resulted in enhanced protection and survival in tumor-bearing mice (Figure 5.12). Thus, immunotherapeutic approaches that utilize TAP and Tapasin have the potential to restore
the priming and expansion of tumor-specific T cells as well as restoring ability of the tumors to process and present tumor antigens.
Chapter 6: General Discussion

6.1 Summary and conclusions

Several conclusions regarding the expression, localization and function of the tapasin isoforms can be drawn from this thesis:

- The tapasin isoforms are widely expressed in many tissues and cell lines. Mature DCs express the highest levels of mRNA of all three isoforms.
- EBV+ human B cell lines (with the exception of Daudi) express isoform 2 at the protein level, where it may play a role in cross-presentation by B cells.
- All three isoforms of tapasin interact with ERP57, and possibly other PLC components as well.
- All three isoforms co-localize with ER markers, but are also present in smaller amounts in the trans Golgi network, endosomes and/or lysosomes in some cell types where they could recruit other PLC components and assist in loading peptides derived from exogenous proteins onto MHC I.
- Isoform 3 can be detected in cell supernatant, indicating it is secreted to some extent from the cell.
- Isoform 3 plays a role in inhibiting the loading of exogenous peptides onto MHC I molecules in a BFA-dependent manner, potentially functioning to avoid bystander killing of uninfected cells or by chaperoning MHC I molecules prior to cross-presentation.
- Isoform 1 and 2 stabilize mouse TAP1 and TAP2; isoform 3 cannot because it lacks the TAP-stabilizing transmembrane domain.
• Isoform 1 and 2 are very similar in their function in terms of their effect on endogenous MHC I antigen presentation, the quality of peptides loaded onto H-2K\textsuperscript{b} and the kinetics of H-2K\textsuperscript{b} maturation.

• None of the isoforms appears to influence loading of H-2K\textsuperscript{b} or H-2D\textsuperscript{b} with self peptides by the other isoforms in a synergistic or dominant negative fashion, nor do any of the isoforms appear to influence MHC II surface expression levels in DC2.4 cells.

• None of the isoforms appears to impact cross-presentation of soluble ovalbumin-derived peptides onto H-2K\textsuperscript{b} molecules in DC2.4 cells, but could potentially play a role in another DC cell type with another antigen taken up via a different route or presented by different MHC I alleles.

Several conclusions can also be drawn from this thesis about the roles of TAP and tapasin in tumor immunosurveillance:

• Stable transfection of rat or human TAP1 alone in B16F10 melanoma cells restores expression of endogenous mouse tapasin at the protein level, reconstituting the MHC I antigen processing pathway and the presentation of TAA-derived peptides to CTLs. Expression of human tapasin alone in CMT.64 carcinoma cells also achieves this effect but without the concurrent increase in protein levels of other APM components.

• Expression of rat TAP1 from vaccinia viral vectors or human TAP1 from adenoviral vectors in B16F10 tumor-bearing mice, as well as human tapasin from adenoviral vectors in CMT.64 tumor-bearing mice, results in
decreased tumor growth, indicating TAP1 and tapasin may be restoring immune surveillance of the tumor cells leading to T cell-mediated control of tumor growth with the viral vectors acting as adjuvants.

- Immunization of mice with TAP1-expressing B16F10 tumors leads to the generation of CTLs capable of not only recognizing TAP1-expressing tumors, but also non-TAP1 expressing tumors of the same type. Similarly, immunization of mice with tumors infected \textit{ex vivo} with AdhTAP1 led to increased numbers of tumor-specific IFN-\(\gamma\)-secreting T cells. These results indicate that TAP1 expression also increases the immunogenicity of the tumor cells during the initiation of the immune response and that this immune response is capable of generating tumor-specific T cells that may then eliminate even tumors with very low MHC I surface expression, perhaps by helping break immune tolerance.

- Expression of human tapasin in CMT.64 tumor-bearing mice leads to increased numbers of tumor-infiltrating lymphocytes and dendritic cells, which correlates with increased anti-tumor immune responses.

- Combinations of adenoviral treatments expressing both human TAP1 and human tapasin lead to maximal protection of mice from tumor-mediated death, indicating that optimal responses could be achieved by adding back several APM components in tumors lacking these factors.
6.2 Overall model of the possible function of tapasin isoforms in antigen presentation

An overall model for the function of the isoforms given the experimental data collected to date is summarized in Figure 6.1. Isoform 1 and 2 in the ER contribute to classical endogenous MHC I antigen presentation to CD8\(^+\) T cells (Figure 6.1-1). Exogenous antigen is taken up into the DC, escapes to the cytosol (perhaps in a partially-degraded state) and is cross-presented onto MHC I via the TAP pathway and isoforms 1 & 2 in the ER (Figure 6.1-2). Exogenous antigen is taken up into the cell and is degraded into MHC I-binding peptides in the “cross-presentation compartment”, theorized to be either an endo/lysosomal-type vesicle or potentially part of the trans Golgi network. Isoform 1 and 2, and to a lesser extent isoform 3, have been shown to co-localize with markers for these locations, and may assist in peptide loading in this compartment. MHC I itself may reach this compartment either by recycling from the cell surface or could be directed here from the ER. The tapasin isoforms interact with ERP57 and may also recruit other proteins of the peptide loading complex (e.g. calreticulin) to assist in peptide loading in this compartment (Figure 6.1-3). Isoform 3 outside the cell may inhibit the loading of exogenous peptides onto surface MHC I, ensuring that only peptides loaded within the DC interact with CD8\(^+\) T cells, thereby preventing the initiation of potentially autoreactive immune responses or the killing of bystander cells at the site of infection (Figure 6.1-4). Isoform 3 inside the ER could similarly inhibit the loading of exogenous peptides that have entered the ER from outside the cell onto MHC I so that CD8\(^+\) responses are focused on TAP-imported peptides, though how these two pools of peptides could be distinguished from each other in the ER is unknown. Isoforms 1 and 2
are likely anchored to the TAP complex and may therefore preferentially load imported peptides. Isoform 3 is not expected to interact with TAP and could act instead on other peptides taken up from the extracellular environment (Figure 6.1-5).

**Figure 6.1 Model of the potential functions of the tapasin isoforms in MHC Class I antigen presentation in DCs.**

Isoform 1 and 2 in the ER contribute to endogenous MHC I antigen presentation (1) as well as cross-presentation of exogenous antigens that escape to the cytosol and are imported by TAP (2). Isoform 1 and 2 (and possibly isoform 3) also participate in “vacuolar” cross-presentation of exogenous antigens (3). Isoform 3 outside the cell (4) or within the ER (5) inhibits the loading of exogenous peptides onto MHC I (5), potentially so that CD8⁺ responses are focused on TAP-imported peptides processed by the DCs. CNX = calnexin.
6.3 Closing thoughts & future work

6.3.1 Mice as models of the human immune system

If the tapasin isoforms play an important role in MHC I antigen presentation, why were they not then found in mice? It was not possible to consistently detect the existence of isoforms of mouse or rat tapasin, though equivalent forms of monkey isoforms 2 and 3 were detected in COS-1 cells. This does not mean, however, that isoforms of mouse and rat tapasin do not exist. There is a report in the ExPASy SwissProt database of an exon 6-deficient form of mouse tapasin detected in the 129 mouse strain (entry: Q9R233-2). Experimental evidence for the expression of this form of tapasin (as well as exon 7-deficient or exon 6+7-deficient forms more similar to the human tapasin isoforms studied in this thesis) was sought out without consistent success. Closer examination of the mouse and human tapasin sequences reveals that the two genes are very similar: both genes are located in similar regions of the MHC Class II locus on chromosome 6 in humans and chromosome 17 in mouse (117;188), and both consist of eight exons of similar length - with one notable exception: exon 7 is fourteen amino acids longer in mouse than in human (Figure 6.2). This could potentially explain the differences in C terminal splicing between the two species.
**Human**

Exon 5 (114aa)  Exon 6 (30aa)  Exon 7 (12aa)  Exon 8 (3aa)
...TLEVA) – (GLSGPSLEDVGLFLSAFLLLGLFKALGWA) – (AVYLSTCKDSKK) – (KAE)

**Mouse**

Exon 5 (114aa)  Exon 6 (30aa)  Exon 7 (26aa)  Exon 8 (3aa)
...TLEVA) – (GFSGPSIEDGIGLFLSAFLLLGLLKVLGWL) – (AAYWTIPEVSKEKATAASLTIPRNSK) – (KSQ)

Figure 6.2 Comparison of mouse and human tapasin sequences at the C terminus.
Exon 7 in mouse tapasin contains 14 more amino acids compared to human tapasin. Both contain the dilysine ER retention motif at their C termini.

Finally, while the mouse immune system is generally a good model for the human one, it is not perfect nor is it identical in its function (253). There are many examples of differences between the mouse and human systems, including within the MHC I antigen processing and presentation pathway. There is evidence in murine MHC I assembly that the heavy chain-β2m complex may remain bound to calnexin even after β2m association, while human MHC I is thought to be immediately released (254-256). This may be partly due to differing affinities of human and mouse MHC I molecules for β2m, with mouse MHC I having a weaker affinity for mouse β2m than the corresponding human proteins (197;257). This may mean that mouse β2m/MHC I may require a longer association with calnexin to stabilize the interaction. Also, human MHC molecules have been shown to stably interact with Bip (255), an association not observed to date in mice. It is possible, therefore, that the isoforms do play a role unique to human (and other primate) immunity that has no precise equivalent in the mouse, but which nevertheless could be important in human immunity in general or in interactions with specific pathogens but not others.
6.3.2 Tapasin-related protein

A gene very similar to tapasin was identified in 2002 in a locus paralogous to the MHC II locus on chromosome 12p13.3 of humans (190) and was called “Tapasin-related” (TpnR). The corresponding protein is also a member of the immunoglobulin superfamily and has similar overall structure to tapasin, but does not have an ER-retention motif at the C-terminus (190). Instead, the cytoplasmic tail contains a dileucine motif, a known internalization and intracellular sorting signal (258). Nevertheless, a GFP-tagged (C-terminus) version of the protein was found to localize in the ER, but small amounts were also detected at the cell surface (190). The TpnR mRNA was widely expressed in many tissues but, unlike tapasin, expression was not inducible by IFN-γ (190). The role of this protein in immunity is currently unknown. It would be very interesting to assess the role of this protein in antigen presentation, as well as possible interactions with the tapasin isoforms. An animal model deficient in TpnR would go a long way to answering questions regarding its function in immunity and biology in general.

6.3.3 Cross-presentation

*In vivo*, cross-presentation of exogenous antigens to initiate CD8+ T cell-mediated immune responses is a complex process. Antigen is taken up in the periphery by DCs, which then migrate to the draining lymph nodes while undergoing a complex process of maturation that is influenced by the general state of the host as well as by the nature of the encountered antigen itself and which DC toll-like receptors (TLRs) it triggers (251;259-261) and which DC subset internalizes the antigen. It has even been hypothesized that the peripheral DC that takes up the antigen later transfers the antigens
or pieces thereof to lymph node-resident DCs that, in turn, actually activate naïve CD8$^+$ T cells (262,263). Therefore, trying to mimic this process in vitro often fails to approximate the true mechanisms in vivo. To accurately evaluate the role of the tapasin isoforms in vivo, constructs have been made for the generation of transgenic mice (described in Appendix B). In these constructs, the tapasin isoforms are expressed under the control of the endogenous mouse tapasin promoter (264). Mice that have stably integrated the constructs in a germ-line transmissible manner could be crossed with tapasin$^{-/-}$ mice to generate strains expressing only the human isoforms alone or in combinations, on an otherwise tapasin$^{-/-}$ background. These mice could then be challenged with ovalbumin-expressing viruses or bacteria, and the OVA-specific T cell responses measured with SIINFEKL-MHC I tetramers and/or the transfer of naïve CFSE-labeled OT-1 T cells (which have an SIINFEKL-specific TCR like that of B3Z T cells), whose proliferation in response to OVA-presentation by DCs could be measured by flow cytometry based on the decrease in CFSE fluorescence intensity with each cell division. Responses to other types of pathogens and antigens could also be evaluated as reagents to these agents in the context of the appropriate mouse MHC alleles become available. Mouse models would also allow the evaluation of the role of the tapasin isoforms in tumor immunity using tumor challenge experiments.

6.3.4 Nailing down the subcellular localization

The results of this thesis, as well as studies done by others, suggest that the di-lysine motif of tapasin is not entirely necessary for ER-retention, since isoform 2, tapasin-related protein and wild-type tapasin with a mutated dilysine motif were all found to localize mainly in ER despite the lack of the motif (101). In addition, in this thesis
isoform 1 was found to co-localize with endosomal markers despite the presence of the motif, indicating that other factors must also dictate the subcellular localization of tapasin.

To truly resolve the localization of the tapasin isoforms at the subcellular level, epitope tagging of the molecules with GFP or FLAG might provide further insight into their trafficking, since all the studies in this thesis employed a monoclonal antibody against the N-terminal region common to all the isoforms. This necessitated expression of each isoform individually in cells deficient in human tapasin. The disadvantage of this approach is that the localization of more than one isoform in a single cell cannot be evaluated. In addition, any post-translational modifications to the N-terminus of tapasin following ER exit (and the N-terminus of tapasin does contain one glycosylation site that could be subject to addition of more complex carbohydrate moieties in the Golgi), might then abrogate binding of the monoclonal antibody to this subset of the molecules, rendering them undetectable by the methods used here. Non-ER forms of tapasin were found, suggesting that the monoclonal antibody is still capable of detecting tapasin after it has left the ER; however, there is still a possibility that certain subsets were not picked up. This potential problem could be avoided by tagging the isoforms with an epitope tag or GFP moiety unaffected by post-ER protein modifications, though of course there is always a danger in altering the natural properties of the protein by any such tagging approaches. Tagging is especially risky in a protein such as tapasin that interacts with so many other proteins: tagging at the N-terminus could result in disruption of interactions with MHC I and/or ERP57 and calreticulin, while tagging at the C-terminus could disrupt interactions with TAP and/or unknown proteins that might interact with the unique C-
termini of isoforms 2 and 3. However, some groups have reported successful GFP-tagging of tapasin without obvious alteration of its functional properties (101), so the technique is worth considering. Another potential strategy, though technically quite challenging, would be to generate antibodies to the tapasin C-terminal regions that would be capable of detecting each individual isoform from a mixed sample. Based on RNA expression of various cell types, co-expression of several isoforms appears to be the normal situation, as most cell lines and tissues expressed at least two if not all three isoforms concurrently. The availability of antibodies reacting with each isoform specifically without cross-reactivity would allow assessment of each isoform’s localization in the presence of the other two isoforms, as it is not known to what degree the different isoforms might interact with one another. Four tapasin molecules are thought to bind a single TAP heterodimer in the peptide loading complex, with each tapasin molecule recruiting an empty MHC I molecule (Figure 1.10). It is not known whether all the isoforms found in the ER participate in this complex, and in what ratios, or if they differentially interact with other PLC members.

6.3.5 Isoform 3 – what is it doing?

The results presented here strongly suggest that isoform 3 is secreted to some extent from cells. Nevertheless, soluble isoform 3 appears to be naturally secreted in quite small amounts that were undetectable unless the protein was overexpressed by recombinant adenoviruses. It would be interesting to see if isoform 3 is present in the serum where it could be circulating throughout the body. Serum isoform 3 was not found in databases of proteins identified in human blood plasma by mass spectrometry (www.plasmaproteomedatabase.org, www.peptideatlas.org/repository, ...)
These searches did retrieve β2m, another APM component well known to be present in the serum at low levels (265). Serum β2m can in fact become a problem in some dialysis patients in which inefficient elimination of excess β2m from the blood can lead to the formation of painful plaque-like deposits of the protein in joints (266). However, the absence of isoform 3 in the blood proteomics databases does not mean it is not there, but rather that amounts may be below detection limits or that the half-life of isoform 3 in the serum is quite low. In addition, proteomics approaches to document the wealth of proteins found in the blood are relatively recent, and it is likely that many more proteins will be identified over the coming years (267). In the meantime, ELISA assays of human serum could potentially answer the question of whether soluble isoform 3 is present in the blood.

Based on the results of this thesis, a scenario can be envisioned whereby soluble tapasin circulates in the serum, inhibiting the loading of exogenous peptides onto MHC I molecules. This could lower levels of uninfected bystander cell killing at sites of viral infection, for example, or in mucosal tissues where aberrant responses to local flora or food antigens are undesirable. In DCs, isoform 3 could potentially avert autoreactive immune responses caused by MHC I binding of self peptides on the cell surface of activated DCs in lymph nodes during the immune response initiation phase. It would be informative to look at the inhibitory effect of isoform 3 on the loading of other peptides of varying affinities, to see if this effect carries over generally to all peptides or is more specific to certain subsets or with certain alleles of MHC I. SIINFEKL binds to H-2K\(^b\) with a relatively high affinity (268); results could differ with peptides of lower affinities. However, at present, very few reagents like the 25.D1.16 and B3Z T cell line that
recognize specific MHC I + peptide combinations are available. In addition, it will be important in the future to establish where in the cell isoform 3 mediates this inhibitory effect, since it was found both extracellularly and within the ER in this study. To shed light on its mechanism of action, it will also be important to establish whether soluble tapasin mediates its effects alone or through interactions with (or sequestration of) other PLC components such as ERp57 and calreticulin.

6.3.6 Other reasons why tapasin isoforms might exist

In all functional assays performed in this thesis, isoform 1 and isoform 2 behaved very similarly. Both mediated similar effects on MHC I endogenous presentation of self peptides (Figure 4.1), viral peptides (Figure 4.2), cell surface thermostability (Figure 4.6) and maturation rates (Figure 4.5). If not that one is involved in cross-presentation and the other not, what other reasons could there be for the existence of two such similar proteins? Two arguments can be made: one, there are differences between isoform 1 and 2 in the properties described above, but the assays used herein did not detect them, or two, they mediate other effects altogether.

In the former case, the experiments performed could be modified to look for further differences. For example, the thermostability assays could be repeated in the absence of serum, which might contain factors such as bovine β2m that could mask underlying effects from the isoforms. Both this assay and the MHC I maturation rate assay could be performed with other MHC I alleles as well. In addition, the peptides bound to H-2Kb and other alleles could be eluted and sequenced by mass spectrometry to determine the relative quantities and identities loaded by each isoform.
In the latter argument, the differences between the isoforms might lie elsewhere or could be more subtle. For example, isoform 1 and 2 might have different half-lives, allowing differential temporal regulation. One might be translated more easily than the other, or the degree to which one form is spliced relative to the other could differ under varying conditions with unknown functional consequences. Finally, one isoform might influence some MHC I alleles differently than others, or one form could be more resistant to inhibition from pathogenic virulence factors, for example, than the other. In the absence of these specific conditions, the functions of the two isoforms might otherwise appear redundant. Because of the incredible diversity of pathogens and other substances with which the immune system interacts, finding the specific circumstances where subtle differences such as these would be revealed to be important is a challenging task.

6.3.7 Something different altogether?

The only known function for normally-spliced tapasin is in assisting to load peptides onto MHC I. This thesis has not addressed the possibility, however, that the isoforms may interact with other proteins unrelated to MHC I antigen processing and presentation.

Tapasin itself has been shown to bind M10.5, an MHC I-like molecule with an open, empty groove that acts as a pheromone receptor (102); the functional significance of this interaction is unknown. Other MHC I-like molecules that bind the same β2microglobulin subunit as conventional MHC I are the CD1 lipid-presenting molecules (269;270) and the neonatal FcRn receptor (271). CD1 molecules present short lipid chains to NKT cells or CD1-restricted T cells (270). Similarly to peptides, the short lipids are loaded into the lipid-binding groove of these molecules in endocytic compartments
(272;273), a process shown to involve tapasin-like chaperones called saposins (274). FcRn binds IgG antibodies at the maternal-fetal interface and transports them across the placenta to provide passive immunity to the fetus, and also plays a role in protecting IgG from premature degradation in adults (275). The assembly of FcRn in the ER has been shown to be facilitated by many of the same chaperones involved in MHC I assembly, including calnexin and ERp57 (276). Recent evidence suggests that expression of FcRn is not limited to trophoblasts, but that it is also found in antigen-presenting cells such as dendritic cells, where it has been hypothesized to participate in antigen presentation (277;278). It is therefore possible that tapasin or its isoforms might play a role in the assembly and function of these MHC I-like molecules based on potential structural similarities between these molecules and tapasin’s known partner, MHC I.

There are other possible pathways that one can speculate could also potentially involve the tapasin isoforms. Some MHC I molecules have themselves been shown to be soluble and shed from the cell surface in both humans (279) and mice (280) due to alternative splicing. The purpose of this phenomenon is still a matter of some debate. Soluble MHC I has been proposed to play a role in the maintenance of maternal tolerance towards the fetus during pregnancy by altering NK cell activity (281-286), but other roles have also been proposed, including the prevention of aberrant immune responses, suppressing the killing of bystander cells, or acting as a decoy for T or NK cells thereby suppressing their cytolytic effects (286;287). The soluble form of tapasin, given its retention of the MHC I-binding domain and the fact that it is also soluble, could potentially interact with soluble MHC I and mediate its as-yet unclear functions. As previously mentioned, soluble tapasin mRNA was highly expressed in mucosal tissues
where aberrant immune responses against commensal organisms must be avoided; therefore, soluble MHC I and soluble tapasin could act together in regulating immune responses in these areas of the body.

One final idea for the role of the tapasin isoforms is in the recently-described phenomenon of immunogenic apoptosis. An important study has shown that cell surface exposure of calreticulin is a hallmark of immunogenic apoptosis, which can be induced by certain classes of apoptosis-initiating drugs but not others (288). During immunogenic apoptosis, the exposure of calreticulin on the cell surface appears to act as an “eat-me” signal for dendritic cells, which then cross-present antigens derived from the apoptotic cells much more efficiently than antigens from cells undergoing apoptosis via mechanisms that do not lead to calreticulin cell surface exposure (288). Since tapasin is known to interact with calreticulin, it is possible that it or its isoforms form part of a cell surface calreticulin complex necessary for DC uptake and cross-presentation of apoptotic cells.

6.3.8 MHC Class I and tumors

The role of the immune system in controlling the development, growth and metastasis of cancer has long been debated, and has fallen in and out of favour many times. A large body of evidence now exists supporting the role of the immune system in both the progression and elimination of cancer (289). CD8\(^+\) T cells in particular have been shown to play an important part in tumor immunity: a large study recently found that extensive immune infiltrates of CD8\(^+\) T cells in colorectal tumors correlated strongly with positive outcomes in cancer therapy (290) and had better predictive value than
conventional staging methods, providing strong evidence for the role of the immune system – particularly Th1-type responses- in controlling tumor growth and relapse.

To achieve immunologically-mediated destruction of tumors, sufficient numbers of T cells with high avidity TCRs recognizing tumor antigens must be generated \textit{in vivo}. These cells must then traffic to and infiltrate the tumor stroma, often in the face of an immunosuppressive microenvironment. Once at the tumor site, the immune cells must be activated to manifest appropriate effector mechanisms such as direct lysis or cytokine secretion capable of causing tumor destruction. Finally, the tumors themselves must have the ability to process and present sufficient levels of tumor-associated antigens (TAAs) on MHC I molecules to the stimulated T cells for recognition and killing.

As discussed earlier, many tumors fail to present tumor-associated antigens due to defects in APM component expression. A highly attractive approach to cancer immunotherapy is the re-introduction of TAP1 and Tapasin in MHC I-deficient cancers, since these components are not only essential for direct antigen presentation to CD8$^+$ T cells, but are also required for the cross-presentation of exogenous tumor antigens in dendritic cells during the initiation of the anti-tumor T cell response immune response (69,70,291-293). The results of this thesis show that the TAP1/Tapasin approach is indeed effective in restoring tumor immunogenicity and decreasing tumor growth in two different tumor models, and – importantly for the therapeutic setting - is also effective even when the components are not re-expressed in every tumor cell (147,148).

This methodology offers many advantages over other current “personalized” immunotherapy protocols, such as adoptive transfer of TAA-specific T cells (220) or vaccination with specific tumor antigens (294). These approaches often require prior
knowledge of a patient’s haplotype and the nature of the TAAs expressed by the tumor, both of which require considerable time and resources to determine. Other approaches, such as vaccines based on TAA peptides, DNA, dendritic cells or modified tumor cells, have successfully generated TAA-specific immune responses, but do not solve the problem of the low immunogenicity of the tumor cells themselves \textit{in vivo} (144;145;295-297). TAP/Tapasin therapy avoids many of these problems: it is applicable to many patients regardless of HLA type since expression of TAP1 is not MHC-restricted and TAP can transport peptides capable of binding to any MHC I allele. As a result, it has the potential to induce immune responses to multiple tumor antigens, including known and unknown TAAs, and may thus provide an advantage over antigen-specific treatments, since it would minimize the escape of tumors that present unknown TAAs or those that shut off expression of the TAA being targeted. The Jefferies laboratory has also shown in other studies that both TAP1 and tapasin expression induces TAP-dependent cross-priming (146) and results in enhanced memory CD4$^+$ and CD8$^+$ T cell production, which could help in control potential relapses (147). These results strongly suggest that using the TAP1/Tapasin approach to generate anti-tumor immune responses results in effective elimination of tumors and long lasting protection against cancer recurrence. In view of the potential therapeutic benefits to TAP1 treatment, recombinant adenoviruses expressing human TAP1 (AdhTAP1) and human tapasin (AdhTpn) have been constructed for future use in clinical trials. Adenoviruses are amongst the preferred viral vector candidates for gene therapies (298;299). In this thesis, testing of these recombinant viruses has been done in various murine MHC I-deficient tumor models to demonstrate proof of principle. It will be important to test the effect of both human TAP1 and human
Tapasin expression in further carcinoma models to strengthen the universality of their effects and to establish the molecular mechanisms underlying them. It is possible that addition of further APM components could elicit even more potent anti-tumor immune responses, but this remains to be tested.

At the mechanistic level, it would be interesting to investigate further how the presence of TAP1 in the stable B16F10 transfectants is able to increase the CD8\(^+\)-T cell anti-tumor responses \textit{in vivo} that were effective even against untransfected tumor cells expressing very little surface MHC I. The experimental results suggest that the presence of TAP contributes to breaking local immune tolerance mechanisms both systemically and at the tumor site by reducing secretion of immunosuppressive cytokines such as IL-10 (148), such that even MHC I-deficient tumors can be recognized again and killed. TAP1 expression in the tumor cells used as immunogens to prime the immune response must somehow have been better able to present peptides or their protein precursors to the DCs for processing. It is possible that DCs may be accessing TAP-imported peptides in the ER of the tumor cells, perhaps bound to HSPs (300). Alternatively, more peptides in partial states of degradation might persist in the TAP-transfected tumor cells for the DCs to subsequently access. ER lumenal peptides imported by TAP might be more protected from cytosolic proteases; in the absence of TAP, these same peptides with “nowhere to go” could be rapidly degraded to amino acids in the cytosol, yielding few antigens for the DCs to present. It will be important to further investigate what factors mediate the increased immunogenicity of TAP-expressing tumor cells and to see if it can be enhanced even further, as this situation is very relevant to the clinical setting where not every tumor cell in the body can realistically be infected with recombinant viruses, but where
elimination of every tumor cell is the ultimate goal. By harnessing the power of the immune system, this goal is showing great promise of being achievable.
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Appendix A: DNA and amino acid sequences of the tapasin isoforms

DNA sequence of the human Tapasin gene

5’ upstream sequence:
..........tgctgggtagtccaggagggcgctgtaaggctggggtcctgggtgagaa
EXON1: (208bp)
CTGGGAGAGGATCTACCCCGGTCCTGGGAAGTGTTGGAAGTTCGCTGTTGAGGATG
CGGCCGAAAGGGTCCCGGCAAGACGCTCTCGTCGTCGTCGACCCGACGGGCGG
AACCGCCGCCGCCGCGGCCGCCACCCGCTGACCTGACTATCTGAGTACAG
INTRON1/2: (140bp)
gtagcgcacccccggctacccctgcgc.............ccccctctgcggtacatacgcc
EXON2: (171bp)
GCCTGGCCGCGCCCTCTAGCAAGGAGCTCTGGGACGCGCCACCCGACGCC
ACCTGGGAGAGGATCGCTGTCGTCGTCGACCCGACGGGCGG
AACCGCCGCCGCCGCGGCCGCCACCCGCTGACCTGACTATCTGAGTACAG
INTRON2/3: (216bp)
ACCCCGGCGGGCGGCTCCCTGACGGGCTCTGGGACGCGCCACCCGACGCC
ACCTGGGAGAGGATCGCTGTCGTCGTCGACCCGACGGGCGG
AACCGCCGCCGCCGCGGCCGCCACCCGCTGACCTGACTATCTGAGTACAG
EXON3: (261bp)
GCCCTGGCGACCGCCGTCTCAGCAGGACCCGCGGTGATCGAGTGTTGGTTCGTGGAGGATG
CGAGCGGAAAGGGCCTGGCCAAGAGACCCGGTGCACTGCTGTTGCGCCAGGGACCGGGGG
AACCGCCGCCGCCGCGGCC GCCACCCGCTGACCTGACTATCTGAGTACAG
INTRON3/4: (7829bp)
gtagctggggagggagttgagga..............ccccctctgcggtacatacgcc
EXON4: (399bp)
TGTTACCTGACTGTCCTCCTCACCCACACCCCTGGCCCTCCTGAGTGAAGTGGGACAGATGCTC
TGCTGGAGACGCCTCTGCTGAGTGGGACAGATGCTC
CGGGGCTCCCTCTTTGGGCTAGGTTGGGCTC
TCTGGCTGCACCTCTCGGGCTCTGCTGAGTGGGACAGATGCTC
CGCCGGGCTCCCTCTTTGGGCTAGGTTGGGCTC
CATTGGCTGCACCTCTCGGGCTCTGCTGAGTGGGACAGATGCTC
GCTGCTCACTGACTGCACCTCTCGGGCTCTGCTGAGTGGGACAGATGCTC
ACCTGCAGACAGGACAGGTCTGAGGCACCTGGGCTGAGTGGGACAGATGCTC
INTRON4/5: (350bp)
gtagctggggagggagttgagga..............ccccctctgcggtacatacgcc
EXON5: (342bp)
AACCCCCCAAATGTGCCTCGACTGGCAACCGGCCCTGAGTGAAGTGGGACAGATGCTC
CGGGGCTCCCTCTTTGGGCTAGGTTGGGCTC
TCTGGCTGCACCTCTCGGGCTCTGCTGAGTGGGACAGATGCTC
CGCCGGGCTCCCTCTTTGGGCTAGGTTGGGCTC
CATTGGCTGCACCTCTCGGGCTCTGCTGAGTGGGACAGATGCTC
GCTGCTCACTGACTGCACCTCTCGGGCTCTGCTGAGTGGGACAGATGCTC
ACCTGCAGACAGGACAGGTCTGAGGCACCTGGGCTGAGTGGGACAGATGCTC
INTRON5/6: (79bp)
gtagctggggagggagttgagga..............ccccctctgcggtacatacgcc
EXON6: (90bp)
GTCCTTCTGACTGCCCTCTTTGGGCTAGGTTGGGCTC
CGGGGCTCCCTCTTTGGGCTAGGTTGGGCTC
TCTGGCTGCACCTCTCGGGCTCTGCTGAGTGGGACAGATGCTC
INTRON 6/7: (138bp)
gtagctggggagggagttgagga..............ccccctctgcggtacatacgcc
EXON7: (35bp)
GTCCTTCTGACTGCCCTCTTTGGGCTAGGTTGGGCTC
TCTGGCTGCACCTCTCGGGCTCTGCTGAGTGGGACAGATGCTC
INTRON 7/8: (2182bp)
gtagctggggagggagttgagga..............ccccctctgcggtacatacgcc
EXON8: (2075bp)
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AAAGCAGAGTGAGGGCACTCACTGCCATCCTGTGGAAGCCACCATCATCTCTGGCCCAAG
CTTCTGTAGTAGCTCCCTAAAATAATACCCTATCATCTGCTCCTAATCCCTCCAATCTCT
CTCCACTGAGTGGCTGGAATGCTTTTTTTTTTTTCTTTCACTTATATAAGGGATAATTTT
TCTTTTTTTTTTTTTTTTGAGACGGAGTCTCACTCTTCCGCCCAGGCTGCAGTGCAGTGG
CATGATCTTGGCTTACTGCAACCTCCGCCTCCTGGGTTCAAGCAATTCTGTGGCTTCAGC
CTCCGGAGTAGCTGGGATTACAGGCACATGCCACCACACCCAGTGAATTTTTGTATTTTT
AGTAGAGACGGGGTTTCACCATGTTGGCCAGGCTGGTCTTGAATTCCTGACCTCAGGTGA
TCTGCCCACCTCAGCCTCCCAAAGTGCTGGGATTACAGGCGTGAGCCACCACACCAGGCC
CGAGAAATGCTTTTTTAAAAAACACACATCTTATGGCATTCACCTTCTTGGAGCTCTAGG
ACAGTGGTTCTCAAAATTTTTTTCTCTCAGGACCTCTTAAAAATCATCAAGGACCCCAAA
AAGCTTTTGGGTATGTGGGTTATAGCTATCAATATTTATGGTACTAGAACTTAAAAGTGA
GAAAAATTTAAAACACGAGAATACATAGGCACACATTCTATTCATCGTGGGAACCATGGT
GTCAATACATATCATGTAGCTTCTGAAAAACTCCACTGTACACTTATAGAATGAAGAAGG
CAAAAAACTTTTTTTTTTTTTTTTTTGAGACGGAGTCTCGCTCTGTCGCCCAGGCTGGAG
TGCAGTGGCGCGATCTCGGCTCACTGCAAGCTCCGCCTCTCGGGTTCACGCCATTCTCCT
GCCTCAGCCTCCCAAGTAGCTCGGACTACAGGCGTCCTCCACCATGCCTGGCTAATATTT
TGTATTTTTTAGTAGAGACGGGGTTTCACCGTGTTAGCCAGGATGGTCTCGATCTCCTAA
CCTGGTGATCCGCCCGCCTCGGCCTCCCAAAGTATTGGGATTACCCGCGTGAGCCACCGC
GCCCGGCTGCAAATAATCTTTCTTTTTTTCTGAGACAGAGTCTCGCTCTGTTGCCCAGGC
TGGAGTGCAGTGGCACGATCTCGGCTCACGGCACGCTCCGCCTCCCGGGTTCACGCCATT
CTCCTGCCTCAGCTTCCCGAGTAGCTGGGACTACAGGGGCCCGCCACCACGCCCGGCTAA
CTTTTTGTGTTTTTAGTAGAGACGGGGTTTCACCGTGTTAGCCAGGATGGTCTCGATCTC
CTGACCTTGTGATCTGCCCGCCTCGGCCTCCCAAAGTGCTGGGATTACAGGCGTGAGCCA
CCGCGCCCGGCGGCGAAACACGATATTGTACTAACATCTTAATTTTGTTATAAAATCTCA
CAAACCCCCTGACATAGTCTCAGAGATCTGTAGGGCCGAGGTTACATTTGGAGAACCCGT
ACTCTAGGGCCAAATCCATTCTTCTTGCCCTGGCTCACTTGTCCCCCCCACCGCCCCGCG
CTGGAGCCACTGCCTAGTTCTTCAGCCCTAGATGGTGCTCGCCAGACCTCCTCTCAATGC
TCATCACACACAGGGCTATTCCTTTCCTCCAATGAACCAAACGCCTCCCGCCCACCTCCA
GGTCCCAGTCCTCTGTTCCCTTTGCCTGGTCCACCCTTGCCCTCCCTGGGTCGCAGACGA
GGTCGGCCTCGTCATTCCCCGCAGACCGCCGCGCGTCCCTCTTGTGCGGTTCACCACAGT
TGTATTTAAGTGATCGTGTGAGTCGTCGTTAAATGCCTGTCTCCCCGCGGATCATGGGCT
CCTCGAGGACAGGGACTGGCCTGTCTGTCCACTGCTGTAACCCCGCGCCGGCATAGGGAC
CTAAGGCCCACTGGAGGGCGCTCATCAAGTAGCTGCTGGATGTTGACGAAGGAAGCGGCG
GCGCAGCTCAGGGATCTCCGAGTCAGGACGGTCGGCCAGACCCACGGGGTAACGGGTCTA
ATCGTGTAGGAATAAAGCTGTATTCCAGTGCTTCC
3’ downstream sequence:
aaacggttctctcattccaacccctttccaagctcaatgaatattccaat..........

Amino acid sequence isoform 1
EXON1: (12aa)

MKSLSLLLAVAL
EXON2: (57aa)

GLATAVSAGPAVIECWFVEDASGKGLAKRPGALLLRQGPGEPPPRPDLDPELYLSVH
EXON3: (87aa)

DPAGALQAAFRRYPRGAPAPHCEMSRFVPLPASAKWASGLTPAQNCPRALDGAWLMVSISSPVLS
LSSLLRPQPEPQQEPVLITMAT
EXON4: (133aa)

VVLTVLTHTPAPRVRLGQDALLDLSFAYMPPTSEAASSLAPGPPPFGLEWRRQHLGKGHLLLAAT
PGLNGQMPAAQEGAVAFAAWDDDEPWGPWTGNGTFWLPTVQPFQEGTYLATIHLPYLQGQVTLEL
AVY
EXON5: (114aa)

KPPKVSLMPATLARAAPGEAPPELLCLVSHFYPSGGLEVEWELRGGPGGRSQKAEGQRWLSALRH
HSDGSVSLSGHLQPPPVTTEQHGARYACRIHHPSLPASGRSAEVTLEVA
EXON6: (30aa)

GLSGPSLEDSVGLFLSAFLLLGLFKALGWA


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EXON7: (12aa)
AVYLSTCKDSKK

EXON8: (3aa)
KAE

**Amino acid sequence isoform 2**

EXON1: (12aa)
MKSLSLLLAVAL

EXON2: (57aa)
GLATAVSAGPAVIECWFDADEGKGLAKRPGALLLRQPGPGEPPRPDLPDPELYLSVH

EXON3: (87aa)
DPAGALQAAPRRYPRGAPAHCPEMSIFVPLPASAKWASGLTPAQNCPRAIDGAWLMVISISSVLS
LSLRLRPQPEPQEQVIPVLMAT

EXON4: (133aa)
VVLTVLHTTPRVPRLQDLALLDLFAYMPPTSEAASLPMPGPPFGLEWRRQHLSKHLLAAT
PGLNQMPAAQEGAVAFAAWDDDEFWGFWTGNQFTWLPTVQPFEQETYLATIHLPYLGQTVTLELA

EXON5: (114aa)
KPPKVSLMPATLARAPGEAPPELCLVSHFYPSGLEVEWELRGGPGGRSQKAEGQRPWLSALRH
HSDGVSLSGHQLQPPTQTEQHAGARYACRIHHPSLAPGRSAEVTLLEA

EXON6: (30aa)
GLSGPSLEDSDVLGLSLAFLLLGLFKALGA

EXON8: (26aa)
ESRVRALTAILWKPSSLAQASVVAP

**Amino acid sequence isoform 3**

EXON1: (12aa)
MKSLSLLLAVAL

EXON2: (57aa)
GLATAVSAGPAVIECWFDADEGKGLAKRPGALLLRQPGPGEPPRPDLPDPELYLSVH

EXON3: (87aa)
DPAGALQAAPRRYPRGAPAHCPEMSIFVPLPASAKWASGLTPAQNCPRAIDGAWLMVISISSVLS
LSLRLRPQPEPQEQVIPVLMAT

EXON4: (133aa)
VVLTVLHTTPRVPRLQDLALLDLFAYMPPTSEAASLPMPGPPFGLEWRRQHLSKHLLAAT
PGLNQMPAAQEGAVAFAAWDDDEFWGFWTGNQFTWLPTVQPFEQETYLATIHLPYLGQTVTLELA

EXON5: (114aa)
KPPKVSLMPATLARAPGEAPPELCLVSHFYPSGLEVEWELRGGPGGRSQKAEGQRPWLSALRH
HSDGVSLSGHQLQPPTQTEQHAGARYACRIHHPSLAPGRSAEVTLLEA

EXON8: (26aa)
ESRVRALTAILWKPSSLAQASVVAP
Appendix B: Constructs for the generation of transgenic mice expressing tapasin isoforms

To evaluate the role of the tapasin isoforms in vivo, constructs to be used in the generation of transgenic mice were made as follows: the mouse tapasin promoter sequence (264) was amplified by PCR using forward primer 5’-GCA TCC TAG AGT CAC AGA GC-3’ and reverse primer 5’-GGT GCT ACG ATC TCT CCT AC-3’. Genomic DNA obtained from a murine lung carcinoma cell line, CMT.64, was used as a template, and PCR was performed with Taq polymerase 95°C for 3 min, followed by 35 cycles of 95°C 1min, 60°C 1min, 72°C 1min, with a final 10min 72°C extension step generating a PCR product of 625bp. The PCR product was inserted into the pCR-BluntII-TOPO vector and sequenced to ensure a match with the published tapasin promoter sequence. The promoter sequence was subsequently removed from the TOPO vector by EcoRI digestion and gel purification, and ligated into the EcoRI site of the pEGFP promoterless vector (Clontech), yielding a tapasin promoter-EGFP reporter construct. The EGFP cassette was removed from the construct by digestion with AgeI and NotI, and T4 DNA Polymerase was used to make blunt ends which were subsequently re-ligated back together. Next, the tapasin isoforms were cut from the pCR-Blunt II-TOPO vectors generated during their initial cloning with PstI and KpnI and ligated into the complementary sites of the tapasin promoter/pEGFP construct lacking the EGFP cassette. Finally, the construct (promoter-tapasin isoform-poly A tail) was linearized and isolated from the vector backbone with BglII and AflIII to remove as much vector DNA as possible in preparation for pronuclear injection.
To test the constructs’ ability to express each isoform under the native murine tapasin promoter, murine Ltk⁻ fibroblasts (which express mouse tapasin) were transfected in 6 well plates with 2.5 μg of digested and gel-purified constructs or mock control with Lipofectamine 2000 (Invitrogen) according to manufacturer’s instructions. Forty-eight hours later, cells were harvested and RNA extracted with Trizol (Invitrogen), digested with Dnase-I (Invitrogen), and 2 μg was converted to cDNA with SuperScript II (Invitrogen) according to manufacturer’s instructions. PCR for human tapasin was performed using an exon5 forward primer 5’-AGG TCA CCC TGG AGG TAG CA-3’ and exon 8 reverse primer 5’-GGG ATT AGG AGC AGA TGA TAG GGT A-3’ and 5μl of cDNA template in a 50μl reaction with Taq polymerase 95°C for 3 min, followed by 35 cycles of 95°C 1min, 60°C 1min, 72°C 1min, with a final 10min 72°C extension step. pMXpie plasmids containing each isoform were used as positive controls. Fifteen microlitres of PCR product were run on a 2% agarose gel for 30 min, and stained with ethidium bromide for viewing with UV light. As a loading control, 5 μl of template was also amplified under the same conditions with S15 rRNA-specific primers (forward: 5’-TTC CGC AAG TTC ACC TAC C-3’, reverse: 5’-CGG GCC GGC CAT GCT TTA CG-3’).
Figure B.1 Constructs for the generation of tapasin isoform-expressing transgenic mice.
(a) The pEGFP-1 promoterless vector (Clontech PT3026-5) was used as a backbone for insertion of the mouse tapasin promoter and human isoforms. The EGFP cassette was later deleted. (b) Test transfection of Ltk- murine fibroblasts shows that the plasmid vectors for the generation of transgenic mice expressing the tapasin isoforms do express the appropriate isoforms at the RNA level.
Appendix C: Construction of recombinant non-replication adenoviruses encoding human tapasin isoforms

To express the tapasin isoforms in tapasin\(^{-/-}\) DCs or MHC I-deficient tumors, recombinant adenoviruses encoding each isoform were created based on the method of Hardy et al. (160). FirstChoice\textsuperscript{TM} Total RNA from human spleen was obtained from Ambion Inc. (Austin, TX). cDNA was synthesized using RETROscript\textsuperscript{R} First Strand synthesis kit for RT-PCR (Ambion Inc.) using Oligo(dT) primers as per the manufacturer’s instructions. Tpn cDNA was amplified using primers designed based on the sequence of human Tpn transcript variant 1 (NM\_003190) using Pfu DNA polymerase (Stratagene, La Jolla, CA). The primer sequences used were as follows: forward primer 5’-GCCATGAAGTCCCTGTC TCTG-3’ and reverse primer 5’-GGGATTAGGAGCAGATGATAGGGTA-3’. The insert was cloned in pCR-Blunt II -TOPO vector (Invitrogen Life Technologies, Carlsbad, CA) and both strands were sequenced to ensure no mutations were present. HTpn was digested from TOPO/hTpn with Pst I and BamHI and then cloned into a Pst I- and BamHI- digested shuttle vector, pAdlox plasmid (301). The resulting vector, pAd/hTpn, was isolated and sequenced to ensure the sequence fidelity. The AdhTpn was generated as previously described (302).

Briefly, the pAd/hTpn, linearized with SfiI, was co-transfected along with \(\Psi\)5 DNA into CRE8 cells using LipofectAMINE PLUS\textsuperscript{TM} Reagent (Invitrogen Life Technologies) to generate AdhTpn. AdhTpn recombinant viral clones were identified by immunofluorescence assay and plaque purified three times in HEK 293 cells. CRE8 cells
have a β-actin-based expression cassette driving a Cre recombinase gene with an N-terminal nuclear localization signal stably integrated into HEK 293 cells. Ψ5 virus is an E1 and E3 deleted version of Ad5 containing loxP sites flanking the packaging site. Ψ5 and recombinant adenovirus were propagated and titred in HEK 293 cells.

The recombinant virus was amplified in large-scale stock in HEK 293 cells, purified by CsCl density gradient centrifugation, and titred in HEK 293 cells. The identity of AdhTpn was confirmed by PCR and DNA sequencing of purified viral DNA using primers specific for Tpn and adenovirus DNA flanking either side of the Tpn gene. The primer sequences were as follows: forward primer 5’-AAG AGC ATG CAT GAA GTC CCT GTC TCT G -3’ and reverse primer 5’- AAT AAG TCG ACC AGT GAG TGC CCT CAC TCT GCT GCT TTC -3’ for amplification of Tpn; forward primer 5’- GTG TTA CTC ATA GCG CGT AA-3’ and reverse primer 5’CCA TCA AAC GAG TTG GTG CTC-3’ for amplification of adenoviral flanking sequence.
Figure C.1 Construction of recombinant adenoviruses expressing the tapasin isoforms.
Recombinant adenoviruses were generated in the Cre-expressing 293 cell line CRE8. The tapasin isoforms’ cDNAs were subcloned into the pAdlox shuttle vector in the PstI and BamHI sites of the multiple cloning region under the control of a CMV promoter, which is flanked by loxP sites and has the ψ5 packaging signal at the 5’ end. These recombine in the presence of Cre recombinase with the loxP sites of the empty viral vector package, called ψ5, resulting in production of recombinant viruses that are then plaque purified 3 times to isolate them from wild-type virus. Because the ψ5 virus has the E1 and E3 regions deleted, it does not replicate in any cells except 293, which stably express the E1 region genes needed for viral replication. Reproduced from (160) with permission.
Figure C.2 Purity test of recombinant adenoviruses expressing the tapasin isoforms by PCR using primers flanking the viral multiple cloning region.
Genomic viral DNA purified from the recombinant viruses and was tested for the presence of wild-type (empty) ψ5 virus after plaque purification with PCR primers flanking the multiple cloning regions. All three recombinant viruses yielded higher molecular weight PCR products than the ψ5 virus due to insertion of the isoform cDNA into the viral genome. No band from ψ5 was detected in any of the recombinant stocks, indicating they consist of pure recombinant virus with no contaminating wild-type virus.

Figure C.3 RNA expression test of recombinant adenoviruses expressing the tapasin isoforms by PCR using tapasin-specific primers.
Genomic viral DNA purified from the recombinant viruses and cDNA derived from tapasin−/− fibroblasts infected with the viruses by RT-PCR were tested for expression of each isoform using PCR primers spanning exon 5 (fwd) and exon 8 (rev) of human tapasin. Results showed that each virus expresses the correct isoform at both the genomic DNA and RNA levels, with bands of 256bp for isoform 1, 221bp for isoform 2, and 131bp for isoform 3.
Figure C.4 Protein expression test of recombinant adenoviruses expressing the tapasin isoforms by Western blot.

Tapasin−/− fibroblasts were infected with each recombinant retrovirus or empty ψ5 vector alone virus at an MOI of 10. Lysates were generated after 48 hours and tested for expression of human tapasin by Western blot using the Rgp48N antibody that recognizes the N terminal region of all three forms. Raji B cell lysate, which expresses both isoform 2 (top band) and isoform 1/3 (bottom band), was used as a control. All viruses expressed the appropriate isoform at the protein level.