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Post-translational Regulation of the NKG2D Ligand Mult1
in Response to Cell Stress

by

Timothy James Nice

A dissertation submitted in partial satisfaction of the
requirements for the degree of

Doctor of Philosophy

in

Molecular and Cell Biology

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor David H. Raulet, Chair
Professor Mark S. Schlissel
Professor Gregory M. Barton
Professor Brian J. Staskawicz

Fall 2009

This dissertation of Timothy James Nice, titled Post-translational Regulation of the
NKG2D Ligand Mult1 in response to cell stress is approved:

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University of California, Berkeley

Abstract

Post-translational Regulation of the NKG2D Ligand Mult1 in Response to Cell Stress

by

Timothy James Nice

Doctor of Philosophy in Molecular and Cell Biology

University of California, Berkeley

Professor David H. Raulet, Chair

NKG2D is a major stimulatory receptor expressed by natural killer (NK) cells and some T cells. The receptor recognizes MHC class I-like cell surface ligands that are poorly expressed by normal tissues but often induced in transformed and infected cells. The existence of several NKG2D ligands in each individual, some with strikingly divergent protein sequences, raises the possibility that different ligands are regulated by distinct disease-associated stresses. The transcripts for some ligands, including murine UL16-binding protein-like transcript 1 (Mult1), are abundant in certain normal tissues where cell surface expression is absent suggesting the existence of translational or post-translational regulation.

This dissertation establishes that Mult1 is ubiquitinated on lysine residues within its cytoplasmic tail, targeting the protein for degradation. Inhibition of lysosomal acidification leads to accumulation of Mult1 at the cell surface and within lysosomes indicating that they are the primary site for Mult1 degradation. This pathway may explain the lack of inappropriate Mult1 expression in healthy tissues where high levels of Mult1 transcripts are present.

Mult1 degradation and ubiquitination is reduced in response to stress imparted by ultraviolet (UV) irradiation. Other genotoxic agents do not induce Mult1 at the protein level, indicating that this pathway is distinct from the previously identified regulation of NKG2D ligands by genotoxic stress. The heat shock pathway also induces Mult1 at the protein level, suggesting that Mult1 may be specifically induced by protein folding stresses.

Membrane-associated RING-CH (MARCH)-4 and MARCH-9 are capable of regulating Mult1 expression in response to heat shock, but may overlap with other E3 ligases in this regulatory role. The MARCH family of E3 ligases appears to have an important role in controlling expression of a broad range of transmembrane proteins with relevance to the immune system. The finding that MARCH-9 is involved in Mult1 regulation extends the immuno-regulatory role of these E3 ligases to cover NKG2D ligands.

These findings represent a novel mechanism for stress-mediated cellular control of NKG2D ligand expression. There is now evidence for regulation of NKG2D ligands at

multiple stages of gene expression, suggesting that integration of multiple pathways is required for activation of lymphocytes through NKG2D. Protein degradation is a final layer of Mult1 regulation, only allowing Mult1 to accumulate at the cell surface if certain stresses are present. It will be of interest in the future to determine which cells and pathological conditions utilize this regulatory mechanism to activate the immune system.

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Chapter 1: Introduction

General introduction to the immune system

The vertebrate immune system is commonly divided into two separate arms, innate and adaptive. These two parts have clearly distinguishing characteristics but cooperate in their efforts to protect against invasion from pathogens. In general, the innate immune system is able to act rapidly and with comparatively less discrimination in response to signs of a foreign presence. Innate immunity is evolutionarily ancient and is present in some form in most eukaryotic organisms. The adaptive immune system, by contrast, evolved more recently and is confined to vertebrates. Addition of adaptive immunity into the mix allows for exquisitely pathogen-specific responses and, as its name suggests, the ability to remember those particular pathogens that are present in the local environment. While often considered as separate entities, the two arms (rapid, low-specificity innate immunity, and slower but highly specific adaptive immunity) are intertwined during an immune response, and work together to keep the sea of microbes at bay.

The innate immune system, from pathogen-eating phagocytes to the barriers provided by epithelial layers and anti-microbial molecules, is essential for survival in a world filled with microbes looking for an easy source of nutrients. Without the protection provided by these innate immune components, we would quickly succumb to our miniscule neighbors. Physical barriers, such as the skin, provide the first line of defense against pathogens, but cells of the innate immune system quickly become involved should a pathogen get past these barriers. Several of these innate immune cell types are phagocytic, meaning that they are capable of engulfing large particles including pathogens. An initial wave of phagocytic cells, such as neutrophils and macrophages, are attracted by inflammatory signals to the site of infection where they eat pathogens and clean up debris from dead or dying cells. Another phagocytic cell of the innate immune system, the dendritic cell (DC), is a messenger that relays signals between the innate and adaptive phases of the immune response. DCs are resident in most tissues and upon recognition of pathogens they will carry their bounty to draining lymph nodes where initiation of adaptive responses can be initiated. An innate immune cell of particular importance to this dissertation is the Natural Killer (NK) cell. Unlike macrophages and DCs, NK cells are not phagocytic and instead have a different set of tools at their disposal. These tools allow NK cells to directly kill cells that have been infected, thus preventing pathogens from growing within. The mechanism of this killing is dependent on pore-forming proteins and proteases contained within the NK cell that can be released towards infected cells and initiate a cell death program. These actions of NK cells, macrophages and neutrophils keep the pathogen under control until the adaptive immune response initiated by the DCs is functional.

The two heavy-hitters of the adaptive immune system are T cells and B cells. The defining characteristic of these adaptive immune cells is their amazing diversity. Unlike cells of the innate immune system, which are relatively similar to their brethren in pathogen recognition capabilities, no two developing T or B cells are the same. T and B cells rearrange segments of their DNA to create a unique receptor that has the potential to recognize a non-self molecule. The cumulative effect of this rearrangement process during development is a pool of T or B cells that have the capability of recognizing

virtually any non-self molecule (including pathogen-associated ones) with exquisite specificity. DCs that have traveled to the lymph node from the site of infection present bits of their digested pathogen to the pool of T cells, and a T cell that can recognize a molecular component of the pathogen will become activated and multiply itself to create an army of pathogen-specific T cells. T cells and other cells within the lymph node selectively activate antigen-relevant B cells in a similar manner. It is not surprising that this complex process takes some time, underscoring the importance of a rapid innate immune response.

Once the capable T and B cells have been selected and activated, there is a very powerful array of activities carried out by these cells. T cell responses can be divided into two main categories: secretion of molecular signals to direct the path of the adaptive immune response (helper T cells) and eradication of infected cells in a manner similar to (though more robust than) that described for NK cells (cytotoxic T cells). B cells respond to activation by producing a soluble form of their receptor, called an antibody, which can bind to the pathogen that initiated the specific B cell response. This binding of antibodies can neutralize pathogen activity as well as enhance their clearance by phagocytic and/or cytolytic cells. The powerful and specific response provided by the adaptive immune system allows for the eventual control of all but the most pathogenic microbes.

NK cells and the stimulatory receptor NKG2D

Natural Killer Cells

Natural killer (NK) cells are lymphocytes of the innate immune system that lack the extreme diversity of T-cells or B cells. Consequently, NK cells are able to respond more rapidly, enabling infections to be controlled in the early stages (Herberman and Ortaldo 1981; Bukowski, Woda et al. 1984; Trinchieri 1989). An individual NK cell's decision to initiate a response or remain tolerant is dictated by a combination of receptor-derived signals provided by ligands on target cells and the extent to which they have been pre-activated by inflammatory cytokines. Inflammatory cytokines, such as IL-12 and type I interferon, are produced by other innate immune cells during the early stages of infection and raise the responsiveness of NK cells, increasing their ability to aid in resolving the infection (Biron 1994). Two major responses provided by NK cells are cytotoxicity of target cells and production of cytokines such as interferon gamma, which is important for activation of other cells within the immune system (Trinchieri 1989).

Determination of targets for cytotoxicity clearly must be tightly controlled to prevent elimination of healthy cells. How NK cells are able to distinguish self from non-self has become increasingly clear as knowledge of NK cell biology has increased. Particularly important has been the identification of NK cell receptors (which are often also expressed on some T cell subsets, reflecting the close evolutionary relationship between these two cell types) (Lanier 1998; Raulet 2004). The receptors come in two major classes distinguished by their ability to initiate either stimulatory or inhibitory signaling cascades. The balance between inhibitory and stimulatory signals originating from ligand-bound surface receptors dictates the response of an individual NK cell. When stimulatory signals outweigh the inhibitory ones and pass a critical threshold, NK cells respond (Raulet and Vance 2006).

Negative regulation of NK cell activity is provided by a panel of inhibitory surface receptors that recognize MHC class I proteins, enabling NK cells to preferentially attack cells that decrease expression of MHC class I molecules (Karre, Ljunggren et al. 1986). MHC class I is commonly downregulated by viruses and lost by tumor cells in order to evade CD8 T cell immune responses (Garrido and Ruiz-Cabello 1991; Jonjic, Babic et al. 2008). The presence of the inhibitory MHC class I receptors on NK cells means that this CD8 T cell evasion has the consequence of increasing susceptibility to NK cell attack. The recognition of MHC class I is mediated primarily by the Ly49 family of receptors in mice and the killer immunoglobulin receptor (KIR) family in humans. In addition, CD94/NKG2A heterodimers indirectly recognize the presence of MHC via presentation of a class I peptide by a non-classical class I molecule (Qa-1b in mice and HLA-E in humans) (Raulet and Vance 2006). Both the Ly49 and KIR families exist as a multi-genic complex encoding an array of receptors, each with affinity for a subset of MHC class I molecules. It has been shown that the expression of these receptors is variegated within the NK cell pool, resulting in expression of only a subset of the genes by any one NK cell (Raulet, Vance et al. 2001). Importantly, it was recently shown that a developing NK cell's capacity for stimulation is set depending on the level of inhibitory signal received through the set of inhibitory receptors expressed (Brodin, Lakshmikanth et al. 2009; Joncker, Fernandez et al. 2009). This ensures that an NK cell with few

interactions with self-MHC will remain tolerant to self-tissues. While Ly49/KIR inhibitory interactions with MHC are clearly important, not all inhibitory receptors recognize MHC molecules. The ligands for many of these non-MHC inhibitory receptors have not been identified, but two that have known ligands are NKR-P1d, which recognizes the broadly expressed protein Clr-b (Iizuka, Naidenko et al. 2003; Carlyle, Jamieson et al. 2004), and Klrg-1, which recognizes cadherin molecules (Grundemann, Bauer et al. 2006; Ito, Maruyama et al. 2006). Signals from the target cell through the set of inhibitory receptors expressed by an NK cell result in a cumulative negative signal. The magnitude of this signal must be offset by stimulatory signals to result in NK cell activation.

Stimulatory signals come from several distinct surface receptors, only some of which have defined ligands. One of the most extensively studied stimulatory NK cell receptors is NKG2D, which will be discussed in more detail below. Another relatively well understood stimulatory receptor is DNAM-1. It belongs to the Ig superfamily and recognizes the cell surface proteins nectin-2 (CD155) and poliovirus receptor (PVR, CD112) (Bottino, Castriconi et al. 2003). One of the first identified markers of NK cells in C57Bl/6 mice, the NK1.1 antigen, is actually the stimulatory receptor NKR-P1c. This receptor is part of the same family as the inhibitory NKR-P1d, but pairs with FcεRIγ, thus initiating a stimulatory signaling cascade. The ligand for NKR-P1c has not been identified, but NKR-P1f (also stimulatory) binds to Clrg, a protein of the same family as the NKR-P1d ligand but with a more restricted pattern of expression (Iizuka, Naidenko et al. 2003). Several members of the SLAM family of receptors are expressed by NK cells, the most extensively studied being 2B4. There has been some controversy over the nature of the signal provided by 2B4 (inhibitory or stimulatory), and the answer may depend on which adaptors are present (Veillette 2006). Analysis of NK cells lacking all three adaptors, however, suggests that stimulation through 2B4 and other SLAM family receptors may be important in elimination of unwanted cells of the hematopoietic lineage (Dong, Cruz-Munoz et al. 2009). A final mention goes to a group of stimulatory receptors called the natural cytotoxicity receptors (NCRs), which are believed to participate in killing of many tumor cells as shown by blocking the receptors with antibodies in lysis assays (Moretta, Bottino et al. 2001). This group includes NKp30, NKp44, and NKp46. While staining with multivalent NCRs has shown that their ligands are expressed on tumor cells, the identification of these ligands has been elusive (Moretta, Bottino et al. 2001). Signaling through all of the stimulatory receptors is integrated and weighed against that from the inhibitory receptors. If the magnitude of stimulation overcomes the basal inhibition, the NK cell initiates a response.

Role of Natural Killer cells in immune responses

NK cells were initially defined in the 1970s as lymphocytes with an ability to kill tumor cells but without immunological memory (Herberman and Ortaldo 1981). Since their discovery, much effort has gone into understanding the mechanisms and extent of their tumor recognition capabilities. As is the case with any immune response to tumors, there is a fine line between an anti-tumor response and an autoimmune response since the

tumor was at one time part of a healthy self-tissue. However, early on NK cells were shown to clearly and specifically restrict tumor growth and metastasis in transplantable tumor models in vivo (Talmadge, Meyers et al. 1980; Hanna and Burton 1981). A major mechanism by which NK cells recognize tumor cells specifically is based on low MHC class I expression, which often occurs in cancer. The discovery of this mode of NK cell recognition was in fact made in the context of anti-tumor responses. In comparison to NK cell resistant and MHCI-sufficient parental tumor cells, variants with low MHCI expression were shown to be sensitive to NK cell-mediated rejection (Ljunggren and Karre 1985; Karre, Ljunggren et al. 1986). It has subsequently been shown using perforin knockout mice that killing via perforin/granzyme is the primary mechanism by which NK cells kill MHCI-low tumor cells (Smyth, Thia et al. 1999). While initially informative, studies of transferable tumors are flawed in that they don't recapitulate the complex evolution of a cancer in the context of the host immune response. Study of mice with genetic predisposition to specific cancers or induction of tumors with chemical carcinogens is a more accurate representation of the natural ways that tumors develop. Using these methods, genetic deficiency in either of the NK receptors DNAM-1 or NKG2D has been shown to predispose mice to tumor formation (Guerra, Tan et al. 2008; Iguchi-Manaka, Kai et al. 2008), although in these cases the relative roles of NK cells and NK receptor-expressing T cells have not been teased apart. The lack of a good model for specific NK cell deficiency has prevented the study of the NK cell's role in physiological models tumor development. This is because development of a tumor is by nature a long process and is therefore not amenable to antibody-mediated NK cell depletion. However, the identification and genetic disruption of the transcription factor E4BP4, which is essential for NK cell development (Gascoyne, Long et al. 2009) may allow more definitive studies in this area.

NK cell responses to pathogens are generally at a peak during the first few days after infection. This means that unlike studies of tumorigenesis, infectious models have allowed analysis of the role of NK cells by their depletion with antibodies. The importance of NK cells is particularly striking during the early response to viral infection (Biron, Nguyen et al. 1999). Depletion of NK cells prior to infection and/or the use of T and B cell deficient mice has indicated the protective role of NK cells in response to mouse cytomegalovirus (MCMV), herpes simplex virus (HSV-1), vaccinia, coxsackie virus, influenza, and ectromelia virus (Bukowski, Woda et al. 1983; Habu, Akamatsu et al. 1984; Godeny and Gauntt 1986; Jacoby, Bhatt et al. 1989). The most extensively studied of these infection models has been MCMV, where NK cells respond within hours to days after infection by producing IFN γ and killing infected cells. MCMV is clearly under selective pressure to dampen the NK cell response and encodes multiple proteins that selectively downregulate ligands for NKG2D from the surface of infected cells (reviewed in (Lenac, Arapovic et al. 2008)). NK cells also appear to play a significant role in responses to intracellular bacteria. A recent study has suggested that NK cells protect against *Salmonella enterica* Serovar Typhimurium colonization of the gut and dissemination to the spleen (Ashkar, Reid et al. 2009). It is not yet clear if the protection is mediated by killing, IFN γ production or both, but there have been reports that NK cells are able to kill *Salmonella* infected cells in vitro (Griggs and Smith 1994). The literature is not in consensus regarding NK cell involvement in protection to another intracellular bacterium, *Listeria monocytogenes*, but it is clear that NK cells are activated and produce

IFN γ during the first few days after infection (Cheers and Wood 1984; Dunn and North 1991; Teixeira and Kaufmann 1994). Early production of IFN γ is critical during the immune response to the protozoan parasites *Toxoplasma gondii* and *Leishmania major*. NK and NKT cells were shown to be the critical source of this IFN γ production through depletion studies showing increased pathogen numbers and disease severity in their absence (Denkers, Gazzinelli et al. 1993; Schariton and Scott 1993). The high levels of IFN γ appear to be important for promoting a productive type of the adaptive immune response (Scott and Trinchieri 1995). These studies show that NK cells become activated in response to a wide variety of pathogens, but their relative contribution to a productive immune response can vary.

NKG2D and its ligands

NKG2D is a homodimeric type II transmembrane c-type lectin and is found on almost all NK cells as well as activated CD8 T cells, and subsets of $\gamma\delta$ T cells, NKT cells and CD4 T cells. Engagement of NKG2D by its ligands on target cells results in NK cell-dependent killing of tumor cells in vivo (Diefenbach, Jamieson et al. 2000; Diefenbach, Jensen et al. 2001), and if expression of ligands is high, stimulation through NKG2D can overcome inhibitory signaling due to MHC class I expression (Raulet 2003). Additionally, engagement of NKG2D on T cells generally enhances T cell responses (Diefenbach, Jensen et al. 2001; Groh, Rhinehart et al. 2001).

The ligands for NKG2D are a family of MHC class I-related molecules, which are generally poorly expressed by normal cells and upregulated on diseased cells (Bauer, Groh et al. 1999; Cerwenka, Bakker et al. 2000; Diefenbach, Jamieson et al. 2000; Cosman, Mullberg et al. 2001; Raulet 2003). The homology to MHC class one is limited to the $\alpha 1$ and $\alpha 2$ domains with the exception of MICA and MICB in humans, which also encodes an $\alpha 3$ domain but doesn't pair with β -2-microglobulin. The evidence shows that NKG2D ligands need not bind peptide in the region homologous to MHC's peptide binding groove, and, in fact, structural analysis has shown the region to be too narrow to support peptide binding (Li, Morris et al. 2001; Radaev, Rostro et al. 2001). The homology between individual NKG2D ligands is quite low and affinities with NKG2D vary (Raulet 2003). This may have the consequence of determining the amount of each individual ligand that is tolerated without activating NK cells, though few studies have addressed this possibility.

The number of discovered ligands for NKG2D continues to grow in both humans and mice. As of the writing of this dissertation, there are nine known murine ligands (Rae1 α –Rae1 ϵ , H60a–H60c, and Mult1) and nine known human ligands (RAET1a–RAET1g (also known as ULPBs), MICA, and MICB), but due to their relatively rapid rate of evolution no direct lines of homology can be drawn between individual mouse and human ligands (Raulet 2003; Bacon, Eagle et al. 2004; Takada, Yoshida et al. 2008). The existence of so many ligands for a single receptor raises the question why so many are needed. One explanation may be that viral evasion of NKG2D-mediated recognition led to selective pressure for ligand redundancy (Lenac, Arapovic et al. 2008). Alternatively, distinct ligands may have selective tissue distribution and/or be differentially regulated,

providing the system with the capacity of responding to a greater range of disease-induced insults (Groh, Bahram et al. 1996; Whang, Guerra et al. 2009). Alternative modes of regulation could operate at numerous levels, including transcription, translation, or by controlling protein or RNA stability or localization.

Regulation of NKG2D ligands

Expression of NKG2D ligands has been shown to initiate immune responses, particularly cytolytic activity of NK cells. These findings illustrate the need for strict regulatory mechanisms controlling NKG2D ligand expression, assuring that only unwanted cells upregulate the ligands at the cell surface. In agreement with this idea, most normal cells lack ligand expression while many tumor cell lines and primary tumors are positive (Groh, Rhinehart et al. 1999; Cerwenka, Bakker et al. 2000; Diefenbach, Jamieson et al. 2000; Pende, Cantoni et al. 2001; Guerra, Tan et al. 2008). Ligand expression also has been shown to increase during infections with certain pathogens (Groh, Rhinehart et al. 2001; Tieng, Le Bouguenec et al. 2002). These observations led to the idea that ligands are upregulated in response to activation of cellular stress pathways, and this increased expression leads to elimination of the stressed cells by NK cells and in some cases T cells.

Use of the term “stress” to describe the signals leading to induction of NKG2D ligands is not very descriptive due to the wide variety of possible stress pathways. Therefore, much effort has been put toward gaining a more detailed understanding of the specific signals associated with the disease states where NKG2D ligands are induced. Induction of stress pathways in cell culture using cell lines with low levels of NKG2D ligand expression has been an invaluable approach to focus research in this area on specific pathways. In this way, heat shock and the DNA damage pathway have been shown to play a role in regulation of NKG2D ligands.

The first reported pathway leading to increased expression of an NKG2D ligand was the heat shock pathway. Two ligands in humans, MICA and MICB, were shown to be transcriptionally upregulated by heat shock (Groh, Bahram et al. 1996; Venkataraman, Suci et al. 2007). Using reporter assays it was shown that there is a functional heat shock response element (HSRE) in the MICA/B promoter region that most likely controls this induction by heat shock (Venkataraman, Suci et al. 2007). While heat shock was studied as a pathway specific to the regulation of the MICA and MICB ligands, genotoxic stress has been identified as a pathway that induces a broad range of NKG2D ligands (Gasser, Orsulic et al. 2005). In the initial study, various drugs and treatments that activate the DNA damage pathway induced ligands in cultured human and mouse fibroblasts. This induction was found to correlate with an increase in RNA and be dependent on initiating kinases of the DNA damage pathway (ATM, ATR, and chk1), though the factors that act directly on NKG2D ligand gene expression have not been identified. Importantly, the DNA damage pathway was found to also be critical for maintenance of ligand expression in tumor cell lines (Gasser, Orsulic et al. 2005). Subsequently, the DNA damage pathway has been implicated as the root cause of

regulation in other scenarios where NKG2D ligands are induced such as on proliferating T cells and on HIV infected cells (Cerboni, Zingoni et al. 2007; Ward, Davis et al. 2009).

Other pathways have also been suggested to play a role in induction of NKG2D ligands, though the evidence is less solid than that for the heat shock and DNA damage pathways. Toll-like receptor (TLR) signaling in macrophages was suggested to induce Rae1 transcripts as well as surface protein (Hamerman, Ogasawara et al. 2004). Although it is not disputed that transcripts are induced upon lipopolysaccharide (LPS) stimulation through TLR4, protein may not actually be present at the cell surface (D.H. Raulet and A.M. Jamieson, unpublished data). A distinct type of stress was recently shown to act specifically on the epidermally expressed H60c. When keratinocytes are cultured in high oxygen or skin is wounded (causing an increase in oxygen tension among other things), H60c levels were shown to increase (Whang, Guerra et al. 2009). This induction may aid in stimulation of immune cells at the wound site and aid in healing. These results are intriguing, but more research is clearly needed in this area to get a complete picture of the networks regulating NKG2D ligand expression.

Disease related stress

NKG2D ligands can be thought of as signals displayed by diseased cells marking them for elimination by the immune system. Therefore, when the goal is to define the mechanisms of NKG2D ligand regulation, it is helpful to first understand the alterations in cellular physiology associated with diseases where NKG2D plays a role. Implicated diseases are not limited to a specific category, with examples of infections, autoimmune conditions, and cancers activating the immune response through NKG2D (Krmptotic, Busch et al. 2002; Ogasawara, Hamerman et al. 2003; Guerra, Tan et al. 2008). Each of these categories is clearly very broad in scope and NKG2D will certainly not be the major component of the response to every type of infection, autoimmune condition, or cancer. However, the fact remains that examples of specific diseases that induce NKG2D ligands cut across these broad categories, suggesting that general cell stress pathways activated in common among these conditions may play an important role. Alternatively, individual ligands for NKG2D may be uniquely regulated. The latter hypothesis would explain both the large number of ligands present in the genome and the wide range of diseases where NKG2D is involved. In any case, it is helpful to understand the broad range of disease related stresses when considering NKG2D ligand regulation.

Pathways activated during infection

There has been a renaissance in the last several decades of interest in microbial detection by the innate immune system. During this time a multitude of cell surface and cytoplasmic “sensors” (most famously the TLRs) have been identified that recognize the presence of common microbial molecules (Medzhitov 2007). These sensors, or pattern recognition receptors (PRRs), are positioned, both in terms of tissue distribution and localization within cellular compartments that maximize their ability to discriminate dangerous from beneficial microbes. Downstream signaling by these PRRs leads to activation of IRF family transcription factors and/or NF- κ B which upregulate genes involved in microbial defense and initiate inflammation (Kopp and Ghosh 1995; Taniguchi, Ogasawara et al. 2001).

Signaling downstream of PRRs can vary significantly, but the IKK $\alpha\beta\gamma$ complex is a common node for pathways leading to NF- κ B. IKK phosphorylates the inhibitory binding partner of NF- κ B, I κ B, leading to ubiquitination and degradation of I κ B. In the absence of its inhibitor, NF- κ B translocates to the nucleus and activates a plethora of genes including the inflammatory cytokines IL-1, IL-6, and TNF- α (Akira and Takeda 2004). A node for signaling pathways leading to interferon production is the activation of a complex including TBK-1 and IKK ϵ . This complex causes phosphorylation and nuclear localization of the transcription factors IRF3 and IRF7. These transcription factors initiate transcription of the type I interferon genes (several IFN α s, production of which varies by cell type, and the single IFN β) (Perry, Chen et al. 2005).

Signaling of type I interferon through its receptor induces a transcriptional program that varies depending on the cell type. In epithelial cells, which are common

gateways for infection, interferon results in what is commonly referred to as an “anti-viral state”, although it is protective for some species of bacterial pathogens as well (Decker, Muller et al. 2005). Importantly, NK cells and other cells of the innate immune system are activated by type I interferon, leading to protective responses (except in the case of certain bacterial infections including *Listeria monocytogenes* and *Mycobacterium sp.* (Manca, Tsenova et al. 2001; Auerbuch, Brockstedt et al. 2004; Carrero, Calderon et al. 2004; O'Connell, Saha et al. 2004)) and initiation of adaptive immunity.

In addition to signaling pathways initiated by sensors of microbial molecules, there are distinct perturbations to cellular homeostasis caused by pathogenic manipulation that may be linked to activation of the immune system (Lin, Walter et al. 2008; Vance, Isberg et al. 2009). Intracellular bacteria must manipulate a range of host cell components in order to secure themselves a space to grow. These components include cellular membranes (uptake and entry into the cytosol), the actin cytoskeleton (uptake and motility), and multiple metabolic pathways (growth) (Vance, Isberg et al. 2009). Viruses also must perturb host metabolic pathways during infection because they require the host's transcription, processing, and translation machinery for propagation. In fact, viruses commonly shut down host protein synthesis (Schneider and Shenk 1987). This benefits the virus both by freeing up translational machinery for its own use as well as preventing anti-viral gene expression. Inhibition of host protein synthesis also appears to be a bacterial pathogenesis strategy (McCusker, Braaten et al. 1991), suggesting that this could be a more general signature of infection and also indicating the usefulness of post-translational immuno-stimulatory mechanisms.

The domination of the host machinery by viruses is often so successful that production of viral coat glycoproteins exceeds the capacity of ER chaperones in the secretory pathway triggering the unfolded protein response (Lin, Walter et al. 2008). This response leads to both inhibition of general translation (which viruses themselves are able to avoid) and increased expression of chaperones. Intriguingly, the unfolded protein response is also becoming appreciated as a pathway that is capable of promoting an inflammatory response (Lin, Walter et al. 2008). The coupling of established sensors or novel signaling components to pathogen-associated disturbances in cellular homeostasis may be essential for distinguishing pathogens from beneficial microbes or endogenous PRR ligands (e.g. nucleic acids) thus maximizing the ratio of beneficial to harmful immune response.

Pathways activated during tumorigenesis

The immune response to a tumor differs from that to a pathogen in that there is no overtly foreign substance against which to respond. Also problematic for the strategy of tumor immunosurveillance is the fact that tumors vary greatly in the sets of changes that lead to their formation. There are, however, defining characteristics that are required for the progression from normal cells to abnormal growth to cancer (Hanahan and Weinberg 2000). The cellular alterations that lead to some of these hallmarks of cancer may also be linked to activation of NK cells and the immune system as a whole. This would allow selective activation in response to abnormal and potentially dangerous cells.

The number of mutations required for progression from normal cell to tumor cell is not consistent with the mutation rate seen in normal cells. To explain how a cancer is able to develop within the human lifespan, it has been suggested that there first must be inactivation of the repair machinery or chromosomal maintenance machinery in normal cells, thus leading to a “mutator phenotype” (Lengauer, Kinzler et al. 1998). This can be thought of as an initiating event that leads to an exponentially faster progression towards cancer. Another pathway that must be disrupted at an early stage of tumorigenesis is the DNA damage pathway. Of particular importance is the famous tumor suppressor p53, which is mutated in over 50 percent of human cancers (Levine 1997). The DNA damage pathway is initiated by sensors of damage including the PI3 kinase-like kinase (PIKK) family members ATM and ATR, amplified by the signal transducers Chk1 and Chk2, and carried out by the effectors p53 and cdc25 (Sancar, Lindsey-Boltz et al. 2004). The importance of a functional DNA damage pathway for the prevention of cancer is strikingly exemplified by the number of heritable mutations in proteins within the pathway that lead to cancer predisposition syndromes (Srivastava, Zou et al. 1990; Savitsky, Bar-Shira et al. 1995; Bell, Varley et al. 1999; O'Driscoll, Ruiz-Perez et al. 2003). The DNA damage pathway protects against cancer by causing cell cycle arrest and/or apoptosis when genomic integrity is compromised (Sancar, Lindsey-Boltz et al. 2004). Thus, cells that are prone to becoming cancer cells are prevented from doing so.

The DNA damage pathway has recently been linked to anti-tumor responses by immune system. As I already mentioned, induction of NKG2D ligands is linked to the DNA damage response (Gasser, Orsulic et al. 2005). Also, study of p53 reactivation in a model of liver cancer showed that the tumor regression after p53 reactivation is accelerated by innate immune cells including NK cells (Xue, Zender et al. 2007). This suggests that, in addition to inducing apoptosis, the DNA damage response can mark genetically unstable cells for elimination by the immune system.

Other evidence for immunosurveillance by the immune system comes from studies of pre-cancerous senescent cells. Senescence is a term used to describe irreversible exit from cell cycle in response to stress including DNA damage (Xue, Zender et al. 2007). Studies have shown that the DNA damage pathway is in fact active in precancerous lesions, perhaps preventing their progression to malignancy (Bartkova, Horejsi et al. 2005; Gorgoulis, Vassiliou et al. 2005). In addition to replicative blockage, DNA damage pathway signaling in senescent cells leads to upregulation of inflammatory mediators, particularly IL-6 (Rodier, Coppe et al. 2009). These signals may attract and activate immune cells to clear the senescent cells, thereby preventing a potential tumor from forming. Evidence for a role of NK cells in particular comes from a study of senescent stellate cells in the liver. Microarray analysis showed that these senescent cells upregulated ligands for NKG2D, and that depletion of NK cells with antibody specific for asialo-GM1 (which also depletes other immune cell subsets) led to a reduction in the rate of stellate cell clearance (Krizhanovsky, Yon et al. 2008). These observations in liver stellate cells may have relevance to senescence in general, but additional studies will need to be undertaken to determine if this response is more generally applicable.

Another type of stress that is a unifying theme between the diverse forms of cancer is stress related to improper protein folding. The increased metabolic output required to support rapidly proliferating cancer cells places a burden on the protein folding machinery. This burden leads to a response commonly referred to as the heat

shock response (Hendrick and Hartl 1993). The major consequence of the heat shock response is increased production of heat shock proteins (HSPs, most of which are protein folding chaperones or cofactors), and if the burden of unfolded proteins cannot be resolved, the cell will undergo apoptosis. Since the heat shock proteins facilitate an increased metabolic output and also protect against apoptosis, it is not surprising that many of these proteins are also overexpressed in cancer cells (Jolly and Morimoto 2000; Whitesell and Lindquist 2005).

In addition to facilitating of the folding of normal proteins, HSPs can also promote the stability of otherwise labile oncogenic mutant proteins. The first example of this activity was the stabilization of oncogenic SRC by HSP90. Mutations to SRC that confer transforming activity prevent the interaction of the c-terminal regulatory domain with its SH2 domain, permanently activating it but also making it less stable. HSP90 was shown to be important for stabilizing the mutant SRC as well as other mutant proteins common in tumors including mutant p53 (Whitesell and Lindquist 2005). The most convincing evidence that the heat shock pathway is involved in cancer is provided by studies of the heat shock factor 1 (HSF1) knockout mouse. HSF1 is a central transcription factor controlling the induction of HSPs in response to protein folding stress. In both genetic and chemically induced models of tumorigenesis, there was found to be a reduced tumor burden in the absence of HSF1. Underlying the role of HSF1 in tumorigenesis, its transcriptional programs were found to alter a range of metabolic processes that allow increased proliferation and survival (Dai, Whitesell et al. 2007).

Post-translational regulatory mechanisms

As mentioned during the discussion of pathways activated during infection, pathogens commonly inhibit host gene expression. When considering the stimulation of an immune response through induction of NKG2D ligands, this is clearly a block that must be overcome if the ligands are to be induced on infected cells. Intriguingly, post-transcriptional regulation is likely to exist for several ligands, based on findings that cell surface expression of ligands in certain cells often does not correlate with the amounts of the corresponding transcripts (Cosman, Mullberg et al. 2001; Carayannopoulos, Naidenko et al. 2002; Radosavljevic, Cuillerier et al. 2002; Diefenbach, Hsia et al. 2003). Since normal cells express little or no ligand at the cell surface, these transcripts must be either translationally inactive or the encoded protein must be prevented from accumulating at the cell surface. This dissertation concerns regulation of a NKG2D ligand at the protein level, so this section will focus on only post-translational mechanisms of gene regulation.

Even if a transcript is being actively translated into protein, there are multiple ways in which the protein itself can be regulated. Post-translational mechanisms can be roughly divided into two classes: those that regulate the amount of protein present in the cell and those that prevent the protein that is present from functioning. The specific possibilities will vary depending on the particular protein in question. Since NKG2D ligands are transmembrane proteins, only post-translational mechanisms that act within the secretory pathway have the potential to regulate their expression.

Turnover of transmembrane proteins

Turnover of cytosolic proteins is carried out by ubiquitin-directed targeting to the proteasome, where degradation occurs. Since the proteasome is located in the cytosol, it was somewhat surprising when the discovery was made that misfolded proteins in the ER can also be degraded by the proteasome (Jensen, Loo et al. 1995; Ward, Omura et al. 1995). This process is called ER-associated degradation (ERAD), and involves ubiquitination, deglycosylation, retrotranslocation, and degradation by proteasomes (not necessarily in this order) (Romisch 2005). The major role of ERAD seems to be in the removal of misfolded proteins in general. There are, however, examples showing that the ERAD machinery can be more selectively involved in regulated turnover of specific proteins. The first example of a protein specifically regulated by ERAD was HMG-coA reductase (HMGR), an enzyme in the mevalonate pathway that produces sterols and isoprenoids. HMGR stability was shown to be selectively regulated by a feedback mechanism sensitive to perturbations in the mevalonate pathway (Hampton and Rine 1994). A well studied example of selective ERAD targeting that has relevance to antiviral immunity is the HCMV proteins, US11 and US2, which are capable of selectively targeting MHC class I for ERAD (Wiertz, Jones et al. 1996). These examples show that proteins can be targeted for destruction by ERAD in a dynamic manner that does not necessarily require misfolding. A possible mechanism for selective inhibition of ERAD

was suggested in a recent study characterizing a novel ER-associated deubiquitinase. This enzyme, USP19, appears to be regulated by the unfolded protein response (UPR) and shows some target selectivity (Hassink, Zhao et al. 2009). There is, in fact, a coordination between ERAD and the UPR showing that ERAD can be regulated under stressful conditions such as insufficient folding capacity in the ER (Travers, Patil et al. 2000).

Transmembrane proteins destined for the cell surface leave the ER and travel through the golgi *en route* to their final destination. Once proteins proceed past the golgi apparatus, however, ERAD is no longer possible. Nonetheless, ubiquitination of mature transmembrane proteins also leads to their degradation. The degradation of transmembrane proteins at this late stage in their biogenesis is proteasome-independent and instead occurs in the lysosome. This was first shown in yeast, where ubiquitination of a GPCR, upon binding of its ligand, led to internalization and degradation by the vacuole/lysosome (Hicke and Riezman 1996). This pathway was extended to mammalian cells through study of the EGF receptor (Felder, Miller et al. 1990) and receptor tyrosine kinases in general. Upon activation and transphosphorylation, RTKs are ubiquitinated by the E3 ubiquitin ligase Cbl and internalized (Joazeiro, Wing et al. 1999). Internalization is not entirely dependent on ubiquitination, but targeting to the lysosome and degradation is. In this way, the receptor is specifically downregulated upon its activation as a mechanism for controlling the duration of the signal.

The decision whether to recycle an endocytosed transmembrane protein or send it to the lysosome occurs in structures called multivesicular bodies (MVBs, also sometimes referred to as late endosomes). The default pathway is recycling to the cell surface, but ubiquitination redirects proteins into luminal MVB vesicles and subsequently the lysosome (Katzmann, Odorizzi et al. 2002). Some proteins in fact may never make it to the cell surface with the default pathway being ubiquitination and trafficking to the lysosome. This is true of the amino acid permease, Gap1, whose ubiquitination is decreased and cell surface expression is induced during growth in the presence of a poor source of nitrogen (Helliwell, Losko et al. 2001). A similar pathway controls MHC class II expression in antigen presenting cells. MHC II is ubiquitinated in resting cells, leading to low levels of protein with a predominantly intracellular localization. Upon activation with LPS, MHC II ubiquitination is reduced and it accumulates at the cell surface (Shin, Ebersold et al. 2006). In general, even proteins that have been ubiquitinated and sent to the MVB can be deubiquitinated by endosomally localized ubiquitin-specific proteases and allowed back into circulation (Katzmann, Odorizzi et al. 2002). This deubiquitination pathway and the other pathways regulating traffic to the lysosome collectively make regulated turnover of transmembrane proteins possible.

Localization of transmembrane proteins

Ubiquitination and other endocytic signals at the plasma membrane lead to a reduction in the amount of cell surface protein. In some cases, degradation of these proteins is not necessary to achieve the desired regulatory effect. Controlling the location of a stable pool of proteins within the cell is also a common mechanism of post-translational regulation, especially when extremely rapid results are required. The glucose

transporter, GLUT4, is rapidly induced in muscle and adipose tissue when metabolic demands increase (e.g. during exercise). Under resting conditions, GLUT4 is present in a combination of endosomes, the trans-golgi network, and specialized storage vesicles. Upon insulin stimulation, GLUT4 containing vesicles rapidly fuse with the plasma membrane, thus allowing increased glucose uptake to meet cellular demands (Bryant, Govers et al. 2002). An example of regulation by protein sequestration at an earlier stage of the secretory pathway is provided by ATF6, a critical unfolded protein response mediator. It is retained in a functionally inactive state within the endoplasmic reticulum by the chaperone BiP. Upon an increase in ER misfolding, ATF6 is released by BiP, travels to the golgi apparatus and is cleaved by site-1 protease (S1P). This cleavage releases a transcription factor encoded by the ATF6 cytoplasmic domain that travels to the nucleus and activates transcription of UPR genes (Shen, Chen et al. 2002). There are many possible variations on these two examples of regulated sequestration, and some proteins may be regulated by a combination of the mechanisms discussed in this section.

For the most part, post-translational mechanisms have not been documented in the regulation of NKG2D ligands. The one exception is the proteolytic cleavage of MICA and MICB, which releases the extracellular domain as a soluble fragment (Groh, Wu et al. 2002). It is postulated that this shedding is an escape mechanism used by tumor cells, but how and if this process is actually regulated are only beginning to be determined (Kaiser, Yim et al. 2007; Waldhauer, Goehlsdorf et al. 2008; Boutet, Aguera-Gonzalez et al. 2009). The relative lack of knowledge regarding NKG2D ligand regulation at the protein level, as well as the aforementioned evidence for regulation of several ligands at a late stage of expression, initiated the body of work described in this dissertation.

Chapter 2: Methodology

Procedures

Cells, antibodies, and reagents

Mouse secondary fibroblasts were prepared as described (Gasser, Orsulic et al. 2005). All cells were cultured in complete DMEM, consisting of Dulbecco's modified Eagle medium (Gibco), 10% fetal calf serum (Omega Scientific), 100 U/ml penicillin (Gibco), 100 µg/ml streptomycin (Gibco), 0.2 mg/ml glutamine (Sigma), 10 µg/ml gentamycin sulfate (Lonza), 20 mM Hepes (Fisher), and 50 µM 2-mercaptoethanol (EMD biosciences).

Monoclonal Mult1 antibody (R+D Systems, clone 237104), monoclonal H60a antibody (R+D Systems, clone 205326), monoclonal pan-Rae1 antibody (R+D Systems, clone MAB17582), PE conjugated goat F(ab')₂ fragment to rat IgG (Jackson ImmunoResearch), PE conjugated goat antibody to mouse IgG1 (Southern Biotech), PE conjugated MHC-I (D^b) antibody (ebioscience, clone 28-14-8), PE conjugated K^b antibody (ebioscience, clone AF6-88.5), and PE conjugated ICAM-1 (ebioscience, clone YN1/1.7.4) were used for flow cytometric analysis. Monoclonal FLAG antibody (Stratagene, clone M2) was used for flow cytometry and western blotting. Monoclonal Mult1 antibody (clone 1D6, kind gift of S. Jonjic (Krmptic, Hasan et al. 2005)) was used to detect Mult1 by immunofluorescence and for immunoprecipitations. Monoclonal ubiquitin antibody (clone P4D1, Santa Cruz), monoclonal α-tubulin antibody (Calbiochem) horseradish peroxidase-conjugated goat anti-mouse IgG (Pierce), and peroxidase-conjugated goat anti-mouse light chain (Jackson ImmunoResearch) were used for western blots. NKG2D tetramer was prepared as previously described (Diefenbach, Jamieson et al. 2000).

Mitomycin C (M-4287), *cis*-diammineplatinum(II) dichloride (P4394), 5-fluorouracil (F6627), caffeine (C0750), chloroquine (C6628), and MG132 (C2211) were purchased from Sigma. SB218078 (559402) was purchased from Calbiochem.

UV and heat shock treatments

Prior to UV irradiation, culture dishes of adherent cells were washed in PBS, and the PBS was completely removed by aspiration. Suspension cells were spun down onto culture dishes and media was completely removed. Cells were exposed to UV-C light in a UV stratalinker (Stratagene), the media was replaced, and cells were cultured for the indicated time. Confluent or sub-confluent cultures were heat shocked by incubation in a waterbath or incubator set to 45 degrees Celsius for 30 minutes followed by subsequent incubation at 37 degrees Celsius for the indicated time.

Quantitative RT-PCR

Total RNA was isolated using Trizol LS (Invitrogen) followed by digestion of contaminating DNA using DNA-free (Ambion) according to manufacture's protocol. RNA was reverse transcribed using Superscript III reverse transcriptase (Invitrogen), and the resulting cDNA was used for qPCR. Duplicate or triplicate amplification mixtures were prepared with 0.01-1 µg cDNA, SYBR GreenER SuperMix (Invitrogen), and 200 nM forward and reverse primers, and cycled using the ABI 7300 Real-Time PCR system. Cycling parameters used were 50 °C for 2 minutes, 95 °C for 10 minutes, and 40 cycles

of 95 °C for 15 seconds and 60 °C for 60 seconds. The following primers were used: Mult1-5', 5'-CAATGTCTCTGTCCTCGGAA-3'; Mult1-3', 5'-CTGAACACGTCTCAGGCACT-3'; GAPDH-5', 5'-GAAGGTCGGTGTGAACGGA-3'; GAPDH-3', 5'-GTTAGTGGGGTCTCGCTCCT-3'; 18s-5', 5'-GTCTGTGATGCCCTTAGATG-3'; 18s-3', 5'-AGCTTATGACCCGCACTTAC-3'; MARCH-4-5', 5'-CTCTGGGAAGTAGCTTGGAC-3'; MARCH-4-3', 5'-CTGAACCATCACAACGGCATG-3'; MARCH-9-5', 5'-TCATGGAGCTGTGAGCTTTG-3'; MARCH-9-3', 5'-CAGGACGATAGCAGCAATCT-3'; RPS29-5', 5'-AGCAGCTCTACTGGAGTCACC-3'; RPS29-3', 5'-AGGTCGCTTAGTCCAACTTAATG-3'.

Retroviral constructs and transduction of cells

Mult1 and H60c were Flag-tagged by inserting open reading frames without signal peptide coding sequence adjacent to CD8 signal peptide and FLAG tag coding sequences. H60a and Flag-tagged Mult1 and H60c were subcloned into the pMSCV2.2-IRES-GFP retroviral vector (Ranganath, Ouyang et al. 1998) using XhoI and NotI restriction sites. Myc-tagged MARCH-4 and MARCH-9 were subcloned into pMSCV-IRES-Thy1.1 using NotI and PmeI restriction sites. Deletion mutants of Mult1 were made using the following reverse primers: Δ239, 5'-

CCAAAGCGGCCGCTCAACGTAAATGTCTTAATTTACAAGAGAC-3'; Δ253, 5'- CCAAAGCGGCCGCTCCAAGAGAGGTGGTGGTGAGGACTGCA-3'; Δ298, 5'-CCAAAGCGGCCGCTCACAACAGAGGTGGTGCTGAGGACTGCA-3'. The H60a-Mult1 chimeras were constructed using the following primers: H60a EC reverse, 5'-CCAAAGCGGCCGCCAGGTGACACTCAGACCCT-3'; Mult1 TM forward, 5'-CCAAAGCGGCCGCTTCTTTTCAGTACCTTGATGATTATTCTA-3'; H60a forward, 5'-ATCTCTCGAGCCACCATGGCAAAGGGAGCCACCAGCA-3'; H60a TM reverse, 5'-CCAAAGCGGCCGCCAGCATAACCAAGCGAATACCATGA-3'; Mult1 tail forward, 5'-CCAAAGCGGCCGCTTGTAATTAAGACATTTACGTACAAA-3'.

Lysine to arginine mutations were made using the QuikChange protocol (Stratagene) and the following primers: KK310RR(+), 5'-GTGCCTGAGACGTGTTTCAGTACGGCGAGAAGATGAATTTCCGACAG-3'; KK310RR(-), 5'-CTGTCCGAAATTCATCTTCTCGCCGTACTGAACACGTCTCAGGCAC-3'; KLK278RLR(+), 5'-GAATGTCTCTGTCCTCGGCGGTTGCGGTCACCTGTGTTTATGCAG-3'; KLK278RLR(-), 5'-CTGCATAAACACAGGTGACCGCAACCGCCGAGGACAGAGACATTC-3'; KK240RR(+), 5'-AATTAAGACATTTACGTACACGACGGATTGGCCTGCAGTCCTCACC-3'; KK240RR(-), 5'-GGTGAGGACTGCAGGCCAATCCGTCGTGTACGTAAATGTCTTAATT-3'.

Retroviral supernatants were generated by co-transfecting 293T cells with plasmids encoding VSV gag/pol and env and pMSCV2.2 retroviral constructs

using Lipofectamine 2000 (invitrogen). Culture supernatants collected 48 hours after transfection were added directly to actively proliferating fibroblasts and transduced cells were sorted based on GFP expression.

Immunofluorescence microscopy

Cells were allowed to adhere to coverslips in tissue culture dishes or to tissue culture treated glass slides. Cells were fixed in 4% paraformaldehyde in PBS for 10 minutes, rinsed in PBS, and permeabilized in 0.2% triton X-100 for 6 minutes. After blocking in PBS with 5% BSA, cells were incubated with Mult1 antibody (clone 1D6, culture supernatant) diluted 1 in 10 in PBS with 5% BSA for 1 hour. Unbound primary antibody was washed out by rinsing 3 times in PBS. As indicated in figure legend, PE or Alexa Fluor 555 conjugated goat anti-rat F(ab')₂ was diluted 1 in 100 in PBS with 5% BSA and added to cells for 1 hour. Unbound secondary antibody was washed out by rinsing 3 times in PBS. Coverslips were mounted on slides with vectashield with DAPI (Vector Labs) and fluorescent signal was visualized using a Nikon eclipse E800 microscope.

Metabolic labeling and immunoprecipitation

WEHI 7.1 cells were starved of methionine and cysteine for 30 minutes in 3 ml methionine and cysteine-free complete DMEM supplemented with 10% dialyzed FCS (Invitrogen). Cells were then labeled with 0.2 mCi/ml Easy Tag EXPRESS ³⁵S protein labeling mix, (Perkin Elmer) for 30 minutes followed by addition of 15 ml DMEM containing a 10-fold molar excess of cold methionine and cysteine. At each time point, 3 ml of media was removed and cells were washed with 10 ml cold PBS before immunoprecipitation.

Lysates were prepared by resuspending cell pellets in 1 ml of either TNT lysis buffer (1% Triton X-100, 50 mM Tris pH 7.4, 300 mM NaCl), RIPA lysis buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 50 mM Tris pH 7.4, 150 mM NaCl), or co-immunoprecipitation buffer (0.5% NP-40, 150 mM NaCl, 20 mM Tris pH 7.4, 10% glycerol) as indicated in Figure Legends. All buffers contained protease inhibitor cocktail tablets (Roche) added immediately before use. When assessing ubiquitination of Mult1, 20 mM N-ethylmaleimide was included in the lysis buffer. When scraping cells into RIPA lysis buffer, DNA was sheared by forcing lysates through 25g needles. Cells were lysed on ice for 30 minutes and cleared by centrifugation at 16,000xg for 15 minutes. Lysates were pre-cleared twice with protein G PLUS agarose beads (Santa Cruz) bound with rat IgG2a isotype control antibodies (eBioscience) and once with uncoupled protein G agarose beads. Pre-cleared lysates were incubated with 1 µg Mult1 antibody (1D6) overnight followed by protein G agarose beads for 2 hours. Beads were washed three times in lysis buffer and once in TBS. Immunoprecipitates were boiled in SDS sample buffer and resolved by SDS-PAGE. For metabolic labeling experiments, gels were dried, exposed to phosphorimager cassettes (Amersham), and imaged on Amersham's Typhoon imager.

Western Blotting and quantitation

Immunoprecipitates were boiled in SDS sample buffer, resolved by SDS-PAGE, and transferred to nitrocellulose membranes. Membranes were blocked in 5% milk and

incubated with primary antibodies followed by horseradish peroxidase-coupled secondary antibodies. Following incubation with western lightning chemiluminescence reagent (Perkin Elmer), membranes were exposed to film and developed. Film images were scanned and analyzed using ImageJ software (NIH). For quantitation of high molecular weight ubiquitin-Mult1 conjugates, the signal above background of species larger than the size of unconjugated Mult1 (~55 kDa) was measured.

Knockdown of MARCH-9

Relative levels of MARCH-4 and MARCH-9 were determined using the primers MARCH-4+9-5' (5'-CAGTGGCAGGCCAT-3') and MARCH-4+9-3'(5'-ACTTTCCACTGCTGGTT-3') followed by digestion with Sall which selectively digests MARCH-4 amplicons.

50,000 secondary fibroblasts were transfected with 100 pmol siRNA oligo using Lipofectamine 2000. The following oligos were used: GFP-22 (Qiagen), control oligo (Invitrogen), and three pre-designed MARCH-9 oligos (Invitrogen). 72 hours later cells were lifted and used for analysis of Mult1 surface expression and MARCH-9 quantitative PCR.

Chapter 3: Ubiquitination and degradation of Mult1

Expression of Mult1

Analysis of Mult1 transcript tissue distribution

In an analysis of Mult1 transcript levels in various tissues, it was observed that Mult1 transcript levels are high in several tissues, including the thymus, relative to the Mult1 negative tumor cell line RMA. Mult1 RNA levels in the thymus approached the level of the Mult1-expressing tumor cell lines YAC-1 and WEHI 7.1 (Fig. 1-1). In contrast, cell surface expression was very low on thymocytes but substantial on YAC-1 (Fig. 1-1). These data suggested that Mult1 cell surface expression is controlled post-transcriptionally, either at the translational or protein level.

Ectopic expression of Mult1

To test for protein level regulation, the open reading frames of Mult1, or of a distantly related NKG2D ligand, H60c (Takada, Yoshida et al. 2008), were N-terminally FLAG tagged and cloned into a retroviral expression vector in the absence of their untranslated regions, which might contain *cis*-acting elements that regulate mRNA stability. The vector used for this analysis encoded a downstream IRES-GFP cassette, such that GFP expression could be used as a marker to indicate that cells had been successfully transduced and expressed the bicistronic Mult1-GFP mRNA (Fig. 1-2). Fibroblasts established from ear tissues of adult mice cultured after the crisis period were used as representative non-transformed cells with low basal Mult1 expression (Gasser, Orsulic et al. 2005). Cells transduced with Mult1 or H60c exhibited a similar intensity of GFP staining, indicating comparable levels of the corresponding bicistronic-transcripts. When GFP-positive cells were analyzed for cell surface protein expression by FLAG staining, however, it was clear that cell surface expression of Mult1 was severely reduced compared to that of H60c (Fig. 1-2). Because the Mult1 coding sequences are included on the same transcript as the abundantly expressed GFP, these data demonstrated that Mult1 cell surface expression is under post-transcriptional control.

Figure 1-1. Mult1 transcript doesn't correlate with surface expression

A. Quantitative RT-PCR for Mult1 was performed on cDNA prepared from the indicated tissues and cell lines. The relative amount of Mult1 RNA was determined by normalizing to GAPDH transcript levels. Data shown is mean $\pm$ SD, n=3. B. Thymocytes and YAC-1 cells were stained with Mult1 antibody and analyzed by flow cytometry. Data in this figure is representative of at least three independent experiments.

A.

B.

Figure 1-2. Mult1 protein is inefficiently expressed

A. Schematic of retroviral vector. LTR=long terminal repeat, SS=signal sequence, ORF=open reading frame, IRES=internal ribosome entry site. B. Fibroblasts were transduced with a retroviral vector encoding the indicated FLAG-tagged protein and stained with FLAG antibody for analysis of gated GFP⁺ cells by flow cytometry. Data in this figure is representative of at least three independent experiments.

Role of the Mult1 cytoplasmic domain

Mult1 cytoplasmic domain deletion mutants

Mult1, unlike H60c or the other murine ligands, has a large cytoplasmic tail (Fig. 1-3). Because cytoplasmic tails often contain motifs that regulate protein stability or localization (Coscoy, Sanchez et al. 2001; Shin, Ebersold et al. 2006), we tested the role of the Mult1 cytoplasmic tail in regulating cell surface expression. Deletion of amino acids 298-334 had no effect on Mult1 expression (not shown) and deletion of amino acids 253-334, comprising most of the cytoplasmic tail, resulted in only a slight increase ($\Delta 253$ mutant, Fig. 1-4). However, deleting amino acids 239-334, comprising nearly the entire cytoplasmic tail, resulted in greatly increased expression of the transduced Mult1 protein at the cell surface ($\Delta 239$ mutant, Fig. 1-4). When staining with Mult1 antibody, relatively low basal expression of endogenous Mult1 was detected on cells transduced with the control vector, but greatly increased staining was evident on cells transduced with the $\Delta 239$ mutant, compared to cells transduced with wildtype Mult1 (Fig. 1-4, right). These data indicated that amino acids in the interval of 239-253 in the cytoplasmic tail were capable of preventing cell surface expression of Mult1, but it remained possible that parts of the protein other than the cytoplasmic tail also play a role.

Mult1 transmembrane and cytoplasmic regions

Figure 1-3. Mult1 transmembrane and cytoplasmic regions

Amino acid sequence of the Mult1 transmembrane (underlined) and cytoplasmic domains with lysines shown in bold. The boxes indicate duplicated amino acid sequences within the cytoplasmic tail, and arrows show the location of the C-termini of the various deletion mutants.

Figure 1-4. Mult1 cytoplasmic domain is required for downregulation

Fibroblasts were transduced using the same approach as in Fig. 1-2 with WT Mult1 or Mult1 truncation mutants and stained with the indicated antibodies. Histograms were gated on cells with similar GFP expression. Data in this figure is representative of at least three independent experiments.

H60-Mult1 chimera

To further test the role of the cytoplasmic tail in preventing cell surface expression, fibroblasts were transduced with vectors encoding chimeric proteins between Mult1 and the heterologous NKG2D ligand, H60a. Replacing the cytoplasmic tail of H60a with that of Mult1 resulted in a small (about two-fold) reduction in H60a staining (Fig. 1-5, IC tail only). However, when both the transmembrane and cytoplasmic domains of H60a were replaced with the corresponding Mult1 domains, cell surface expression of the resulting chimeric protein was five-fold lower than wildtype H60a (Fig. 1-5, TM+IC). Taken together, these data showed that the Mult1 cytoplasmic tail was necessary but not sufficient for appreciable suppression of cell surface expression, which required sequences in both the cytoplasmic tail and transmembrane domain of the protein.

Lysine residues are required for Mult1 degradation

The reduced cell surface expression of Mult1 dependent on its cytoplasmic tail could reflect the degradation of Mult1, or the retention of the intact protein intracellularly. To determine whether Mult1 was being sequestered in an intracellular compartment, transduced cells were permeabilized and stained with Mult1 antibody. Immunofluorescence microscopy revealed that whereas the $\Delta 239$ deletion mutant protein was readily detectable in transduced cells, the intact Mult1 protein was not, arguing that the full length protein is subject to degradation, as opposed to simply being retained intracellularly (Fig. 1-6).

We examined the possibility that intact Mult1 is regulated by a common pathway for transmembrane protein turnover, which depends on ubiquitination of the protein, usually on lysine residues, and subsequent degradation (Bonifacino and Traub 2003; Hicke and Dunn 2003). A mutant Mult1 protein in which the six lysine residues in the Mult1 cytoplasmic tail were mutated to arginines was prepared to test the importance of these residues. Substitution of the six lysines in the cytoplasmic tail of Mult1 resulted in highly elevated cell surface expression, indistinguishable from that obtained with a cytoplasmic tail deletion (Fig. 1-7). These data strongly suggested that Mult1 expression is regulated by ubiquitination.

Figure 1-5. Mult1 transmembrane and cytoplasmic domains are sufficient for downregulation

Fibroblasts were transduced using the same approach as in Fig. 1-2 with chimeras consisting of the H60a extracellular domain linked to the TM (transmembrane) and IC (intracellular) domains of Mult1 (TM+IC), or to the H60a TM domain and the Mult1 IC domain (IC tail only), and stained with the indicated antibodies. Histograms were gated on cells with similar GFP expression. Data in this figure is representative of at least three independent experiments.

Figure 1-6. WT Mult1 is not sequestered intracellularly

Fibroblasts were grown on cover slips followed by fixation, permeabilization, and staining with Mult1 antibodies. Scale bar is 10μm. Data in this figure is representative of at least three independent experiments.

Figure 1-7. Lysines in Mult1 cytoplasmic domain are required for downregulation

Fibroblasts were transduced using the same approach as in Fig. 1-2 with WT Mult1, Mult1 truncation or KR mutants and stained with the indicated antibodies. Histograms were gated on cells with similar GFP expression. Data in this figure is representative of at least three independent experiments.

Mechanism of Mult1 degradation

Ubiquitination of Mult1

To directly test whether Mult1 is ubiquitinated, Mult1 was immunoprecipitated from Mult1-transduced cells and western blotted with ubiquitin antibodies. FLAG antibody immunoprecipitation proved to be unsuitable for the analysis due to a relatively poor signal to noise ratio and low levels of Mult1-ubiquitin conjugates. Therefore, Mult1 antibodies were used for the immunoprecipitation. High molecular weight ubiquitin species, suggestive of polyubiquitination, were clearly more abundant in immunoprecipitates from cells transduced with wildtype Mult1 than in immunoprecipitates from cells transduced with empty vector (Fig. 1-8). The low level of ubiquitinated Mult1 present in cells transduced with empty vector most likely corresponded to the basal level of endogenous Mult1 present in the cells used for transduction (Fig. 1-4, right panel). The near absence of bands in the control antibody precipitates confirmed the identity of the ubiquitinated species as Mult1 (Fig. 1-8). Cells transduced with the K to R Mult1 mutant exhibited a dramatic increase in Mult1 protein levels compared to the wildtype, as revealed by the anti-FLAG Western blot (Fig. 1-8, bottom), but the levels of polyubiquitinated Mult1 were not increased compared to cells receiving the empty vector (Fig. 1-8). Hence, polyubiquitination of Mult1 was dependent on the lysines in the cytoplasmic tail. Furthermore, the much reduced amount of FLAG-tagged Mult1 in cells transduced with wildtype Mult1, as compared to K to R substituted Mult1, confirms that ubiquitination is associated with dramatically reduced amounts of the protein in transduced cells, suggesting that ubiquitination leads to protein degradation.

Polyubiquitinated transmembrane proteins may be targeted for degradation via the proteasome (ERAD) or lysosome (Bonifacino and Traub 2003; Hicke and Dunn 2003). MG132 inhibits proteasomal degradation, but as this results in depletion of free ubiquitin pools (Schubert, Ott et al. 2000), ubiquitin-dependent targeting to the lysosome may also be inhibited by MG132. MG132 treatment of WT Mult1-transduced cells led to significantly increased staining of the transduced Mult1 at the cell surface, confirming the role of a ubiquitin-dependent process (either proteasomal or lysosomal) in degrading WT Mult1 (Fig. 1-9). MG132 had little effect on the already high surface expression of the KR mutant Mult1 (Fig. 1-9). Treatment with chloroquine, which specifically inhibits lysosomal degradation without effecting proteasomal degradation, also led to an increase in WT Mult1 with little effect on KR Mult1 expression (Fig. 1-9), indicating that most WT Mult1 is degraded in the lysosome.

Localization of Mult1

Immunofluorescence microscopy of WT Mult1-transduced cells treated with either MG132 or chloroquine showed that the accumulation of Mult1 resulted in a

punctate localization, perhaps representing accumulation within the lysosome (Fig. 1-10). Mult1 accumulation also occurred in untransduced cells, albeit to a lesser extent, indicating that endogenous Mult1 is similarly affected by chloroquine or MG132 treatments. As expected, KR mutant Mult1 was detectable in untreated cells, and was not much affected by the inhibitors. This data is consistent with degradation of WT Mult1 predominantly in the lysosome.

To determine if, in fact, the punctate structures were lysosomes, WT Mult1 transduced cells were treated with MG132 or chloroquine followed by staining with LAMP1 (a resident protein of late endosomes and lysosomes) and Mult1 antibodies. The Mult1 that accumulated with either treatment strikingly co-localized with LAMP1 indicating that the compartments were lysosomes (Fig. 1-11). Very little Mult1 was detected in untreated cells, but most of the Mult1 signal was punctate and localized to LAMP1-positive vesicles (Fig. 1-11). This suggests that, under normal conditions, WT Mult1 also is targeted to the lysosome for degradation.

The effect seen with chloroquine treatment is not surprising since it inhibits degradation within lysosomes, but is not clear why proteasome inhibition also led to lysosomal accumulation since there is not an established connection between the proteasome and lysosomal degradation. It is possible that both proteasome-dependent and lysosome-dependent pathways regulate Mult1, and the accumulation seen with proteasome inhibition in these cells reflects an increase in total protein rather than a defect in lysosomal degradation. Even if this is the case, these experiments clearly show that a significant portion of Mult1 degradation occurs in the lysosome.

Figure 1-8. Mult1 is polyubiquitinated

Fibroblasts transduced with FLAG-tagged Mult1 were lysed in RIPA buffer and Mult1 was immunoprecipitated with Mult1 or control antibody followed by Western blotting with either ubiquitin or FLAG antibodies. Iso. = isotype control, Vec. = empty vector transduced, WT = WT Mult1 transduced, and KR = Lys to Arg mutant Mult1 transduced. Data in this figure is representative of at least four independent experiments.

Figure 1-9. Inhibitors of both proteasomal and lysosomal degradation induce WT Mult1

Untransduced, WT Mult1 transduced, or KR Mult1 transduced fibroblasts were treated with either DMSO, 20uM MG132, or 100uM chloroquine for 6hr followed by staining with FLAG antibody and analysis by flow cytometry. Data in this figure is representative of at least four independent experiments.

Figure 1-10. Mult1 accumulates in a punctate pattern

Untransduced, WT Mult1 transduced or KR Mult1 transduced fibroblasts were grown on tissue culture-treated glass slides followed by treatment with DMSO, 20uM MG132, or 100uM chloroquine for 6 hours. Cells were permeabilized, stained with Mult1 antibodies followed by Alexa Fluor 555 conjugated secondary antibodies, and DAPI, and analyzed by immunofluorescence microscopy. Scale bar is 10 μ m. Data in this figure is representative of at least four independent experiments.

Figure 1-11. Mult1 accumulates in lysosomes

WT Mult1 transduced fibroblasts were grown on tissue culture-treated glass slides followed by treatment with DMSO, 20uM MG132, or 100uM chloroquine for 4 hours. Cells were permeabilized, stained with Mult1 antibodies followed by Alexa Fluor 555 conjugated secondary antibodies, biotinylated LAMP-1 antibodies followed by APC conjugated streptavidin, and DAPI, and analyzed by immunofluorescence microscopy. Scale bar is 10 μ m. Data in this figure is representative of two independent experiments.

Regulation of Mult1 on thymocytes

The regulation of Mult1 expression on thymocytes, which represent an ex-vivo subset of cells that have high levels of Mult1 transcripts but little protein expression, was also analyzed. More detailed analysis showed that larger thymocytes, defined as cells with high forward scatter (FSC-high cells), expressed a modest amount of Mult1 at the cell surface, perhaps related to the proliferative status of these cells. To investigate the mechanism of Mult1 protein-level regulation in these cells, thymocytes were treated with MG132 and chloroquine. Treatment of thymocytes with chloroquine led to an increase in the amount of Mult1 protein on the cell surface of large thymocytes with no change in Mult1 RNA levels, suggesting that lysosomal degradation was regulating Mult1 protein expression in these cells (Fig. 1-12). MG132 treatment, however, resulted in a small and inconsistent increase in Mult1 expression, and this increase in Mult1 may not be due to protein level regulation because it was accompanied by an increase in Mult1 RNA levels (Fig. 1-12). This probably reflects the wide range of pathways perturbed in the presence of proteasome inhibitors, and may provide an explanation for the increase in lysosomal Mult1 seen earlier in cells treated with MG132 (Fig. 1-11). Taken together, these data point towards the lysosome as a primary site for Mult1 degradation and show that this mechanism suppresses Mult1 expression on normal cells.

Figure 1-12. Chloroquine but not MG132 post-translationally induces Mult1 on thymocytes

B6 thymocytes were either treated with 20 μ M MG132, 100 μ M chloroquine as described in Materials and Methods, followed by 37-degree incubation for 6 hours and staining with Mult1 antibody. Large (forward scatter-high (FSC-high)) or small (FSC-low) cells were analyzed for Mult1 expression as indicated. Solid gray histograms and dashed gray lines represent isotype staining of untreated and treated cells respectively. Identically treated cells were used for Mult1 quantitative RT-PCR and normalized to 18S ribosomal RNA. Data shown is mean $\pm$ SD, n=3. Data is representative of three independent experiments.

Chapter 3 summary

NKG2D is a potent stimulatory immunoreceptor, so cell surface expression of NKG2D ligands must be tightly regulated to ensure that only diseased or distressed cells display them. Therefore, it is somewhat surprising that normal tissues often contain abundant transcripts for some ligands, including Mult1 and several human orthologs (Cosman, Mullberg et al. 2001; Radosavljevic, Cuillerier et al. 2002; Diefenbach, Hsia et al. 2003). In this chapter, a novel mechanism for regulating cell surface expression of Mult1 at the protein level is described. In cultured cells under normal conditions, Mult1 protein was rapidly degraded by a mechanism dependent on cytoplasmic lysine residues that is associated with poly-ubiquitination of the protein. Under these conditions, Mult1 protein was severely depleted from both the cell surface and intracellular compartments, indicating that it was degraded, rather than relocated within the cell. Inhibitors of lysosomal acidification blocked this depletion, causing accumulation of Mult1 within lysosomes as well as at the cell surface. This degradative pathway may explain the discrepancy between transcript levels and protein levels seen in normal tissues, such as the thymus.

Chapter 4: Regulation of Mult1 degradation by stress pathways

UV irradiation, but not the DNA damage pathway, induces Mult1 post-translationally

UV irradiation induces Mult1

The degradation of Mult1 protein described in the preceding chapter may be important for preventing its inappropriate appearance on healthy cells. If so, it remained to be determined how this inhibition is lifted in diseased cells and the basis of its regulation. Previous studies have shown that the DNA damage response, which is initiated by genotoxic stress, induces cell surface expression of ligands for NKG2D on cultured fibroblasts by increasing transcript levels (Gasser, Orsulic et al. 2005). To determine whether activation of the DNA damage response pathway also induced Mult1 at the protein level, Mult1 transduced cells were exposed to various genotoxic agents. The only treatment of this type that led to an increase in transduced Mult1 protein was UV irradiation (Fig. 2-1). Mult1 was not induced in this system by several agents other than UV that activate the DNA damage response, including ionizing radiation, 5-FU, cisplatin and mitomycin C.

To characterize the specificity of induction by UV irradiation to Mult1, the regulation of another transmembrane NKG2D ligand, H60a, and the H60a-Mult1 chimera containing the transmembrane and cytoplasmic domains of Mult1 were analyzed. UV treatment caused a cell surface induction of both endogenous Mult1 and the H60a-Mult1 chimera with no change in the amounts of the corresponding transcripts (Fig. 2-2). In contrast, surface levels of the intact H60a were unchanged by UV treatment (Fig. 2-2). This result shows that induction by UV at the protein level is not generally applicable to all NKG2D ligands and depends on sequences within the Mult1 TM and cytoplasmic domains.

Figure 2-1. UV irradiation induces ectopically expressed Mult1

Fibroblasts transduced with FLAG tagged WT Mult1 were exposed to the indicated genotoxic stress treatments for 16 hours followed by staining with FLAG antibody and flow cytometry. Solid gray histograms and dashed gray lines represent isotype staining of untreated and treated cells respectively. Data is representative of three independent experiments.

Figure 2-2. Mult1 transmembrane and cytoplasmic domains are sufficient for induction by UV irradiation

Secondary fibroblasts transduced with H60a or the H60a-Mult1 chimera (the H60a extracellular domain with the TM and IC domains of Mult1) were UV irradiated as in Fig. 2-1 followed by staining with Mult1 or H60a antibody at 6 hours after treatment. Parallel cultures of cells transduced with the H60a-Mult1 chimera were used for semi-quantitative RT-PCR to quantify transcripts of the chimeric protein, endogenous Mult1, and HPRT. Solid gray histograms and dashed gray lines represent isotype staining of untreated and treated cells respectively.

DNA damage pathway is not required for induction by UV

The finding that ectopically expressed Mult1 was not induced by most agents that activate the DNA damage response suggested that UV-induced expression might not be mediated by the DNA damage pathway. Consistent with this interpretation, inhibitors of critical protein kinases in the DNA damage response pathway, such as a Chk1, ATR and ATM, failed to inhibit protein level induction of Mult1 by UV radiation (Fig. 2-3). Furthermore, whereas we previously observed that Mult1 induction by treatment with the DNA damage response inducer aphidicolin is correlated with increased levels of Mult1 transcripts peaking at 24 hours (Gasser, Orsulic et al. 2005), UV irradiation led to a maximal increase in Mult1 at the cell surface between 4 and 8 hours without significantly increasing the abundance of Mult1 transcripts in transduced cells (Fig. 2-4), suggesting that UV acted at a distinct stage in Mult1 biogenesis. In the transduced cells, transcription is directed by the retroviral vector promoter and the transcripts lack untranslated regions that may be subject to regulation, which could potentially account for the failure of UV to upregulate the abundance of the transcripts after genotoxic stress. Alternatively, considering that the effect of UV on Mult1 mRNA levels was not previously tested (Gasser, Orsulic et al. 2005), it was possible that UV differs from other genotoxic agents in this regard. Indeed, we found that the amounts of endogenous Mult1 transcripts in untransduced cells were decreased rather than increased in cells treated with UV (Fig. 2-4), suggesting that UV prevents transcription or induces degradation of endogenous Mult1 transcripts. Despite the decreased amounts of transcripts, endogenous Mult1 cell surface expression was modestly augmented in cells treated with UV (Fig. 2-2, 2-4). Together, these data suggested that a UV activated pathway distinct from the DNA damage response pathway is responsible for induction of Mult1 expression at the protein level.

Figure 2-3. DNA damage pathway is not required for post-translational induction of Mult1 by UV

Fibroblasts transduced with FLAG tagged WT Mult1 were pretreated for one hour with either 2mM caffeine (top right panel) or 150 nM Chk1 inhibitor (SB218078, bottom right panel) followed by exposure to UV-C light and staining with FLAG antibodies 16 hours later for analysis by flow cytometry. Solid gray histograms and dashed gray lines represent isotype staining of untreated and treated cells respectively. Data is representative of two independent experiments.

Figure 2-4. Kinetics of post-translational Mult1 induction by UV irradiation

A. Fibroblasts transduced with FLAG-tagged Mult1 were exposed to 50 J/M² UV-C irradiation. After UV treatment for 4 or 8 hours, cells were stained with FLAG antibody and analyzed by flow cytometry. Cells treated in duplicate were used for Mult1 quantitative PCR and normalized to GAPDH. Data shown is mean $\pm$ range, n=2. B. Untransduced fibroblasts were UV irradiated as in A followed by staining with Mult1 antibody at the indicated time post-UV. Cells treated in duplicate were used for Mult1 quantitative PCR and normalized to GAPDH.

Mult1 protein expression is induced by heat shock

Considering the evidence that the protein level regulation of Mult1 by UV was not mediated by the DNA damage response, it was of interest that the cellular response to UV overlaps with a number of other stress-related pathways, including inflammatory cytokine signaling and the heat shock response (Maytin 1992; Rosette and Karin 1996). Whereas inflammatory cytokines did not appreciably induce cell surface expression of transduced or endogenous Mult1 in fibroblasts (not shown), heat shock at 45 degrees Celsius did boost Mult1 cell surface expression in transduced cells (Fig. 2-5). Additionally, heat shock increased the cell surface expression of endogenous Mult1 in secondary fibroblasts and WEHI 7.1 thymoma cells (Fig. 2-5). In none of these cases did heat shock induce appreciable increases in the amounts of Mult1 transcripts in these cells (Fig. 2-5).

Figure 2-5. Mult1 is post-translationally regulated by heat shock

A. Fibroblasts transduced with FLAG-tagged Mult1 were shifted to 45 degrees Celsius for 30 minutes followed by incubation at 37 degrees Celsius for 4hr. Cells were lifted with 5mM EDTA and stained with FLAG antibody for flow cytometry. Cells treated in duplicate were used for Mult1 quantitative PCR and normalized to GAPDH. Data shown is mean $\pm$ range, n=2. B. Heat shock of untransduced fibroblasts and WEHI 7.1 cells was performed as in Fig. 6A followed by 37-degree incubation for the indicated time. Endogenous Mult1 levels were measured by flow cytometry. Cells treated in duplicate were used for Mult1 quantitative PCR as described above. HS = heat shock.

Mechanism of post-translational Mult1 regulation

Regulation at the protein level is unique to Mult1

As shown earlier (Fig. 2-2), UV irradiation had no effect on the already robust expression of H60a. This finding suggested that post-translational regulation may be unique to Mult1. Mouse secondary fibroblasts do not express H60a, but do express at least one of the Rae1 isoforms. While transduced Mult1 was induced on these cells by treatment with MG132, chloroquine, UV irradiation, or heat shock, Rae1 levels did not change (Fig. 2-6). Expression of ICAM1, a transmembrane molecule involved in cell adhesion, also remained unchanged by these treatments (not shown). Therefore, these treatments did not induce a global change in trafficking of transmembrane proteins or even NKG2D ligands other than Mult1. Instead, this type of regulation appears to be quite specific to Mult1.

Since Mult1 is the only known NKG2D ligand in these cells that is induced by these treatments, the staining of treated cells with fluorescent NKG2D tetramers is expected to reflect the capacity of the induced Mult1 to bind the NKG2D receptor. Increased detection of Mult1 with antibody was always accompanied by an increase in NKG2D tetramer binding (Fig. 2-6), showing that induced Mult1 could be effectively recognized. It was important to demonstrate that the induced Mult1 on the cell surface can bind its receptor, because there is evidence that Mult1 can be proteolytically cleaved within its extracellular domain (not shown), which might prevent the induced Mult1 from binding its receptor.

Localization of Mult1 following heat shock

A significant fraction of Mult1 is targeted to lysosomes in unmanipulated cells (Fig. 1-11). Reduction of Mult1 targeting to the lysosome or a redirection of lysosome-targeted Mult1 to the cell surface are plausible mechanisms for its post-translational induction. As a rough comparison of the proportion of lysosomal Mult1 before and after heat shock, cells were co-stained with Mult1 and LAMP-1 antibodies and analyzed by immunofluorescence microscopy. As expected, there was an increase in total Mult1 signal following heat shock treatment, but there was not a substantial decrease in Mult1 within LAMP-1 positive vesicles from that seen in untreated cells (Fig. 2-7). While most cells still showed some Mult1 in lysosomes following heat shock, there was a relative increase in Mult1 signal at the perimeter of the cell compared to untreated cells, most likely representing residence at the plasma membrane (Fig. 2-7, arrows). Therefore, even though levels of lysosome-resident Mult1 are not obviously different, the ratio of cell surface Mult1 to lysosomal Mult1 is increased following heat shock. These observations do not rule out any mechanism, but they are suggestive of an alteration in Mult1 trafficking, with conditions after heat shock treatment being more permissive of transit to the plasma membrane.

Figure 2-6. Regulation at the protein level is unique to Mult1 and leads to increased binding of NKG2D receptor

WT Mult1 transduced fibroblasts were treated with 20 μ M MG132, 100 μ M chloroquine UV-C irradiation, or heat shock as described in Materials and Methods section. After 6 hours, cells were lifted with 5 mM EDTA and stained with NKG2D tetramer, pan-Rae1 antibodies, or FLAG antibodies and analyzed by flow cytometry. Solid gray histograms and dashed gray lines represent control staining of untreated and treated cells respectively. Data is representative of three independent experiments.

Figure 2-7. Localization of Mult1 following heat shock

WT Mult1 transduced fibroblasts grown on tissue culture-treated glass slides were incubated at 37 or 45 degrees for 30 minutes followed by further incubation at 37 degrees for four hours. Cells were permeabilized, stained with Mult1 antibodies followed by Alexa Fluor 555 conjugated secondary antibodies, biotinylated LAMP-1 antibodies followed by APC conjugated streptavidin, and DAPI, and analyzed by immunofluorescence microscopy. Arrows indicate Mult1 staining at the perimeter of cells following heat shock.

Role of ubiquitination in Mult1 induction

To address whether altered ubiquitination of Mult1 is involved in regulation by UV and heat shock, Mult1-ubiquitination was compared by immunoprecipitating Mult1 and immunoblotting with ubiquitin antibodies. Because heat shock and UV treatment lead to an increase in the total amount of Mult1 protein as evidenced by increased signal in the FLAG western blot (55 kDa species, Fig. 2-8), the change in the extent of Mult1 ubiquitination was revealed by normalizing the difference in ubiquitin signal to the increase in FLAG signal. Although it is difficult to accurately quantify ECL western blotting data, a calculation based on multiple experiments suggested that the fraction of polyubiquitinated Mult1 in UV-treated or heat shocked cells was reduced at least 3 fold (Fig. 2-8). This data showed that the inefficient expression of Mult1 protein was countered by a stress-induced reduction in ubiquitination, correlating with increased Mult1 expression.

Cells	WT Mult1 transduced cells		
	None	UV	HS
FLAG	2207	9516	8386
Ubiquitin	35068	46172	36732
Normalized ubiquitination	1.00	0.31	0.28

Figure 2-8. Induction of Mult1 correlates with reduced ubiquitination

Fibroblasts transduced with the indicated retroviral construct were treated with 50 J/M² UV-C irradiation or cultured at 45 degrees Celsius for 30 minutes and scraped into RIPA buffer 6 hours after treatment. Cleared lysates were immunoprecipitated with Mult1 antibody and used in western blots with FLAG or ubiquitin antibodies. WT = WT Mult1 transduced, KR = Lys to Arg mutant Mult1 transduced cells, and HS = heat shock. Quantitation was performed as described in Materials and Methods. Data are representative of three independent experiments.

Determination of Mult1 half-life

In the context of the preceding data (Fig. 2-8), which show a role of Mult1 ubiquitination in regulating protein expression, it seemed likely that the mechanism of regulation involved alterations in the stability of the Mult1 protein. To quantify the degree of Mult1 protein stabilization in response to heat shock, pulse-chase experiments were carried out with WEHI 7.1 cells that had, or had not, been heat-shocked. Heat shock leads to a decrease in translation (Duncan and Hershey 1984; Cuesta, Laroia et al. 2000), preventing efficient metabolic labeling of Mult1 after heat shock treatment. Therefore, WEHI 7.1 cells were pulsed with radioactive methionine immediately prior to exposing cells to heat shock, or not, and residual Mult1 was quantified starting at one hour after treatment, when the induction of Mult1 had been set in motion. Mult1 was clearly stabilized after heat shock, with an observed increase in half-life from 4 hours in untreated cells to 12 hours in heat shocked cells (Fig. 2-9). This alteration in Mult1 half-life definitively showed that the induction of Mult1 at the cell surface in response to heat shock was due to protein stabilization.

Regulation of endogenous Mult1 on thymocytes

It was of interest to test whether Mult1 expression was induced by heat shock and UV irradiation in thymocytes, which represent normal cells in which post-translational degradation of Mult1 occurs (Fig. 1-12). Similar to the effect seen with chloroquine treatment, the modest basal expression in large thymocytes was boosted substantially by heat shock. Smaller thymocytes, in contrast, expressed little Mult1 before or after heat shock (Fig. 2-10). The increased Mult1 on large, heat shocked thymocytes was not due to increased transcription or mRNA stabilization, because mRNA levels were markedly decreased in heat shocked cells compared to untreated cells (Fig. 2-10). Together, these results suggest that expression of endogenous Mult1, like transduced Mult1, is regulated at the protein level by activation of the heat shock pathway. It remains unclear why large but not small thymocytes are capable of inducing Mult1 expression, but it may be significant that large thymocytes are proliferating (Hoffman, Passoni et al. 1996), as are WEHI 7.1 thymoma cells. In both cases this proliferative status correlated with detectable Mult1 expression that could be induced to higher levels by heat shock (Fig. 2-5, Fig. 2-10). It is possible that non-proliferating small thymocytes lack Mult1 mRNA. Though efforts to isolate pure populations of small and large thymocytes for mRNA analysis were prevented by technical limitations, a crude measurement suggests that large thymocytes probably have higher levels of Mult1 mRNA than small thymocytes (not shown),

Unlike heat shock, UV irradiation did not alter Mult1 expression on thymocytes (Fig. 2-10). UV irradiation and heat shock both post-translationally induced Mult1 in fibroblasts, but the signaling pathways leading to this common outcome are not necessarily the same. The finding that heat shock but not UV induced the expression of Mult1 by thymocytes raises the possibility that distinct pathways are involved. It is equally possible that thymocytes, unlike skin-derived fibroblasts, specifically lack a component that couples UV light-induced signals to a common pathway for Mult1 induction. This possibility would fit with the fact that thymocytes, unlike skin cells, are not exposed to the damaging effects of UV light.

Figure 2-10. Heat shock but not UV irradiation induces Mult1 on thymocytes

B6 thymocytes exposed to UV irradiation or heat shocked as described in Materials and Methods, followed by 37-degree incubation for 6 hours and staining with Mult1 antibody. Large (forward scatter-high (FSC-high)) or small (FSC-low) cells were analyzed for Mult1 expression as indicated. Solid gray histograms and dashed gray lines represent isotype staining of untreated and treated cells respectively. Identically treated cells were used for Mult1 quantitative PCR and normalized to 18S ribosomal RNA. Data shown is mean $\pm$ SD, n=3. Data is representative of three independent experiments.

Chapter 4 Summary

There are multiple roles that protein turnover could play in gene expression. Protein degradation could act as a static inhibitor of expression that must be overcome by induction at an earlier level. Alternatively, the degradation itself could be regulated (either increased or decreased), while transcript levels and translation of the protein remain constant. The data in this chapter demonstrate that the basal rate of Mult1 degradation seen in normal cells is dampened in response to UV irradiation and heat shock, resulting in less ubiquitination, increased overall levels of Mult1 in the cells, and substantially elevated cell surface expression.

Mult1 induction at the protein level could facilitate the anti-tumor immune response since the heat shock response is known to be activated in some cancer cells (Jolly and Morimoto 2000; Garrido, Brunet et al. 2006). In this regard, it is notable that Mult1 expression occurs frequently on primary lymphomas and adenocarcinomas, as demonstrated in a recent study (Guerra, Tan et al. 2008). Infection by some viruses also induces the heat shock response (Lindquist 1986; Wu, Hurst et al. 1986), which may participate in the induction of NKG2D ligands and thus trigger NKG2D-mediated antiviral responses. Coupling expression of NKG2D ligands to the heat shock response could be beneficial in countering these advantages and eliminating the corresponding threat.

Chapter 5: Role of MARCH family E3 ligases in Mult1 regulation

Introduction to the MARCH family of E3 ligases

In order to better understand the molecular mechanism of post-translational Mult1 regulation, it was important to identify the E3 ubiquitin ligase that targets Mult1 for degradation. E3 ubiquitin ligases are, in most cases, non-catalytic scaffolds that bring a ubiquitin-bearing E2 ubiquitin conjugating enzyme in close proximity to a target allowing the transfer of ubiquitin to occur (Pickart 2001). Thus, target specificity of ubiquitination reactions are most often determined by an E3.

The membrane-associated RING-CH (MARCH) family of E3 ligases (Ohmura-Hoshino, Goto et al. 2006) represented a promising set of candidate Mult1 regulatory proteins for several reasons. MARCH proteins are transmembrane proteins, and most of their targets identified so far are also transmembrane proteins, many with significance to the immune system (Bartee, Mansouri et al. 2004; Lehner, Hoer et al. 2005; Nakamura, Fukuda et al. 2005; Hoer, Smith et al. 2007; De Gassart, Camosseto et al. 2008). A particularly interesting example is the regulation of MHC class I by MARCH-4 and MARCH-9 as well as virally encoded homologues of the MARCH proteins (Coscoy, Sanchez et al. 2001; Bartee, Mansouri et al. 2004). MHC class II is regulated by distinct MARCH proteins, and the purpose of this regulation has been more completely characterized than that for MHC class I as described earlier (pg. 31) (Shin, Ebersold et al. 2006; De Gassart, Camosseto et al. 2008).

While the mechanism determining substrate recognition has not been determined for all the endogenous MARCH proteins, the substrate specificity of the viral MARCH homologues has been shown through domain swapping to be dependent on their membrane spanning regions (Sanchez, Coscoy et al. 2002). Since the transmembrane domain of Mult1 is involved in its protein-level regulation (Fig. 1-5) and Mult1 has homology to MHC class I, the MARCH proteins were prime candidates for Mult1 E3 ligases. This initial suspicion for a role of MARCH proteins in regulating Mult1 was bolstered by subsequent evidence that one of the virally encoded MARCH homologues downregulates the human NKG2D ligands MICA and MICB (Thomas, Boname et al. 2008).

Screen of a set of cloned human March proteins

A set of HA-tagged MARCH proteins cloned from human cDNA (L. Coscoy) was used to screen for the ability to regulate Mult1 expression. 293T cells were transiently co-transfected with individual March plasmids or control plasmid together with the retroviral plasmid encoding Mult1-IRES-GFP (Fig. 1-2). Cells were stained 24 hours later with Mult1 antibodies and transfected cells were selectively analyzed by gating on GFP expressing cells. Strikingly, co-transfection of Mult1 with either of two closely related family members, MARCH-4 and MARCH-9, prevented Mult1 from being expressed (Fig. 3-1). This effect was specific to MARCH-4 and MARCH-9 because no other MARCH family members caused notable variations in Mult1 expression compared

to control cells. Interestingly, these two proteins were the same MARCH proteins reported to downregulate MHC class I molecules.

Lysines in the Mult1 cytoplasmic tail were shown to be responsible for its degradation (Fig. 1-7, Fig. 1-8), and the co-transfection of MARCH-4 and 9 with Mult1 led to increased ubiquitination of Mult1 (W. Deng, not shown). To determine the role of the cytoplasmic tail in Mult1 regulation by these E3 ligases, 293T cells were co-transfected with Mult1 cytoplasmic tail mutants together with MARCH-9 or control plasmid. In the absence of MARCH-9, the mutant Mult1 proteins were all increased in cell surface expression compared to WT Mult1. The mutant truncated after amino acid 253 (Δ 253) was modestly increased in expression, whereas marked increases in expression were observed with the mutant truncated after amino acid 239 (Δ 239), as well as the mutant in which lysines were substituted with arginines (KR). This indicates that 293T cells may express an endogenous E3 ligase capable of regulating WT Mult1, and the effect of MARCH-9 co-expression must be considered in the context of these variations in basal Mult1 expression. Co-expression of MARCH-9 with the transfected Mult1 proteins caused a substantial downregulation of WT Mult1 and Δ 253 Mult1, indicating that amino acids in the interval 253-334 are not required for interaction with MARCH-9. In contrast, MARCH-9 co-expression had little or no effect on expression of the Δ 239 mutant, which lacks most of the cytoplasmic tail, including the six lysine residues that are optimal targets for ubiquitination. Interestingly, the KR mutant lacking all lysine residues in the cytoplasmic tail was partially downregulated when co-expressed with MARCH-9. This partial effect may be due to ubiquitination of Mult1 on non-lysine residues within the cytoplasmic tail, a phenomenon that was demonstrated in the case of ubiquitination targeted by one of the viral homologues of MARCH proteins, MIR1 (Cadwell and Coscoy 2005). Together, these results show that the Mult1 cytoplasmic domain is critical for regulation by MARCH-9, and that the lysines within this domain appear to be the primary but not only requirement for degradation targeted by MARCH-9.

The transmembrane and cytoplasmic domains of Mult1, when attached to the extracellular domain of H60a, were shown to be sufficient for allowing post-translational regulation (Fig. 1-5, Fig. 2-2). To determine if the transmembrane and cytoplasmic domains of Mult1 were sufficient for regulation by MARCH-9, 293T cells were co-transfected with H60a or H60-Mult1 chimera together with MARCH-9 or empty vector. The H60a-Mult1 chimera showed less surface staining than unmanipulated H60a in the absence of MARCH-9, but was reduced even further when MARCH-9 was co-expressed (Fig. 3-2). Somewhat unexpectedly, expression of H60a with its own transmembrane and cytoplasmic domains was also decreased in the presence of MARCH-9 (Fig. 3-2), indicating that H60a may also be a MARCH-9 target. H60a has several lysine residues at the N-terminal end of its relatively short cytoplasmic domain, perhaps providing a target for MARCH-9 mediated ubiquitination. Notably, the earlier analysis of Mult1 and H60a regulation (Fig. 2-2) suggested that the endogenous E3 ligase that targets Mult1 in fibroblasts does not target H60a. It remains unclear whether this discrepancy is due to overexpression of MARCH-9 in the current experiments, the action of a distinct E3 ligase in fibroblasts that is more specific for Mult1, or some other factor. The data do raise the possibility that cells that naturally express high levels of MARCH-9 may downregulate H60a and possibly other NKG2D ligands in addition to Mult1.

Figure 3-1. MARCH-4 and MARCH-9 specifically prevent Mult1 surface expression

293T cells were co-transfected with plasmid encoding WT Mult1-IRES-GFP and plasmid encoding the indicated MARCH proteins in a 1:3 ratio. 24 hours later cells GFP positive cells were analyzed by flow cytometry for Mult1 expression. Shaded grey histograms represent staining of cells transfected with plasmid encoding IRES-GFP only.

Figure 3-2. MARCH-9 downregulation is dependent on residues within cytoplasmic domain

A. 293T cells were co-transfected with the indicated Mult1-IRES-GFP plasmids and control (solid line) or MARCH-9 (dashed line) plasmids in a 1:3 ratio. 24 hours later GFP positive cells were analyzed for Mult1 expression by flow cytometry. B. Cells were co-transfected as in A using H60 or H60-Mult1 chimera encoding plasmids instead of Mult1 encoding plasmids. Cells were analyzed 24 hours later for H60 surface expression by flow cytometry. Data in this figure are representative of at least two experiments.

MARCH-9 and Mult1 regulation by heat shock

Mouse MARCH-4 and MARCH-9 downregulate endogenous Mult1

Following the analysis using human MARCH proteins, it was of interest to examine the effects of mouse MARCH-4 and 9 on endogenous Mult1. Murine versions of MARCH-4 and 9 were amplified from cDNA, N-terminally tagged with the myc epitope, and cloned into a MSCV retroviral expression vector. C1498 cells were used for experiments with ectopically expressed MARCH-4 and 9 because they have high basal levels of Mult1 even in the absence of heat shock treatment. Consistent with the results from the co-transfection experiments, stable expression of either MARCH-4 or 9 in these cells led to a decrease in endogenous Mult1 at the cell surface (Fig. 3-3). There was a greater downregulation of Mult1 seen in MARCH-9 expressing cells that correlated with higher levels of retroviral transduction. However, gating on the cells within the population that had the highest MARCH-4 expression using a marker of retroviral transduction, Thy1.1, showed that MARCH-4 was nearly as efficient as MARCH-9 in downregulating Mult1. The lower level of transduction seen with MARCH-4 retroviruses may reflect toxicity of high MARCH-4 levels in these cells.

Because MARCH-4 and 9 have been reported to downregulate human MHC-I and ICAM-1 (Bartee, Mansouri et al. 2004; Hoer, Smith et al. 2007), the surface expression of these proteins in MARCH-expressing C1498 cells was also examined. While there was no effect seen on ICAM-1 or K^b expression levels, D^b was slightly reduced in the cells expressing MARCH-9 (Fig. 3-3). MARCH-9 regulation of murine transmembrane molecules other than these (such as other haplotypes of MHC-I) may be more dramatic but this remains to be determined. In any case, these results show that regulation of Mult1 by MARCH-9 expression in C1498 cells is quite specific and most likely not an artifact of forced expression.

MARCH-9 expression is sufficient for Mult1 regulation

C1498 cells are one of a few cell lines analyzed that fail to significantly induce Mult1 upon heat shock treatment. This may be either because they lack a component of the Mult1 regulatory pathway or because the pathway is constitutively active. Introduction of MARCH-9 into these cells reduced the high basal level of Mult1, raising the possibility that MARCH-9 substituted for a missing component (E3 ligase) in the pathway regulating Mult1. Indeed, when MARCH-9 expressing C1498 cells were heat shocked, significant levels of Mult1 were restored to the cell surface whereas little change in Mult1 expression was seen on parental C1498 cells (Fig. 3-4). These data suggests that the parental cells lack an E3 ligase for Mult1, and this defect is rescued by ectopic expression of MARCH-9. Interestingly, D^b was not induced by heat shock in the MARCH-9 transduced cells. In fact, heat shock caused a reduction in D^b levels at the cell surface in both MARCH-9-expressing and parental C1498 cells (Fig. 3-4). From this

finding, it can be concluded that not all MARCH-9 regulated proteins are induced by heat shock. Instead, there must be other factors that uniquely effect regulation of Mult1 by this E3 ligase.

Dynamic association of Mult1 with MARCH-9

The lack of D^b induction by heat shock in MARCH-9 expressing cells suggested that a global change in MARCH-9 activity may not be the cause of Mult1 regulation. To test this possibility, RNA and protein levels were analyzed before and after heat shock. As expected, transcript levels for MARCH-9 were much higher in MARCH-9 transduced cells compared to parental C1498 cells, but there was only a slight reduction in transcript levels following heat shock (Fig. 3-5). Similarly, the transduced MARCH-9 protein, detected by western blotting with Myc epitope antibodies, was not detectably depleted following heat shock (Fig. 3-5). These data argue strongly against the possibility that heat shock stimulates Mult1 expression by reducing MARCH-9 expression.

An alternative explanation for Mult1 regulation is that the interaction between Mult1 and MARCH-9 is reduced following heat shock, thus leading to stabilization of Mult1. This hypothesis was tested with co-immunoprecipitation experiments. Mult1 immunoprecipitates from C1498 or C1498-MARCH-9 cells with or without heat shock were immunoblotted with myc antibodies to detect associated MARCH-9. MARCH-9 was observed specifically in immunoprecipitates from the MARCH-9 transduced cells at the expected size for MARCH-9 (~40 kDa), indicating an interaction with Mult1. The interaction of MARCH-9 with Mult1 was substantially reduced following heat shock, correlating with the increased display of Mult1 on the cell surface (Fig. 3-6). These data are consistent with a dynamic Mult1-MARCH interaction and, since MARCH-9 levels are not regulated by heat shock, suggest that an unidentified heat shock-sensitive event controls the extent of this interaction. The exact nature of this regulation by heat shock remains to be determined.

Figure 3-3. Mouse MARCH-9 downregulates endogenous Mult1

C1498 cells were transduced with retrovirus encoding MARCH-4 or MARCH-9 and IRES-Thy1.1 as a marker of transduction. Thy1.1 positive cells were sorted and analyzed for expression of Mult1 (A). MARCH-9 expressing cells were further analyzed for expression of K^b, D^b, or ICAM-1 (B).

Figure 3-4. MARCH-9 is sufficient for Mult1 regulation

C1498 cells and MARCH-9 transduced C1498 cells were heat shocked at 45 degrees for 30 minutes followed by a four-hour incubation at 37 degrees. Expression of Mult1 and D^b were analyzed by flow cytometry.

Figure 3-5. MARCH-9 levels are unchanged by heat shock

Cells treated as described in figure 3-4 were used for MARCH-9 quantitative PCR or myc western blot. MARCH-9 transcripts were normalized to RPS29. The band in the Myc blot indicated by the * is a non-specific background band, below which myc-MARCH-9 was visible. C-MARCH9 = C1498 cells transduced with MARCH-9, HS = heat shock, ctrl = control.

Figure 3-6. The interaction between Mult1 and MARCH-9 is regulated by heat shock

Cells treated as in figure 3-4 were lysed in co-immunoprecipitation buffer and Mult1 was immunoprecipitated. Isolated proteins were separated by electrophoresis and Mult1-associated MARCH-9 was detected by western blot with myc antibodies. Histograms show detection of Mult1 on cells prior to immunoprecipitation.

Knockdown of endogenous MARCH-9

Expression level of MARCH-4 and MARCH-9 in cell lines and tissues

While overexpression of MARCH-4 and MARCH-9 clearly resulted in suppression of Mult1 expression, it was important to determine the role of the proteins at their endogenous levels of expression. Because both MARCH-4 and MARCH-9 lead to downregulation of Mult1, it was important to be aware of their relative expression when studying the role of endogenous MARCH proteins in Mult1 regulation. To this end, a panel of mouse cell lines and tissues were analyzed by quantitative RT-PCR for expression of MARCH-4 and MARCH-9 (Fig. 3-7). Transcripts for MARCH-9 were represented in a broad range of tissues, but MARCH-4 transcripts were more selectively expressed, most notably in the brain and lung. The tissue distribution observed here is similar to an already published analysis of human MARCH-4 and 9 expression (Bartee, Mansouri et al. 2004), suggesting similar roles for these proteins in mice and humans. MARCH transcript levels were comparable between all the cell lines analyzed, with Mult1-low WEHI 7.1 cells having similar levels of MARCH-9 transcripts as representative Mult1-high cells (3T3 and C1498) and none having significant amounts of MARCH-4 transcripts (Fig. 3-7).

To directly address the possibility that MARCH-9 is more abundantly expressed than MARCH-4, PCR primers were designed that amplified a homologous region of the highly related MARCH-4 and MARCH-9 sequences that contained a distinguishing Sall restriction enzyme site unique to MARCH-4. Selective digestion of the MARCH-4 amplicons within the PCR product revealed the relative abundance of the two transcripts within cells. Secondary mouse fibroblasts were selected for this more detailed analysis since they had been well characterized in terms of their Mult1 regulation. No detectable digestion product was present when fibroblast cDNA was used as the template, indicating that MARCH-9 is the only transcript present in significant quantity in these cells (Fig. 3-7). Therefore, MARCH-9 could be specifically studied in these cells with the assurance that MARCH-4 had only a very minor role in Mult1 regulation.

A.

B.

Figure 3-7. MARCH-9 is broadly expressed and is predominant in fibroblasts

A. RNA from the indicated cells and tissues was used for MARCH-4 or MARCH-9 quantitative RT-PCR and relative quantity was determined by normalization to RPS29.
 B. Primers binding to a homologous region of MARCH-4 and MARCH-9 were used in a PCR with the indicated template. MARCH-4 sequences were specifically digested with *Sall* and resolved by electrophoresis. Fibro = secondary mouse fibroblasts.

MARCH-9 knockdown has no effect on basal Mult1 expression

Mult1 in fibroblasts is kept at low levels under normal conditions due to ubiquitination and degradation. To determine if the endogenous MARCH-9 in these cells is responsible for the observed regulation, siRNA oligos targeting MARCH-9 were used to deplete MARCH-9 transcripts. Fibroblasts ectopically expressing WT Mult1 and GFP were transfected with GFP oligos, negative control oligos, or three different MARCH-9 oligos. While the GFP oligos efficiently inhibited GFP expression in at least 80 percent of cells, demonstrating the high efficiency of transfection, none of the three different MARCH-9 oligos used for knockdown resulted in appreciable increases in Mult1 expression (Fig. 3-8). Analysis of RNA levels in these cells showed that the MARCH-9 oligos were effective, reducing transcripts by between 70 and 95 percent (Fig. 3-8). To ensure that there was no off target effect on Mult1, which could confound interpretation of the results, Mult1 transcripts were also quantified. While one of the oligos (M9 #3) appeared to reduce Mult1 RNA levels as a side effect, the MARCH-9 oligo that resulted in the best specific knockdown (M9 #1) had no effect on Mult1 transcripts (Fig. 3-8). These results argue that MARCH-9 is not necessary for the basal suppression of Mult1 expression seen in these cells, but may carry out this function redundantly with another E3 ligase.

Figure 3-8. Knockdown of MARCH-9 has no effect on Mult1 expression

Fibroblasts expressing HA-tagged Mult1 were transfected with control oligos (solid line) or the indicated siRNA oligos (dashed line, M9=MARCH-9). Expression of GFP and HA-Mult1 was analyzed 72hrs after transfection. RNA was extracted for QPCR analysis, and relative levels of Mult1 and MARCH-9 transcripts were determined by normalizing to RPS29.

Chapter 5 summary

Before the biological role of Mult1 post-translational regulation can be defined, identification of the proximal regulatory proteins that control Mult1 expression is necessary. The E3 ubiquitin ligases, MARCH-4 and MARCH-9, have been identified here as potential Mult1 regulatory proteins. They are clearly capable of regulating Mult1 in a quite specific way, with other MARCH family members having no effect on Mult1 expression. Even though ectopically expressed MARCH-9 could downregulate Mult1 expression, knockdown of endogenous MARCH-9 failed to induce Mult1 expression in fibroblasts. Additional studies will be necessary to determine whether (1) MARCH-9 functions redundantly with other E3 ligases in fibroblasts to downregulate Mult1; (2) Mult1 is regulated by MARCH-9 and/or MARCH-4 in distinct cell types that express higher levels of these E3 ligases; or (3) that MARCH-4 and MARCH-9 only influence Mult1 expression when overexpressed at super-physiological levels. Note that although the levels of MARCH-4 and MARCH-9 mRNA were much higher in transduced cell lines than in fibroblasts, they were only ~3 fold higher than the levels found in certain other normal cell types, such as brain cells and kidney cells. Therefore, the physiological level of MARCH-9 in some cell types is not dramatically less than the level in the transduced cells. Whether or not MARCH-9 or MARCH-4 regulate Mult1 physiologically, it appears likely that distinct E3 ligases can also regulate Mult1.

While MARCH-4 and MARCH-9 appear to be redundant as E3 ligases for Mult1 in some cells, forced expression of MARCH-9 was sufficient to restore Mult1 regulation by heat shock in cells that were otherwise unresponsive. This suggests that in the absence of any other Mult1 E3 ligase that acts on Mult1, MARCH-9 is compatible with the normal regulation of the protein by heat shock. Analysis of the transduced cells showed that expression of MARCH-9 itself was unchanged by heat shock, but the interaction between MARCH-9 and Mult1 was reduced. Notably, D^b downregulation by MARCH-9 was not reversed by heat shock, raising the possibility that the regulatory mechanism targets Mult1 rather than MARCH-9. Further experiments will be necessary to establish the underlying mechanisms.

Chapter 6: Conclusions

Post-translational regulation of Mult1 in response to heat shock and UV irradiation represents a novel strategy for the regulation of NKG2D ligands. The induction of Mult1 at the protein level, as opposed to transcriptional or mRNA levels, may be specifically advantageous for several reasons. First, it allows a more rapid increase in ligand expression because the transcripts are already present in polysomes and the protein is already being synthesized. Protein-level regulation also allows ligand induction in situations where transcription and perhaps even translation are inhibited. This might be the most significant advantage because of the existence of multiple viral strategies for inhibiting host gene expression at these levels (Lyles 2000; Glaunsinger and Ganem 2004).

Mult1 is unique among the murine NKG2D ligands investigated to date in its induction at the protein level. Rae1 proteins, unlike Mult1, are GPI-linked proteins that lack cytoplasmic tails that are typically necessary for this form of regulation. Another ligand, H60a, has a shorter cytoplasmic tail containing several lysine residues, and could be downregulated by overexpressed MARCH-9. However, in cells that did not overexpress MARCH-9, but did regulate Mult1 in response to heat shock and UV irradiation, H60a was not similarly regulated.

Among the human NKG2D ligands, it was previously demonstrated that MICA is induced by heat shock (Groh, Bahram et al. 1996). Although similar to Mult1 in outcome, it is important to emphasize that the induction of MICA by heat shock has been linked to a completely different mechanism. The MICA gene promoter contains heat shock response elements, and induction of MICA by heat shock is accompanied by increased MICA transcription (Venkataraman, Suciú et al. 2007). As already indicated, heat shock failed to induce Mult1 transcripts in the cells studied here, and often had the opposite effect of reducing Mult1 transcripts. Although previous studies investigated transcriptional induction of MICA by heat shock, it is possible that heat shock also regulates MICA post-transcriptionally. Indeed, post-transcriptional regulation of MICA was recently documented by studies showing that virus-encoded MARCH homologues can ubiquitinate and downregulate MICA from the cell surface (Thomas, Boname et al. 2008). MICA does not appear to be a MARCH-9 substrate (not shown), but it remains possible that some other cellular E3 ligase regulates MICA in a manner similar to that shown for Mult1. The RAET1 family of human NKG2D ligands is less likely to be regulated by ubiquitination as most of them are GPI anchored. However, RAET1G is a transmembrane protein with a single lysine residue within its cytoplasmic tail (Bacon, Eagle et al. 2004), grouping it with the MIC proteins as candidate for ubiquitin-mediated regulation in humans.

As a result of tumorigenesis and other disease processes, numerous stress pathways are often induced, including the heat shock pathway and the DNA damage response (Jolly and Morimoto 2000; Bartkova, Horejsi et al. 2005; Gorgoulis, Vassiliou et al. 2005; Garrido, Brunet et al. 2006). Activation of these pathways may underlie the expression of Mult1 and other ligands in tumor cell lines such as C1498, YAC-1, and in primary tumors (Guerra, Tan et al. 2008). Interestingly, several tumor cell lines, including C1498, showed relatively poor induction of Mult1 by heat shock (Figure 3-4, and data not shown), consistent with the possibility that the relevant stress pathway is constitutively activated in these cells. Alternatively, it is possible that C1498 and the other cell lines mentioned lack a critical signaling component for induction of the Mult1

degradation pathway, perhaps as a result of mutations that accumulated during tumorigenesis or propagation of the cells.

Studies of MARCH-9 provide evidence that there is indeed a missing component in C1498 cells, namely an E3 ligase capable of downregulating Mult1. Introduction of MARCH-9 into C1498 cells lowers basal expression of Mult1 and restores regulation of Mult1 in response to heat shock. Because this regulation is made possible simply by introducing an E3 ligase for Mult1, it is not reasonable to maintain that the heat shock pathway is constitutively activated. Instead, C1498 cells must not be expressing an E3 ligase capable of downregulating Mult1, making induction by heat shock impossible. This does not necessarily mean that expression of the E3 ligase was lost by C1498 cells, however, because they may have never expressed one to begin with. If it is true that certain cell types do not express an E3 ligase for Mult1, then it means that Mult1 protein level regulation is exclusive to particular cell types that express an E3 ligase capable of targeting Mult1, such as MARCH-9.

Most of the experiments on MARCH-4 and 9 were done with cells ectopically expressing these proteins, so concerns about the physiological relevance of the data must be addressed. The downregulation of Mult1 seen with transfected MARCH-4 or 9 (Fig. 3-1) may be due to non-physiological interactions resulting from the abnormally high levels of transfected MARCH proteins. Clearly, however, there is some specificity to this interaction since not all of the MARCH family members downregulated Mult1 when overexpressed (Fig. 3-1). Additionally, stable expression of MARCH-9 in C1498 cells led to decreased surface expression of D^b and Mult1, but did not alter expression of the other potential MARCH-9 targets ICAM-1 and K^b. These demonstrations of specificity, both in terms of which MARCH family members are capable of Mult1 downregulation and the selectivity of Mult1 as a target, argue against a non-specific effect of overexpression. In fact, the level of MARCH-9 RNA in transduced C1498 cells (Fig. 5-5) is only about three times higher than levels seen in the tissues with the most abundant MARCH-9 RNA (e.g. brain, Fig. 5-7), suggesting that the amount of MARCH-9 in C1498-MARCH-9 cells is comparable to physiological expression in cells originating from other tissues.

In the cell lines analyzed, endogenous MARCH-9 expression did not correlate with the ability to regulate Mult1 at the protein level (Fig. 5-5, WEHI 7.1 vs. C1498). This observation may be accounted for by the dynamic nature of the Mult1-MARCH interaction (Fig. 3-6), the extent of which may vary between cell types making the absolute level of MARCH-9 expression less meaningful. Antibodies recognizing the endogenous MARCH-9 protein will be required to determine if this is the case. An alternative explanation for the lack of correlation between MARCH-9 expression level and Mult1 regulation is redundancy of MARCH-9 with as-of-yet unidentified cellular E3 ligases. This redundancy would also explain the lack of a phenotype following knockdown of endogenous MARCH-9 in fibroblasts (Fig. 3-8), which, therefore, must express a distinct E3 ligase for Mult1. The fact that Mult1 is induced by heat shock in these fibroblasts, suggests that multiple E3 ligases can regulate Mult1 in response to heat shock stress. A molecular explanation for this redundancy could be that the heat shock stress targets Mult1 directly, preventing interaction with multiple E3 ligases. This idea is supported by the observation that heat shock treatment does not induce D^b expression on

C1498-MARCH-9 cells (Fig. 3-6). So, while MARCH-9 may be redundant as an E3 ligase for Mult1, these studies have provided insight into the molecular mechanism of Mult1 regulation at the protein level.

Together with previous studies, the findings reported here indicate that there are multiple paths leading to the induction of NKG2D ligands. In some cells or circumstances, these levels of regulation may serve as serial checkpoints, regulated by distinct stimuli, to ensure that ligands are induced only in diseased cells and not in normal cells. In many normal cell types, for example, Mult1 transcripts are rare (Diefenbach, Hsia et al. 2003) (Figure 1). In these cells Mult1 expression requires induction at the mRNA level (which may be accomplished by the DNA damage response and other pathways that regulate transcription) and may require additional induction at the protein level. In other cell types Mult1 transcripts are abundant *ex vivo* and protein level regulation may be the only regulatory checkpoint in place. A subset of thymocytes, and possibly other cell types, fall into this category. Finally, the finding that Mult1 is so far unique among the mouse ligands studied in being induced at the protein level by the UV and heat shock pathways suggests that Mult1 may serve as a specific sentinel for these distinct forms of stress. Other ligands may exhibit some distinct specialization in the stresses that regulate them, providing broad innate immune “coverage” for numerous stresses associated with various diseases. Along with differences in tissue-distribution, unique regulatory control of the various ligands would create a complex warning system that relays various danger signals to immune cells through the NKG2D receptor system. These considerations are likely to provide part of the explanation for the existence in each individual of numerous distinct ligands for NKG2D.

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