Posttranslational regulation of the NKG2D ligand Mult1 in response to cell stress

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NKG2D is a major stimulatory receptor expressed by natural killer (NK) cells and some T cells. The receptor recognizes major histocompatability complex class I–like cell surface ligands that are poorly expressed by normal tissues but are 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 proteinlike transcript 1 (Mult1), are abundant in certain normal tissues where cell surface expression is absent, suggesting the existence of translational or posttranslational regulation. We report here that under normal conditions, Mult1 protein undergoes ubiquitination dependent on lysines in its cytoplasmic tail and lysosomal degradation. Mult1 degradation and ubiquitination is reduced in response to stress imparted by heat shock or ultraviolet irradiation, but not by other forms of genotoxicity, providing a novel mechanism for stress-mediated cellular control of NKG2D ligand expression.

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Abbreviations used: ATM, ataxia-telangiectasia mutated; ATR, ATM and Rad3-related; Chk1, checkpoint kinase 1; H60a, Histocompatibility 60a; MICA, MHC class I chain-related gene A; MICB, MHC class I chain-related gene B; Mult1, Murine UL16-binding protein-like transcript 1; Rae1, Retinoic acid early inducible gene 1.

Natural killer (NK) cells exhibit more restricted recognition capabilities than T or B cells. Consequently, NK cells are less diverse and respond more rapidly, enabling infections to be controlled in the early stages (1–3). As the understanding of NK cell biology has increased, it has become clear that the balance between inhibitory and stimulatory signals originating from surface receptors dictates their response. When stimulatory signals outweigh the inhibitory ones and pass a critical threshold, NK cells respond with cytolytic killing and production of cytokines (4). 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. Stimulatory signals come from several distinct surface receptors, only some of which have defined ligands.

NKG2D is a stimulatory immune receptor found on almost all NK cells, as well as on activated CD8 T cells and subsets of γδ T cells, NKT cells, and CD4 T cells. It recognizes a family of MHC class I–related molecules, which are generally poorly expressed by normal cells and up-regulated on diseased cells (4–8). Engagement of NKG2D by these ligands on target cells results in NK cell–dependant killing of tumor cells in vivo (5, 9), and if expression of ligands is high, stimulation through NKG2D can overcome inhibitory signaling caused by MHC class I expression (4). Engagement of NKG2D on T cells generally enhances T cell responses (9, 10). These findings illustrate the need for strict regulatory mechanisms controlling NKG2D ligand expression, assuring that only unwanted cells up-regulate the ligands at the cell surface. In agreement with this idea, most normal cells lack ligand expression, whereas many tumor cell lines and primary tumors are positive (5, 7, 11–13). Ligand expression has also been shown to increase during infections with certain pathogens (10, 14). This observation led to the idea that ligands are up-regulated 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. The range of stress pathways involved in ligand induction is currently an area of active research. Two of the ligands in humans, MHC class I chain–related gene A and B (MICA and MICB), were shown

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to be transcriptionally up-regulated by heat shock (15, 16) and genotoxic stress was shown to specifically induce cell surface expression of NKG2D ligands in fibroblasts (17).

The number of known ligands for NKG2D continues to grow (18), raising the question of why so many are needed. One explanation may be that viral evasion of NKG2D-mediated recognition led to selective pressure for ligand redundancy. Alternatively, distinct ligands may be differentially regulated, providing the system with the capacity for responding to a greater range of disease-induced insults. The alternative regulatory modes could operate at numerous levels, including transcription, translation, or by controlling protein or RNA stability or localization. Intriguingly, posttranscriptional 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 (6, 19–21).

Abundant levels of transcripts of murine UL16-binding proteinlike transcript 1 (Mult1), which is a murine NKG2D ligand, are found in several normal tissues, most notably the thymus (19, 21). In this study, we have investigated the regulation of Mult1 expression and show that lysines within the cytoplasmic tail of the protein are targets of ubiquitination, which inhibits Mult1 protein expression under normal cellular conditions. Moreover, we provide evidence that this protein-level process is regulated by specific stresses, including heat shock and UV irradiation, but not by other DNA-damaging agents tested. These data suggest that Mult1 expression at the cell surface is regulated by an interplay of stress-induced pathways operating at different stages of Mult1 biogenesis.

RESULTS

Ectopically expressed Mult1 protein is inefficiently displayed at the cell surface

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 A). In contrast, cell surface expression was very low on thymocytes, but substantial on YAC-1 (Fig. 1 B). These data suggested that Mult1 cell surface expression is controlled posttranscriptionally, either at the translational or protein level. To test for protein level regulation, the open reading frames of Mult1 or of a distantly related NKG2D ligand, H60c (18), 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. Fibroblasts established from ear tissues of adult mice cultured after the crisis period were used as representative nontransformed cells with low basal Mult1 expression (17). 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 with that of H60c (Fig. 1 C). 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 posttranscriptional control.

Lysines in the cytoplasmic tail of Mult1 inhibit its surface expression

Mult1, unlike H60c or the other murine ligands, has a large cytoplasmic tail (Fig. 2 A). Because cytoplasmic tails often contain motifs that regulate protein stability or localization (22, 23), 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 (unpublished data), and deletion of amino acids 253–334, comprising most of the cytoplasmic tail, resulted in only a slight increase (Δ253 mutant; Fig. 2 B). 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, as detected by staining with FLAG antibody (Δ239 mutant; Fig. 2 B, left). 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

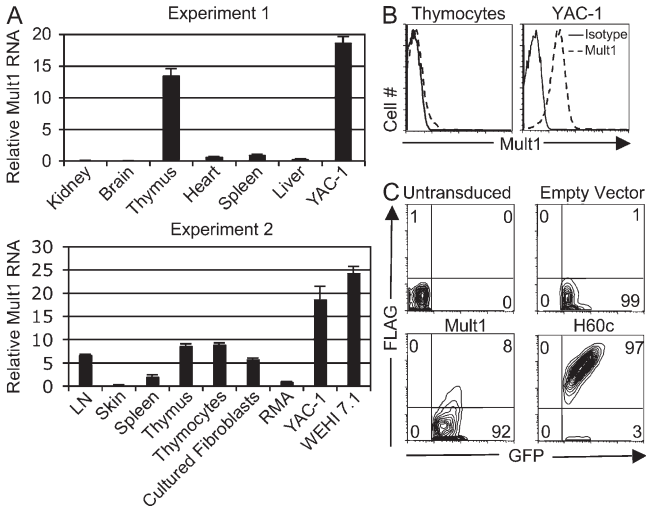

Figure 1. Mult1 protein is inefficiently expressed. (A) Quantitative RT-PCR for Mult1 was performed on cDNA prepared from the indicated tissues and cell lines. The relative amount of Mult1 RNA was determined by normalizing to GAPDH transcript levels. Data shown is mean ± SD; $n = 3$. (B) Thymocytes and YAC-1 cells were stained with Mult1 antibody and analyzed by flow cytometry. (C) B6 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.

transduced with the Δ239 mutant compared with cells transduced with WT Mult1 (Fig. 2 B, 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.

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, histocompatability 60a (H60a).

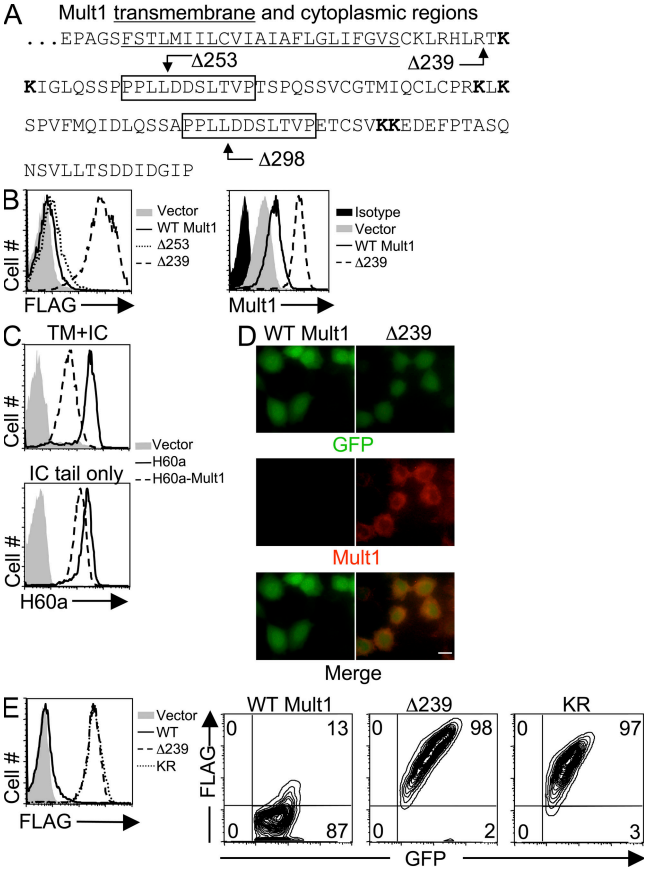

Figure 2. Lysine residues in the Mult1 cytoplasmic tail inhibit protein expression. (A) Amino acid sequence of the Mult1 transmembrane (underlined) and cytoplasmic domains with lysines shown in bold. The boxes indicate duplicated amino acid sequences within the cytoplasmic tail, and arrows show the location of the C termini of the various deletion mutants. (B, C, and E) Fibroblasts were transduced using the same approach as in Fig. 1 C with WT Mult1, Mult1 truncation or KR mutants, or 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. (D) Fibroblasts were grown on coverslips, followed by permeabilization and staining with Mult1 antibody and PE conjugated secondary antibody. The complete absence of detectable Mult1 in cells transduced with WT Mult1 is likely caused by the lower sensitivity of immunofluorescence microscopy, as compared with flow cytometry (B). Bar, 10 μm. Data in this figure is representative of at least three independent experiments.

Replacing the transmembrane and cytoplasmic tail of H60a with the corresponding Mult1 domains resulted in a fivefold decrease in cell surface expression compared with WT H60a (Fig. 2 C, TM+IC). In a separate analysis, replacing only the cytoplasmic tail of H60a with that of Mult1 resulted in a twofold reduction in H60a staining (Fig. 2 C, IC tail only). Taken together, these data showed that the Mult1 cytoplasmic tail was necessary, but not sufficient, for appreciable suppression of cell surface expression, which appeared to require sequences in both the cytoplasmic tail and transmembrane domain of the protein. Sequences in the extracellular domain may also play a role in achieving maximal suppression of cell surface expression.

The reduced cell surface expression of Mult1 dependent on its cytoplasmic tail could reflect the degradation of Mult1 after translation, 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 Δ239 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. 2 D). We therefore examined the possibility that the intact protein is degraded by the most common pathway for protein turnover, which depends on ubiquitination of the protein, usually on lysine residues, and subsequent proteolysis (24, 25). 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. 2 E). These data strongly suggested that Mult1 expression is regulated by ubiquitination.

Ubiquitination is involved in the inhibition of Mult1 expression

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 because of 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 WT Mult1 than in immunoprecipitates from cells transduced with empty vector (Fig. 3). 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. 2 B, right). The near absence of bands in the control antibody precipitates confirmed the identity of the ubiquitinated species as Mult1 (Fig. 3). Cells transduced with the KR Mult1 mutant exhibited a dramatic increase in Mult1 protein levels

compared with the WT, as revealed by the anti-FLAG Western blot (Fig. 3 A, bottom), but the levels of polyubiquitinated Mult1 were not increased compared with cells receiving the empty vector (Fig. 3). 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 WT Mult1, as compared with KR Mult1, confirms that ubiquitination is associated with dramatically reduced amounts of the protein in transduced cells, suggesting that ubiquitination leads to protein degradation.

Polyubiquitinated proteins are typically targeted for degradation via the proteasome or lysosome (24, 25). MG132 inhibits proteasomal degradation, but as this results in depletion of free ubiquitin pools (26), ubiquitin-dependent lysosomal degradation is also inhibited by MG132. Indeed, 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. 3 B). MG132 had little effect on the already high surface expression of the KR mutant Mult1 (Fig. 3 B). 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. 3 B), indicating that most WT Mult1 is degraded in the lysosome. Immunofluorescence microscopy of WT Mult1-transduced cells treated with either MG132 or chloroquine also showed an accumulation of Mult1, in a punctate pattern, perhaps representing accumulation within the lysosome (Fig. 3 C). 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. Parallel flow cytometric analysis of identically treated permeabilized cells stained with Mult1 antibody also showed WT-Mult1 accumulation after both MG132 and chloroquine treatments (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20081335/DC1). Together, these data are most consistent with degradation of WT Mult1 predominantly in the lysosome.

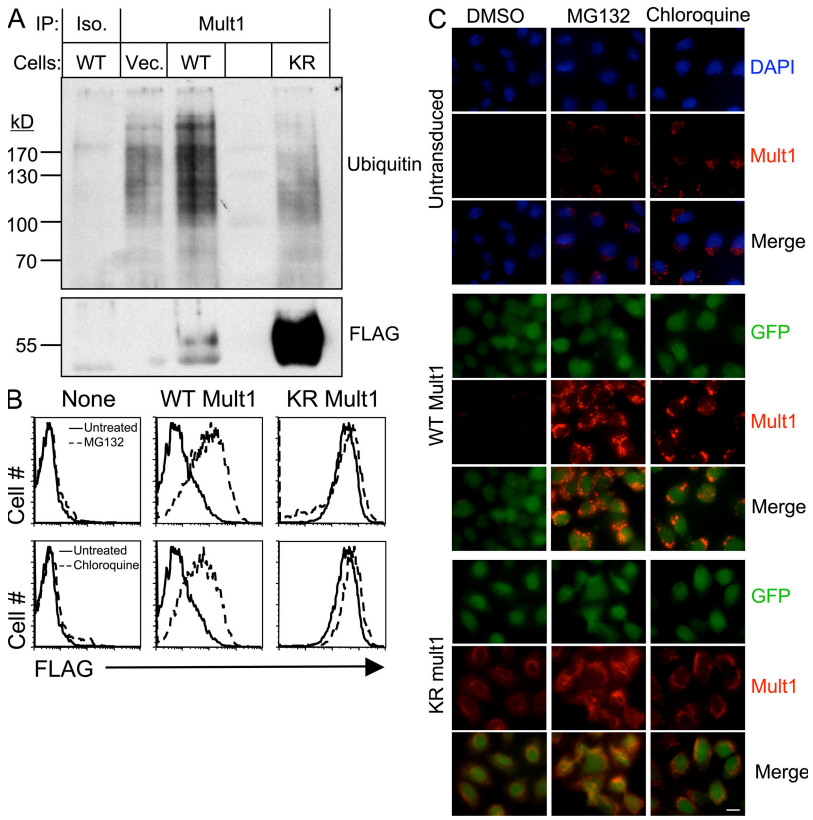

Figure 3. Ubiquitination is involved in the inhibition of Mult1 expression. (A) Fibroblasts transduced with FLAG-tagged Mult1 were lysed in RIPA buffer and Mult1 was immunoprecipitated with Mult1 or control antibody, followed by Western blotting with either ubiquitin or FLAG antibodies. Iso., isotype control; Vec., empty vector transduced; WT, WT Mult1 transduced; KR, Lys to Arg mutant Mult1 transduced. (B) Untransduced, WT Mult1–transduced, or KR Mult1–transduced fibroblasts were treated with either DMSO, 20 µM MG132, or 100 µM chloroquine for 6 h, followed by staining with FLAG antibody and analysis by flow cytometry. (C) Untransduced, WT Mult1–transduced, or KR Mult1–transduced fibroblasts were grown on tissue culture–treated glass slides and treated with DMSO, 20 µM MG132, or 100 µM chloroquine for 6 h. Cells were permeabilized, stained with Mult1 antibodies followed by Alexa Fluor 555–conjugated secondary antibodies and DAPI, and analyzed by immunofluorescence microscopy. Bar, 10 µm. Data in this figure is representative of at least four independent experiments.

Mult1 protein expression is induced by UV irradiation

The inhibition of Mult1 protein expression 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 (17). 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. 4 A). The finding that 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, suggested that UV-induced expression might not be mediated by activation of the DNA damage response.

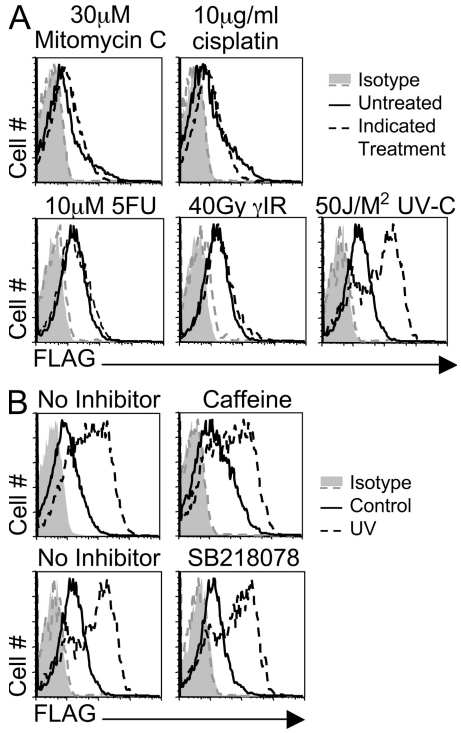

Figure 4. UV irradiation induces transduced Mult1 independently of the DNA damage response pathway. (A) Fibroblasts transduced with FLAG-tagged WT Mult1 were exposed to the indicated genotoxic stress treatments for 16 h, followed by staining with FLAG antibody and flow cytometry. Data are representative of three independent experiments. (B) Fibroblasts transduced with FLAG-tagged WT Mult1 were pretreated for 1 h with either 2 mM caffeine (top right) or 150 nM Chk1 inhibitor (SB218078; bottom right), followed by exposure to UV-C light and staining with FLAG antibodies 16 h later for analysis by flow cytometry. Solid gray histograms and dashed gray lines in A and B represent isotype staining of untreated and treated cells respectively. Data are representative of two independent experiments.

Consistent with this interpretation, inhibitors of critical protein kinases in the DNA damage response pathway, such as Chk1, ataxia-telangiectasia mutated (ATM) and Rad3-related (ATR), and ATM, failed to inhibit protein-level induction of Mult1 by UV radiation (Fig. 4 B). 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 h (17), UV irradiation led to a maximal increase in Mult1 at the cell surface between 4 and 8 h without significantly increasing the abundance of Mult1 transcripts in transduced cells (Fig. 5 A), 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 up-regulate the abundance of the transcripts after genotoxic stress. Alternatively, considering that the effect of UV on Mult1 mRNA levels was not previously tested (17), 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. 5 B), 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. 5, B and C). 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.

The transmembrane and/or cytoplasmic domains of Mult1 appear to play a role in UV-induced cell surface expression, because UV treatment caused a modest cell surface induction of the H60a-Mult1 chimera containing these domains, with no change in the amounts of the corresponding transcripts (Fig. 5 C). In contrast, surface levels of the intact H60a, used as a control, were unchanged by UV treatment (Fig. 5 C). To address whether altered ubiquitination of Mult1 is involved in UV-induced regulation, Mult1-ubiquitination was compared in untreated cells and cells treated with UV by immunoprecipitating Mult1 and immunoblotting with ubiquitin antibodies. Because the UV treatment leads to an increase in the total amount of Mult1 protein as evidenced by increased signal in the FLAG Western blot (55-kD species; Fig. 5 D and Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20081335/DC1), the change in the extent of Mult1 ubiquitination was revealed by normalizing the difference in ubiquitin signal to the increase in FLAG signal. The extent of Mult1 polyubiquitination was reproducibly reduced by UV treatment of the cells. Although it is difficult to accurately quantify ECL Western blotting data, a calculation based on multiple experiments suggested that the fraction of Mult1 that was ubiquitinated in UV-treated cells was reduced between 3- and 20-fold, depending on the experiment (Fig. 5 D, Fig. S2, and not depicted). These data showed that the inefficient expression of Mult1 protein was countered by a UV-induced reduction in ubiquitination, correlating with increased Mult1 expression.

Heat shock induces Mult1 by increasing protein stability

Considering the evidence that the protein level regulation of Mult1 by UV was not mediated by the DNA damage response, it was interesting to find that the cellular response to UV overlaps with a number of other stress-related pathways, including inflammatory cytokine signaling and the heat shock response (27, 28). Whereas inflammatory cytokines did not appreciably induce cell surface expression of transduced or endogenous Mult1 in fibroblasts (unpublished data), heat shock at 45°C did boost Mult1 cell surface expression in

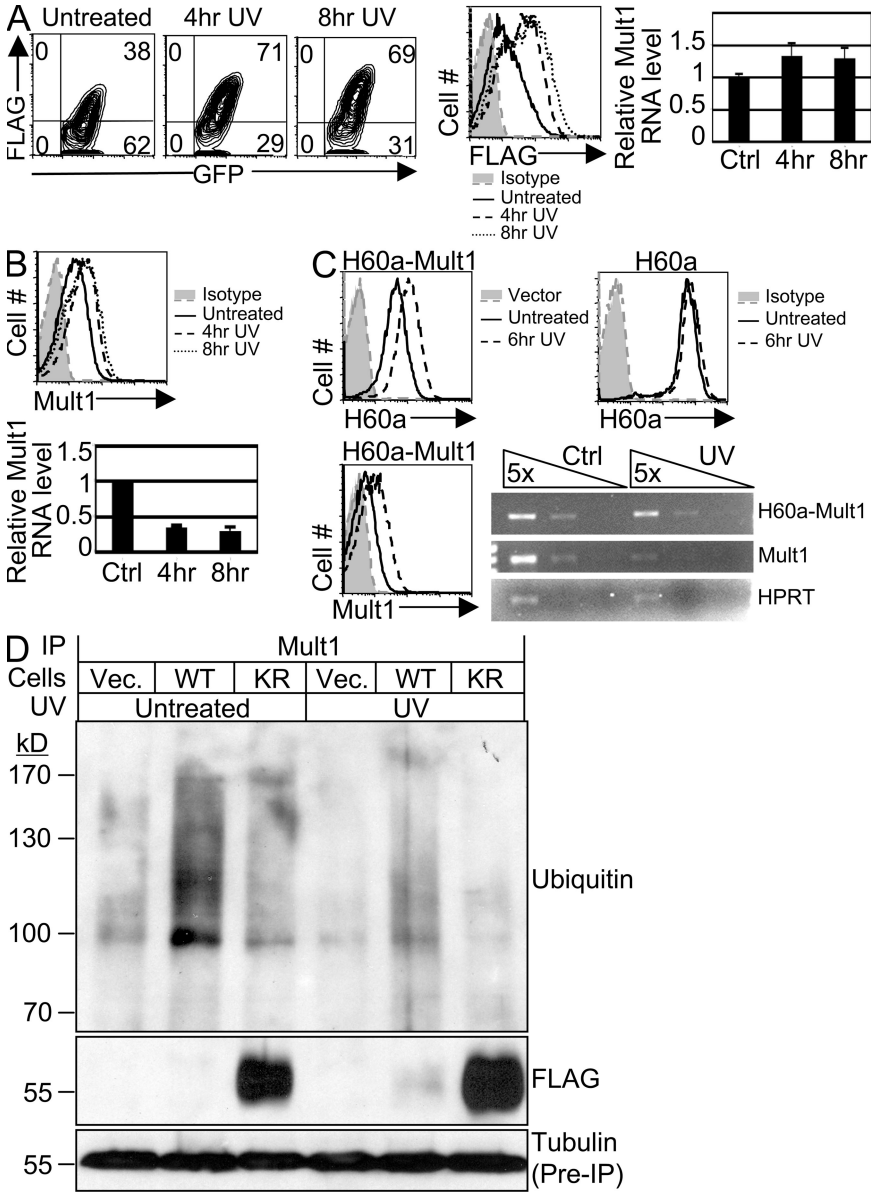

Figure 5. Mult1 induction by UV irradiation is accompanied by a decrease in ubiquitination. (A) Fibroblasts transduced with FLAG-tagged Mult1 were exposed to 50 J/M^2 UV-C irradiation. After UV treatment for 4 or 8 h, cells were stained with FLAG antibody and analyzed by flow cytometry. Cells treated in duplicate were used for Mult1 quantitative PCR and normalized to GAPDH. Data shown is mean ± range; $n = 2$. (B) Untransduced fibroblasts were UV irradiated as in Fig. 1 A, followed by staining with Mult1 antibody at the indicated time after UV treatment. Cells treated in duplicate were used for Mult1 quantitative PCR as in A. (C) B6 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. 1 A, followed by staining with Mult1 or H60a antibody at 6 h after treatment. Parallel cultures of cells transduced with the H60a-Mult1 chimera were used for semiquantitative RT-PCR to quantify transcripts of the chimeric protein, endogenous Mult1, and HPRT. Solid gray histograms and dashed gray lines in A–C represent isotype staining of untreated and treated cells respectively. (D) Fibroblasts transduced with indicated retroviral construct were treated with 50 J/M^2 UV-C irradiation and scraped into RIPA buffer 6 h after UV treatment. Cleared lysates were immunoprecipitated with Mult1 antibody and Western blotted with FLAG or ubiquitin antibodies. Vec., Vector transduced; WT, WT Mult1 transduced; KR, Lys to Arg mutant Mult1 transduced cells. Data in this figure is representative of at least three independent experiments.

transduced cells (Fig. 6 A). As with UV irradiation, this induction by heat shock was correlated with a reduction in the fraction of ubiquitinated Mult1 (Fig. S2). Additionally, heat shock at 45°C boosted cell surface expression of endogenous Mult1 in post-crisis fibroblasts and WEHI 7.1 thymoma cells (Fig. 6 B). In none of these cases did heat shock induce appreciable increases in the amounts of Mult1 transcripts in these cells (Fig. 6, A and B).

It was of interest to test if Mult1 expression was induced by heat shock in normal thymocytes, which contain relatively high levels of preformed Mult1 transcripts (Fig. 1). 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. The modest basal expression in larger thymocytes was boosted substantially by heat shock. Smaller thymocytes, in contrast, expressed little Mult1 before or after heat shock (Fig. 6 C). The increased Mult1 on large, heat-shocked thymocytes was not caused by increased transcription or mRNA stabilization, because mRNA levels were unexpectedly markedly decreased in heat-shocked cells compared with untreated cells (Fig. 6 C). To investigate the mechanism of Mult1 protein level regulation in these cells, thymocytes were treated with MG132 and chloroquine. MG132 treatment resulted in a slight increase in Mult1 expression, but this increase in Mult1 protein may be caused by the pleiotropic effects of MG132 on other levels of gene expression because it was accompanied by an increase in Mult1 RNA levels (Fig. S3, available at http://www.jem.org/cgi/content/full/jem.20081335/DC1). However, treatment

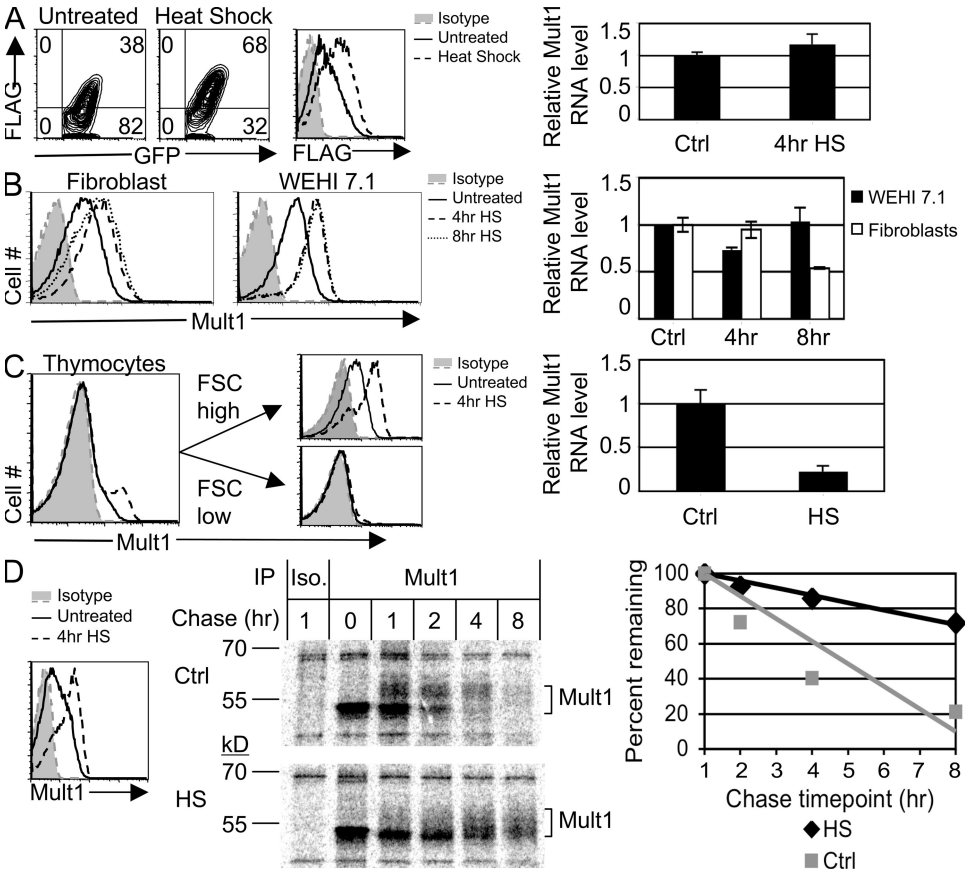

Figure 6. Heat shock induces Mult1 by altering protein half-life. (A) Fibroblasts transduced with FLAG-tagged Mult1 were shifted to 45°C for 30 min, followed by incubation at 37°C for 4 h. Cells were lifted with 5 mM EDTA and stained with FLAG antibody for flow cytometry. Cells treated in duplicate were used for Mult1 quantitative PCR and normalized to GAPDH. Data shown is mean ± range; $n = 2$. (B) Heat shock of untransduced fibroblasts and WEHI 7.1 cells was performed as in A, followed by 37°C 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 in A. HS, heat shock. (C) B6 thymocytes were heat shocked as in A, followed by 37°C incubation for 6 h, staining with Mult1 antibody, and, in some cases, gating on large (forward scatter-high [FSC-high]) or small (FSC-low) cells. Identically treated cells were used for Mult1 quantitative PCR and normalized to 18S ribosomal RNA. Data shown is mean ± SD; $n = 3$. Solid gray histograms and dashed gray lines in A–C represent isotype staining of untreated and treated cells respectively. (D) Analysis of Mult1 protein half-life in heat shocked WEHI 7.1 cells. Cells were pulse labeled with radioactive amino acids and heat shocked at time zero of the chase as in A, followed by incubation at 37°C for the remainder of the chase. Staining of Mult1 on identically treated cells is shown at the 4-h time point. Cells from each time point were lysed in TNT lysis buffer, and Mult1 was immunoprecipitated and resolved by SDS-PAGE. Signal intensity was determined using ImageJ software (NIH). Data in this figure is representative of at least three independent experiments.

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 this subset of cells (Fig. S3). Unlike heat shock, UV irradiation did not alter Mult1 expression on thymocytes (Fig. S3; see Discussion). Together, these results suggest that expression of endogenous Mult1, like transduced Mult1, is inhibited by lysosomal degradation and regulated at the protein level by activation of the heat shock response.

In the context of the preceding data (Fig. 5 D, Fig. S2), which showed a role of Mult1 ubiquitination in regulating protein expression, it seemed likely that the mechanism of regulation by heat shock involved alterations in the stability of Mult1 protein. To quantify the degree of Mult1 protein stabilization in response to heat shock, pulse-chase experiments were performed with WEHI 7.1 cells that had or had not been heat shocked. Heat shock leads to a decrease in translation (29, 30), preventing efficient metabolic labeling of Mult1 after heat shock treatment. Therefore, WEHI 7.1 cells were pulsed with radioactive methionine immediately before heat shock treatment, and Mult1 turnover rate was measured starting at 1 h after heat shock, 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 h in untreated cells to 12 h in heat-shocked cells (Fig. 6 D). This alteration in Mult1 half-life definitively showed that the induction of Mult1 at the cell surface in response to heat shock was caused by protein stabilization.

DISCUSSION

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 UL16-binding proteins, the human orthologues of this family of NKG2D ligands (6, 19, 20). How ligand expression is prevented in these cases has not been previously addressed. In this study, 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 polyubiquitination 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. UV irradiation and heat shock countered this process, resulting in less ubiquitination, increased overall levels of Mult1 in the cells, and substantially elevated cell surface expression.

The protein-level induction by UV documented here cannot be attributed to signaling mediated by the DNA damage response pathway. First, several other agents that reliably induce the DNA damage response, including ionizing radiation, had no effect on protein-level induction of Mult1. Second, induction of Mult1 by UV irradiation was not prevented by inhibitors of Chk1, ATR, and ATM, which are key kinases in the DNA damage response. These inhibitors prevented ligand induction by other genotoxic agents (17). Third, induction of Mult1 by UV resulted in a sharp and rapid decrease in endogenously encoded Mult1 transcripts, whereas induction via the DNA damage response pathway, using aphidicolin and several other agents, resulted in increased amounts of Mult1 transcripts (17). Indeed, of the treatments tested that are known to induce the DNA damage response, UV irradiation was unique in inducing Mult1 at the protein level.

The selective effects of UV irradiation suggest that UV activates other pathways, distinct from the DNA damage response, that induce Mult1 protein stabilization. Previous studies indicate that there is some overlap in the responses to heat shock and UV irradiation (28, 31). In line with these observations, heat shock at 45°C also augmented cell surface expression of Mult1 at the protein level. In addition, both UV irradiation and heat shock specifically induced Mult1, but not Rae1, leading to increased binding by soluble NKG2D receptors (Fig. S4, available at http://www.jem.org/cgi/content/full/jem.20081335/DC1). The latter data confirm that the induced Mult1 binds its physiological ligand.

Mult1 induction at the protein level could facilitate the antitumor immune response because the heat shock response is known to be activated in some cancer cells (32, 33). In this regard, it is notable that Mult1 expression occurs frequently on primary lymphomas and adenocarcinomas, as demonstrated in a recent study (12). Infection by some viruses also induces the heat shock response (34, 35), which may participate in the induction of NKG2D ligands and thus trigger NKG2D-mediated antiviral responses. In both cancer cells and virus-infected cells, activation of the heat shock response underlies increased metabolic output of the cells, enabling tumor growth, viral replication and possibly conferring these dangerous cells with a survival advantage (32, 33). Coupling expression of NKG2D ligands to the heat shock response could be beneficial in countering these advantages and eliminating the corresponding threat.

The induction of NKG2D ligands 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. Consistent with this notion, induction of Mult1 by heat shock peaked after <4 h (Fig. 5 B), whereas peak induction by genotoxic stress was delayed until ~20 h (17). 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 (36, 37).

UV irradiation and heat shock both induced Mult1 protein stabilization, but the signaling pathways leading to this common outcome are not necessarily the same. The finding that heat shock, but not UV-irradiation, induces ligand

expression by thymocytes (Fig. S3) raises the possibility that distinct pathways are involved, but 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 would fit with the notion that thymocytes, unlike skin cells, are not exposed to the damaging effects of UV light. Further experimentation will be necessary to determine if these two types of cell stress operate via a common pathway.

It remains unclear why large, but not small, thymocytes are capable of inducing Mult1 expression. It may be significant that large thymocytes are proliferating (38), as are WEHI 7.1 thymoma cells, and this status correlated with a modest amount of Mult1 expression and Mult1 induction after heat shock (Fig. 6 B, C). The correlation between proliferation and Mult1 expression and inducibility in thymocytes may be accounted for by the observation that the expression of some heat shock proteins is regulated by cell cycle status (39). Stress associated with proliferation (replicative stress) may also contribute to higher Mult1 expression on large thymocytes.

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 (32, 33, 39, 40). Activation of these pathways may underlie the expression of Mult1 and other ligands in tumor cell lines such as YAC-1 and in primary tumors (12). Interestingly, several tumor cell lines, including YAC-1, showed relatively poor induction of Mult1 by heat shock (unpublished data), which is consistent with the possibility that the relevant stress pathway is constitutively activated in these cells. Alternatively, it is possible that YAC-1 and other tumor cells 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.

Mult1 is unique among the murine NKG2D ligands investigated to date in its induction at the protein level in response to heat shock. 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, but nevertheless showed no evidence for this form of protein level regulation (Fig. 2 C and Fig. 5 C). Among the human NKG2D ligands, it was previously demonstrated that MICA is induced by heat shock (16). Although similar to our results 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 (15). 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 posttranscriptionally. Indeed, posttranscriptional regulation of MICA was recently documented by a study showing that virus-encoded proteins can ubiquitinate and down-regulate MICA from the cell surface (41). Whether MICA is similarly regulated by endogenous processes such as those reported here remains to be determined. 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 (42), grouping it with the MIC proteins as candidate for ubiquitin-mediated regulation in humans.

Mult1 degradation in unstressed conditions is inhibited by MG132, which inhibits all ubiquitin-dependent processes, and chloroquine, which specifically inhibits lysosomal degradation. Hence, the data suggest that the lysosome is a primary site for degradation of ubiquitinated Mult1. These data may suggest that ubiquitination of the Mult1 cytoplasmic tail occurs at a post-ER stage of biogenesis, leading to lysosomal targeting and degradation therein. However, it remains possible that the endoplasmic reticulum–associated degradation (ERAD) pathway (43), which involves proteasomal degradation of protein ubiquitinated in the ER, also participates in Mult1 degradation.

Together with previous reports, 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 (Fig. 1) (19). In these cells, Mult1 induction presumably requires induction at the mRNA level (which may be accomplished by the DNA damage response) and in addition induction at the protein level. In other cell types, Mult1 transcripts are abundant ex vivo and protein level regulation may be sufficient for induction. A subset of thymocytes, and possibly other cell types, falls 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.

MATERIALS AND METHODS

Cells, antibodies, and reagents. B6 post-crisis fibroblasts were prepared as previously described (17). All cells were cultured in complete DMEM, consisting of DMEM (Invitrogen), 10% fetal calf serum (Omega Scientific), 100 U/ml penicillin (Invitrogen), 100 μg/ml streptomycin (Invitrogen), 0.2 mg/ml glutamine (Sigma-Aldrich), 10 μg/ml gentamycin sulfate (Lonza), 20 mM Hepes (Thermo Fisher Scientific), and 50 μM 2-mercaptoethanol (EMD Biosciences).

Monoclonal Mult1 antibody (clone 237104; R&D Systems), monoclonal H60a antibody (clone 205326; R&D Systems), monoclonal pan-Rae1 antibody (R&D Systems; clone MAB17582), PE-conjugated goat $F(ab')_2$ fragment to rat IgG (Jackson ImmunoResearch Laboratories), and PE-conjugated goat antibody to mouse IgG1 (SouthernBiotech) were used for flow cytometric analysis. Monoclonal FLAG antibody (clone M2; Stratagene) was used for flow cytometry and Western blotting. Monoclonal Mult1 antibody (clone 1D6; gift from S. Jonjic (University of Rijeka, Rijeka, Croatia) [44]) was used to detect Mult1 by immunofluorescence and for immunoprecipitations. Monoclonal ubiquitin antibody (clone P4D1; Santa Cruz Biotechnology, Inc.), monoclonal α-tubulin antibody (Calbiochem), horseradish peroxidase–conjugated goat anti–mouse IgG (Thermo Fisher Scientific), and peroxidase-conjugated goat anti–mouse light chain (Jackson ImmunoResearch Laboratories) were used for Western blots. NKG2D tetramer was prepared as previously described (5).

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

UV and heat shock treatments. Before 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 subconfluent cultures were heat shocked by incubation in a waterbath or incubator set to 45°C for 30 min, followed by subsequent incubation at 37°C 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 the manufacturer's protocol. RNA was reverse transcribed using Superscript III reverse transcription (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 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and of 60°C for 60 s. 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′; and 18s-3′, 5′-AGCTTATGACCCGCACTTAC-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 from M. Whang and D. Jensen (Univeristy of California, Berkely, Berkeley, CA). H60a and Flag-tagged Mult1 and H60c were subcloned into the pMSCV2.2-IRES-GFP retroviral vector (45) using XhoI and NotI restriction sites. Deletion mutants of Mult1 were made using the following reverse primers: Δ239, 5′-CCAAAGCGGCCGCTCAACGTAAATGTCTTAATTTACAAGAGAC-3′; Δ253, 5′-CCAAAGCGGCCGCTCCAAGAGAGGTGGTGGTGAGGACTGCA-3′; and Δ298, 5′-CCAAAGCGGCCGCTCACAACAGAGGTGGTGCTGAGGACTGCA-3′. The H60a-Mult1 chimeras were constructed using the following primers: H60a EC reverse, 5′-CCAAAGCGGCCGCCCAGGTGACACTCAGACCCT-3′; Mult1 TM forward, 5′-CCAAAGCGGCCGCTTCTTTCAGTACCTTGATGATTATTCTA-3′; H60a forward, 5′-ATCTCTCGAGCCACCATGGCAAAGGGAGCCACCAGCA-3′; H60a TM reverse, 5′-CCAAAGCGGCCGCCAGCATACACCAAGCGAATACCATGA-3′; Mult1 tail forward, 5′-CCAAAGCGGCCGCTTGTAAATTAAGACATTTACGTACAAA-3′. Lysine to arginine mutations were made using the QuikChange protocol (Stratagene) using the following primers: KK310RR(+), 5′-GTGCCTGAGACGTGTTCAGTACGGCGAGAAGATGAATTTCCGACAG-3′; KK310RR(-), 5′-CTGTCGGAAATTCATCTTCTCGCCGTACTGAACACGTCTCAGGCAC-3′; KLK278RLR(+), 5′-GAATGTCTCTGTCCTCGGCGGTTGCGGTCACCTGTGTTTATGCAG-3′; KLK278RLR(-), 5′-CTGCATAAACACAGGTGACCGCAACCGCCGAGGACAGAGACATTC-3′; KK240RR(+), 5′-AATTAAGACATTTACGTACACGACGGATTGGCCTGCAGTCCTCACC-3′; and KK240RR(-), 5′-GGTGAGGACTGCAGGCCAATCCGTCGTGTACGTAAATGTCTTAATT-3′.

Retroviral supernatants were generated by cotransfecting 293T cells with plasmids encoding VSV gag/pol and env and pMSCV2.2 retroviral constructs using Lipofectamine 2000 (Invitrogen). Culture supernatants collected 48 h 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 min, rinsed in PBS, and permeabilized in 0.2% Triton X-100 for 6 min. 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 h. Unbound primary antibody was washed out by rinsing three times in PBS. As indicated in the figure legends, 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 h. Unbound secondary antibody was washed out by rinsing three times in PBS. Coverslips were mounted on slides with Vectashield with DAPI (Vector Laboratories), 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 min 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 [^{35}S] protein labeling mix (PerkinElmer) for 30 min, 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, and 300 mM NaCl) or RIPA lysis buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 7.4, and 150 mM NaCl), as indicated in the figure legends. Both buffers contained protease inhibitor cocktail tablets (Roche). 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 25-gauge needles. Cells were lysed on ice for 30 min and cleared by centrifugation at 16,000 *g* for 15 min. Lysates were precleared twice with protein G PLUS agarose beads (Santa Cruz Biotechnology, Inc.) bound with rat IgG2a isotype control antibodies (eBioscience) and once with uncoupled protein G agarose beads. Precleared lysates were incubated with 1 μg Mult1 antibody (1D6) overnight followed by protein G agarose beads for 2 h. 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 (GE Healthcare), and imaged on GE Healthcare'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. After 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 kD) was measured.

Online supplemental material. Intracellular Mult1 staining of cells treated with MG132 or chloroquine is shown in Fig. S1. A Western blot

detecting polyubiquitinated Mult1 before and after UV or heat shock treatments is shown in Fig. S2. Mult1 levels on thymocytes after UV, heat shock, MG132, or chloroquine treatments are shown in Fig. S3. Surface staining of WT Mult1-transduced fibroblasts with NKG2D tetramer after UV, heat shock, MG132, or chloroquine treatments is shown in Fig. S4. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20081335/DC1.

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