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Cystatin B inhibition of TRAIL-induced apoptosis is associated with the protection of $FLIP_L$ from degradation by the E3 ligase itch in human melanoma cells

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Past studies have identified a number of distinct mechanisms that contribute to the resistance of melanoma cells against apoptosis induced by TNF-related apoptosis-inducing ligand (TRAIL). In this report we show that cystatin B is another endogenous inhibitor of TRAIL-induced apoptosis. Cystatin B-deficient melanoma cell lines established by shRNA knockdown displayed increased apoptosis that was associated with enhanced activation of caspase-8 induced by TRAIL. This was not related to the inhibitory effect of cystatin B on the lysosomal cysteine proteases, cathepsin B and L, as they did not have a role in TRAIL-induced apoptosis in most melanoma cell lines even when cystatin B was inhibited. Instead, sensitization of melanoma cells to TRAIL-induced apoptosis by inhibition of cystatin B appeared associated with decreased stability of $FLIP_L$ as the levels of $FLIP_L$ were reduced because of shortened half-life time in melanoma cells deficient in cystatin B. In contrast, over-expression of cystatin B increased the levels of $FLIP_L$, decreased the amount of the E3 ligase Itch associated with $FLIP_L$, and reduced $FLIP_L$ ubiquitination. Inhibition of Itch by siRNA restored the levels of $FLIP_L$ and blocked sensitization to TRAIL-induced apoptosis associated with deficiency in cystatin B. Taken together, these results indicate that cystatin B regulates Itch-mediated degradation of $FLIP_L$ and thereby TRAIL-induced apoptosis in melanoma cells.
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Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) appears to be a promising candidate for cancer therapeutics because of its ability to preferentially induce apoptosis in malignant cells.[1–3] TRAIL induces apoptosis by binding to two death receptors (Rs), TRAIL-R1 and -R2. This leads to the recruitment of the adaptor protein, Fas-associated death domain (FADD), which in turn recruits the initiator caspase, caspase-8, resulting in the formation of the death-inducing signaling complex (DISC).[1–3] Upon recruitment to the DISC, caspase-8 is activated by auto-proteolytic cleavage, leading to the activation of downstream effector caspases, such as caspase-3, either directly or indirectly by recruitment of the mitochondrial apoptotic pathway through cleavage (activation) of the BH3-only protein, Bid, of the Bcl-2 family, eventually causing apoptotic cell death.[1–3]

We and others have identified a number of distinct mechanisms that contribute to the resistance of melanoma cells to TRAIL-induced apoptosis.[4,5] Among them, the FLICE-like inhibitory protein (FLIP) can interrupt apoptotic signaling at the DISC level by competing with caspase-8 for binding to FADD.[5] FLIP is frequently expressed as two isoforms at the protein level, FLIP long ($FLIP_L$) and FLIP short ($FLIP_S$), which are generated by alternative splicing.[5] Both $FLIP_L$ and $FLIP_S$ contain two N-terminal death effector domains (DED) that allow for interaction with FADD.[5,6] $FLIP_L$ in addition has a C-terminal caspase-like domain and is highly homologous to caspase-8 but has no proteolytic activity.[6] Moreover, $FLIP_L$ can also exert other molecular mechanisms that inhibit TRAIL-induced apoptosis such as activation of NF-κB and Akt.[6]

Besides caspases, other proteases such as calpain and cathepsins are known to have roles in apoptotic signal transduction.[7–9] Under physiological conditions, cathepsins are localized in lysosomes where they function as hydrolases responsible for intralysosomal protein degradation.[10] In response to certain apoptotic stimuli such as TNFα, Fas, and p53, cathepsins are released into the cytosol where some of them, such as the cysteine cathepsins, cathepsin B and L, contribute to the execution of apoptosis either by direct

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Abbreviations: CA074Me, L-3-*trans*-(propylcarbamoyl)oxirane-2-carbonyl]-L-isoleucyl-L-proline methyl ester; DISC, death-inducing signaling complex; FADD, Fas-associated death domain; FLIP, FLICE-like inhibitory protein; MAb, monoclonal antibody; MG132, carbobenzoxy-L-leucyl-L-leucyl-L-leucinal; $\Delta\Psi$m, mitochondrial membrane potential; PI, propidium iodide; shRNA, Short hairpin RNA; siRNA, Small interference RNA; TRAIL, TNF-related apoptosis-inducing ligand; TRAIL-R, TRAIL receptor; z-IETD-fmk, z-Ile-Glu(Ome)-Thr-Asp(Ome)-CH_2F
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cleavage of key cellular substrates, or by acting in concert with caspases.[7–9] Recently, cathepsin B has been shown to be involved in TRAIL-induced apoptosis in various types cells.[11,12] Moreover, it has been reported to have a role in induction of apoptosis of melanoma cells by the antifolate agent pyrimethamine.[13]

The cytosol contains endogenous cysteine cathepsin inhibitors, cystatins, which function as threshold inhibitors to protect cells from detrimental consequences caused by lysosomal release of the cathepsins.[10,14] Among them, cystatin B appears to be of particular interest, in that cystatin B-deficient mice exhibit increased apoptosis of cerebellar granule cells that is associated with the increased expression of apoptosis genes.[10,14] Mutations in cystatin B are responsible for the primary defect in Unverrcht–Lundborg disease (EPM1).[15] Intriguingly, cystatin B is frequently expressed at high levels in cancer cells.[14,16,17] Although the biological significance of the high levels of expression remains to be elucidated, increased cystatin B either on tumor tissues or in serum has been reported to be a biomarker for disease progression in a number of cancers.[16,17]

In view of the potential involvement of cysteine cathepsins in TRAIL-induced apoptosis of melanoma cells, we have tested if cystatin B has a role in the protection of melanoma cells against TRAIL-induced apoptosis. We show in this report that cystatin B contributes to the resistance of melanoma cells to apoptosis induced by TRAIL, but this is, unexpectedly, not due to the inhibition of cysteine proteases, cathepsin B and L, in the majority of melanoma cell lines. We demonstrate that cystatin B stabilizes $FLIP_L$ by preventing its degradation mediated by the E3 ligase Itch, and thus protecting against TRAIL-induced apoptosis in melanoma cells.

Results

Inhibition of cystatin B sensitizes melanoma cells to TRAIL-induced apoptosis. Our initial studies indicated that cystatin B was commonly expressed at relatively high levels in most melanoma cell lines (Figure 1a). To examine if cystatin B has a role in the regulation of sensitivity of melanoma cells to TRAIL-induced apoptosis, we inhibited cystatin B expression in Mel-RM and Mel-FH, two melanoma cell lines that had moderate to high levels of cystatin B and were relatively resistant to TRAIL-induced apoptosis (Figure 1a and Supplementary Figure 1A), with shRNA by lentiviral infections (Figure 1b). Although inhibition of cystatin B enhanced TRAIL-induced activation of caspase-3, cleavage of PARP, and accumulation of sub-G1 DNA contents (Figure 1c and d), it did not have any significant effect on the sensitivity of the cells to apoptosis induced by the DNA-damaging agent cisplatin, and the BH3 mimetic obatoclax, both of which induce apoptosis of melanoma cells independently of the death receptor pathway (Supplementary Figure 1B and data not shown).[18,19] Sensitization of melanoma cells to TRAIL-induced apoptosis by inhibition of cystatin B was confirmed with siRNA knockdown of cystatin B in another two melanoma cell lines (Sk-Mel-28 and IgR3) that are relatively resistant to TRAIL-induced apoptosis (Figure 1e).

Cystatin B-mediated protection against TRAIL-induced apoptosis is not related to its inhibitory effect on cathepsin B in most melanoma cell lines. Cystatin B is known to be an endogenous inhibitor against lysosomal cysteine cathepsins.[10,14] Among the latter, cathepsin B can retain endopeptidase activity at neutral pH in the cytosol upon release from the lysosome, and has been reported to have a role in TRAIL-induced apoptosis in various types of cells.[10,14] Cathepsin B is synthesized as an inactive proenzyme (43 kD) that is processed into active 25 kD and/or 31 kD species.[20] As shown in Figure 2a, cathepsin B in melanoma cell lines was predominantly expressed as the active forms. Of note, ME4405 and Mel-AT, the two melanoma cell lines that were most sensitive to TRAIL-induced apoptosis, appeared to contain the highest levels of cathepsin B (Figure 2a and Supplementary Figure 1A).

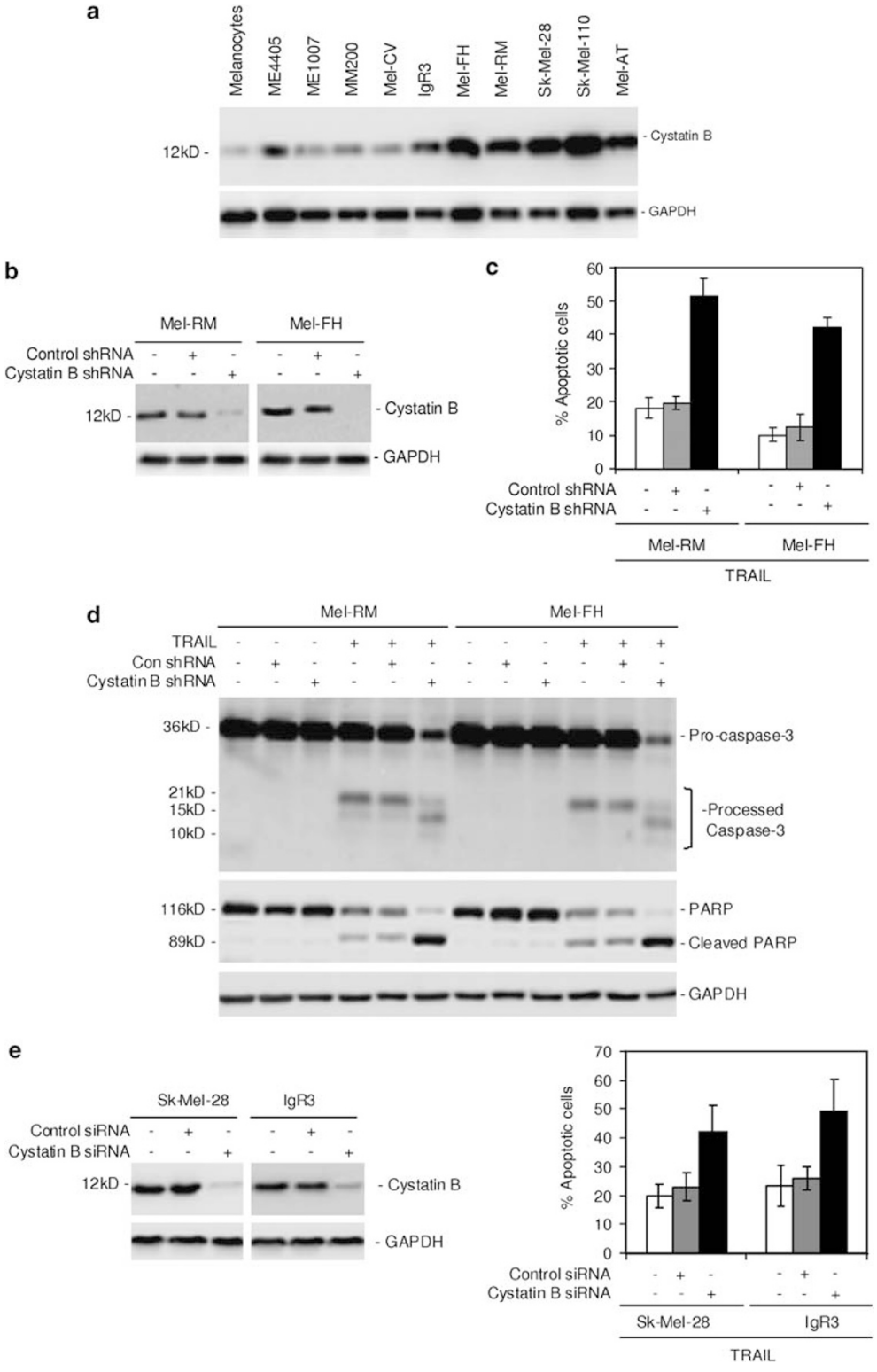
To assess if cathepsin B contributes to TRAIL-induced apoptosis of melanoma cells, the effect of the cathepsin B specific inhibitor CA074Me, as well as the specific inhibitor for another cysteine cathepsin, cathepsin L, z-FF-fmk, on TRAIL-induced apoptosis was examined. Strikingly, although CA074Me partially inhibited TRAIL-induced apoptosis in Me4405 and Mel-AT, the two most sensitive melanoma cell lines, it did not have any notable effect on the sensitivity to TRAIL-induced apoptosis in the other melanoma cell lines (Figure 2b), nor did it inhibit TRAIL-induced apoptosis in Mel-RM and Mel-FH cells with cystatin B knocked down (Figure 2c). Consistently, CA074Me partially blocked TRAIL-induced activation of caspase-3 in ME4405, but not in MM200 cells (Figure 2b). Inhibition of cathepsin L did not alter the sensitivity of melanoma cells to apoptosis induced by TRAIL (Supplementary Figure 2).

To confirm the cell line-dependent effects of inhibition of cathepsin B on TRAIL-induced apoptosis of melanoma cells, we knocked down cathepsin B with siRNA in ME4405 and MM200 cells (Figure 2d). Similar to results with CA074Me, inhibition of cathepsin B by siRNA blocked TRAIL-induced apoptosis in ME4405, but not in MM200 cells. Taken together, these results suggest that although inhibition of cathepsin B by cystatin B may have a role in protection against TRAIL-induced apoptosis in some sensitive melanoma cell lines, for example ME4405 and Mel-AT, it is unlikely to be responsible for inhibition of TRAIL-induced apoptosis by cystatin B in the majority of melanoma cell lines.

Inhibition of cystatin B enhances damage to mitochondria and activation of caspase-8 induced by TRAIL. We focused on investigation of the cathepsin B-independent mechanism(s) by which cystatin B protects melanoma cells against TRAIL-induced apoptosis. As shown in Figure 3a and b, inhibition of cystatin B in Mel-RM and Mel-FH cells with shRNA resulted in increased reduction in mitochondrial membrane potential ($\Delta\Psi m$), and mitochondrial release of cytochrome C, as revealed by the elevated cytosolic levels of cytochrome C, induced by TRAIL. Similarly, TRAIL-induced activation of caspase-9 (Figure 3c) and activation of Bax were enhanced by the inhibition of cystatin B (Supplementary Figure 3).

We also analyzed the activation of caspase-8 induced by TRAIL in Mel-RM and Mel-FH deficient in cystatin B. Figure 3c shows that TRAIL induced increases in caspase-8 activation when cystatin B was inhibited. Of note, the p12 form of cleaved products of caspase-8 was hardly detected in Mel-RM presumably because of relatively low concentrations in the cells.

The importance of enhanced activation of caspase-8 in sensitization of melanoma cells to TRAIL-induced apoptosis by inhibition of cystatin B was confirmed by the inhibitory effect



of the caspase-8 specific inhibitor z-IETC-fmk on TRAIL-induced apoptosis in cystatin B-deficient Mel-RM and Mel-FH cells (Figure 3d). Consistent with this, deficiency in cystatin B resulted in increases in the amount of caspase-8 co-precipitated with FADD, and concomitant reduction in the amount of $FLIP_L$ associated with FADD, induced by TRAIL (Figure 3e), indicating that cystatin B may protect melanoma cells from TRAIL-induced apoptosis by inhibition of recruitment of caspase-8 to the DISC.

Downregulation of $FLIP_L$ in melanoma cells when cystatin B is inhibited. FLICE-like inhibitory protein is an important endogenous inhibitor of activation of caspase-8 induced by death receptors.[4,5] As shown in Figure 4a, inhibition of FLIP by siRNA enhanced TRAIL-induced apoptosis in Mel-RM and Mel-FH cells, whereas overexpression of $FLIP_L$ or $FLIP_S$ protected Mel-CV and MM200 cells from TRAIL-induced apoptosis (Figure 4b), thereby confirming an important role of FLIP in attenuating apoptotic signaling initiated by TRAIL in melanoma cells.

We examined whether FLIP is involved in cystatin B-mediated protection against apoptosis induced by TRAIL. As shown in Figure 4c, the $FLIP_L$ protein levels were noticeably lower in melanoma cells deficient in cystatin B expression. This did not appear to be caused by off-target effects of the shRNA, but due to a post-transcriptional mechanism(s), because shRNA inhibition of cystatin B did not result in any significant change in the levels of FLIP mRNA expression (Supplementary Figure 4). In contrast to inhibition of cystatin B, overexpression of cystatin B increased $FLIP_L$ protein levels as shown in Mel-CV and MM200 cells (Figure 4d). This was associated with the inhibition of TRAIL-induced apoptosis (Figure 4e). Of note, the levels of $FLIP_s$ remained unaltered irrespectively of the levels of cystatin B (Figure 4c and d). Collectively, these results indicate that cystatin B may impinge on $FLIP_L$ expression in melanoma cells.

Cystatin B-mediated protection of melanoma cells against TRAIL-induced apoptosis involves stabilization of $FLIP_L$. Consistent with a rapid turn-over rate of the $FLIP_L$ protein,[6] treatment with the proteasome inhibitor MG-132 could readily increase the $FLIP_L$ levels (Figure 5a). To study whether inhibition of cystatin B can change the stability of $FLIP_L$ in melanoma cells, we treated Mel-RM and Mel-FH cells deficient in cystatin B with cycloheximide for varying periods upto 4 h, and monitored the changes in the levels of $FLIP_L$ expression in western blot analysis. The rate of reduction in the $FLIP_L$ protein after treatment with cycloheximide was accelerated in the cells with cystatin B knocked down by shRNA (Figure 5b). In contrast, when cystatin B was overexpressed in Mel-CV and MM200 cells, the half-life time of the $FLIP_L$ protein was prolonged (Supplementary Figure 5A and B).

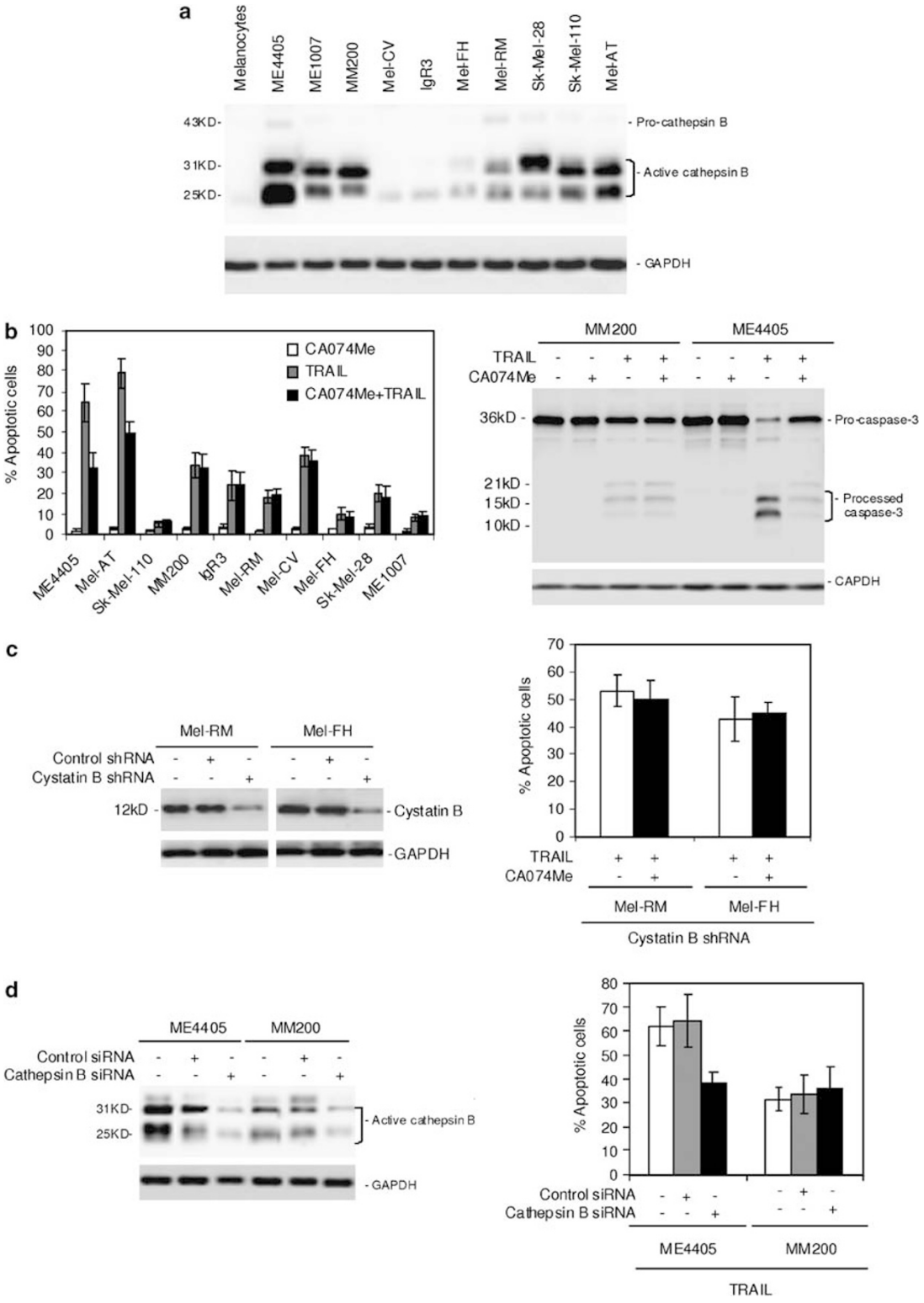
To confirm that decreased $FLIP_L$ contributes to the enhancement of TRAIL-induced apoptosis in melanoma cells deficient in cystatin B, we overexpressed $FLIP_L$ in Mel-RM and Mel-FH cells with cystatin B stably knocked down by shRNA. As shown in Figure 5c and d, overexpression of $FLIP_L$ blocked TRAIL-induced apoptosis and activation of caspase-8 and -3 in the cystatin B-deficient melanoma cells. Of note, there was accumulation of the catalytically inactive p21 form of cleaved caspase-3. This was presumably due to decreased mitochondrial release of Smac/DIABLO as a result of less Bid activation by reduced activation of caspase-8, in that Smac/DIABLO released from the mitochondria is known to be critical for freeing the p21 form of caspsae-3 from inhibition by XIAP in TRAIL-induced apoptosis of melanoma cells.[21] Together, these results indicate that cystatin B stabilizes $FLIP_L$, which in turn protects melanoma cells from TRAIL-induced apoptosis by inhibiting the activation of caspase-8.

Cystatin B prevents $FLIP_L$ from degradation by Itch. As proteasomal degradation of $FLIP_L$ is mediated by the E3 ubiquitin ligase Itch,[22] we examined whether alterations in cystatin B levels may impinge on the interaction between Itch and $FLIP_L$. Figure 6a shows that Itch could be co-precipitated with $FLIP_L$ in melanoma cells, but the amount of Itch in FLIP precipitates from Mel-CV and MM200 cells overexpressing cystatin B was lower than that from the cells transfected with the vector alone (Figure 6b). Consistent with this, overexpression of cystatin B reduced the levels of $FLIP_L$ ubiquitination (Figure 6c). As the levels of $FLIP_L$ expression in cystatin B deficient cells were low, we were unable to compare the amount of Itch associated with $FLIP_L$ expression in these cells with that in the corresponding control cells.

To confirm that the inhibition of interaction between Itch and $FLIP_L$ is responsible for cystatin B-mediated stabilization of

Figure 1 Inhibition of cystatin B sensitizes melanoma cells to TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis. (**a**) Expression of cystatin B in a panel of melanoma cell lines and a melanocyte line. In total 25 μg of total protein of whole cell lysates from a melanocyte line and a panel of melanoma cell lines as indicated was subjected to western blot analysis of cystatin B and GAPDH (as a loading control). The data shown are representative of three individual experiments. (**b**) Inhibition of cystatin B by short hairpin RNA (shRNA). Mel-RM and Mel-FH cells were transduced with the control or cystatin B shRNA. A total of 25 μg of total protein of whole-cell lysates was subjected to western blot analysis of cystatin B and GAPDH (as a loading control). The graphs represent results from cells of clones with lowest cystatin B levels that were expanded and used for subsequent experiments. The data shown are representative of three individual western blot analyses. (**c**) Cystatin B deficient melanoma cells are more sensitive to TRAIL-induced apoptosis. Mel-RM and Mel-FH cells with cystatin B stably inhibited by shRNA as shown in **b** were treated with TRAIL (200 ng/ml) for 24 h before apoptosis was measured by the propidium iodide method using flow cytometry. The data shown are the mean ± S.E. of three individual experiments. (**d**) Inhibition of cystatin B enhances TRAIL-induced activation of casapse-3 and cleavage of PARP. Mel-RM and Mel-FH cells with cystatin B stably inhibited by shRNA as shown in B were treated with TRAIL (200 ng/ml) for 3 h. In all, 25 μg of total protein of whole-cell lysates was subjected to western blot analysis of caspase-3, PARP, and GAPDH (as a loading control). The data shown are representative of three individual Western blot analyses. (**e**) Sensitization of melanoma cells to TRAIL-induced apoptosis by small interfering RNA (siRNA) knockdown of cystatin B. Left panel: Sk-Mel-28 and IgR3 cells were transfected with the control or cystatin B siRNA. Twenty-four hours later, 25 μg of total protein of whole-cell lysates was subjected to western blot analysis of cystatin B and GAPDH (as a loading control). Right panel: Sk-Mel-28 and IgR3 cells were transfected with the control or cystatin B siRNA. Twenty-four hours later, cells were treated with TRAIL (200 ng/ml) for a further 24 h. Apoptosis was measured by the propidium iodide method using flow cytometry. The data shown are either representative (left panel), or the mean ± S.E. (right panel), of three individual experiments

$FLIP_L$, we transfected a siRNA pool for Itch into Mel-RM and Mel-FH cells with cystatin B stably knocked down by shRNA (Figure 6d). These cells expressed reduced levels of $FLIP_L$ in comparison with those transduced with the control shRNA (Figure 4c), but siRNA inhibition of Itch increased the levels of $FLIP_L$, and reduced sensitivity of the cells to TRAIL-induced apoptosis (Figure 6d and e). When Itch was knocked down in Mel-CV and MM200 cells overexpressing cystatin B, it did not



provide further significant protection against TRAIL-induced apoptosis in comparison with overexpression of cystatin B alone (Figure 6b and f).

To determine whether cystatin B blocks the interaction between Itch and $FLIP_L$ by direct binding to these proteins, we carried out GST-pull down assays on whole cell lysates from Mel-RM and Mel-FH cells using either GST-cystatin B or GST as bait. The proteins pulled down were subjected to western blot analysis for Itch and FLIP. Analysis for cathepsin B and L that are known to interact with cystatin B was included as a control. Figure 6g shows that although cathepsin B and L were detected, neither Itch nor FLIP could be observed in the proteins pulled down by GST-cystatin B. Consistently, neither Itch nor FLIP could be co-immunoprecipitated with cystatin B from whole cell lysates of Mel-RM and Mel-FH cells (data not shown).

Discussion

The results above appear to provide several new insights into the regulation of TRAIL-induced apoptosis in melanoma cells. They show that cystatin B, an endogenous cysteine cathepsin inhibitor, protects melanoma cells against TRAIL-induced apoptosis regardless of the absence of involvement of the cathepsins in apoptosis induced by TRAIL. Moreover, they demonstrate that cystatin B stabilizes $FLIP_L$ by interfering with its interaction with the E3 ligase Itch, thus preventing Itch-mediated proteasomal degradation of $FLIP_L$ in melanoma cells.

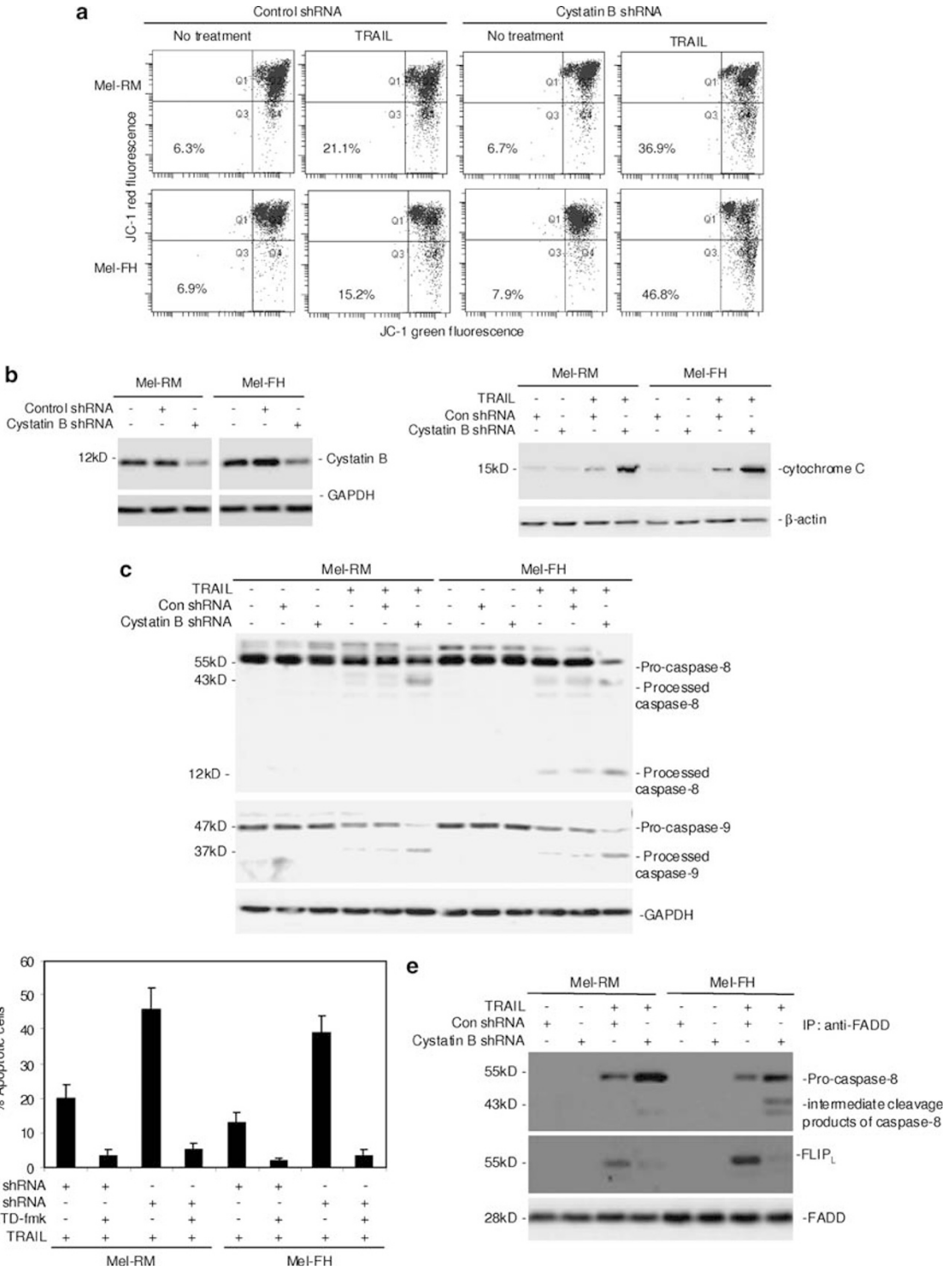
The cysteine proteases, cathepsin B and L, have been reported to contribute to apoptosis induced by various stimuli including TRAIL in a number of cell types.[11–13,23] However, inhibition of cathepsin L did not have any effect on TRAIL-induced apoptosis (Supplementary Figure 2), whereas inhibition of cathepsin B partially blocked apoptosis induced by TRAIL in only two (ME4405 and Mel-AT) of eight melanoma cell lines (Figure 2b). It should be noted that the two lines were most sensitive to TRAIL (Supplementary Figure 1A), even though there was no overall correlation between the levels of cathepsin B expression and sensitivity of melanoma cells to TRAIL-induced apoptosis (data not shown). This puts forward a testable premise that involvement of cathepsin B may enhance the sensitivity of melanoma cells to apoptosis induced by TRAIL. The mechanism by which cathepsin B is involved in TRAIL-induced apoptosis in some but not most melanoma cell lines remains unknown, but it may be related to the levels of cathepsin B expression as ME4405 and Mel-AT contained the highest levels of cathepsin B among the cell lines (Figure 2a). Another possibility is that the mechanism(s) responsible for permeabilizing lysosomes does not operate similarly in melanoma cell lines.[10,12] In this regard, activation of the BH3-only protein, Bim, has been shown to mediate permeabilization of lysosomes in cholangiocarcinoma cells.[12] However, siRNA knockdown of Bim did not block TRAIL-induced apoptosis in ME4405 cells (data not shown), suggesting that failure of cathepsin B to contribute to TRAIL-induced apoptosis is unlikely because of inhibition of Bim. Although it is of interest to address these questions, our focus of this study was to investigate the cathepsin-independent mechanism by which cystatin B protects melanoma cells from TRAIL-induced apoptosis.

Despite the absence of involvement of cathepsin B and L in TRAIL-induced apoptosis in most melanoma cell lines, cystatin B, a well-established endogenous inhibitor against cysteine cathepsins,[10,14] appeared to protect against apoptosis induced by TRAIL. This was demonstrated in two melanoma cell lines deficient in cystatin B established by stable knockdown with shRNA, and was further confirmed by siRNA knockdown of cystatin B in another two melanoma cell lines (Figure 1b, c, and e). Sensitization of melanoma cells to TRAIL-induced apoptosis by inhibition of cystatin B was related to enhanced activation of the mitochondrial apoptotic pathway, and more importantly, related to enhanced activation of caspase-8 and increased association of caspase-8 and FADD (Figure 3), suggesting that cystatin B may inhibit recruitment of caspase-8 to the DISC, thus blocking TRAIL-induced apoptotic signaling transduction.

Cystatin B-mediated inhibition of TRAIL-induced activation of caspase-8 appeared associated with the regulation of $FLIP_L$ expression, in that the levels of $FLIP_L$ were decreased in melanoma cells deficient in cystatin B, but were increased when cystatin B was overexpressed (Figure 4c and d). As a structural homologue of caspase-8, FLIP competes with caspase-8 for binding to FADD, thereby inhibiting recruitment of caspase-8 to the DISC.[5,6] However, our previous studies have shown that there was no general correlation between the

Figure 2 Cystatin B-mediated protection against TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis is not because of its inhibitory effect on cathepsin B in most melanoma cell lines. (**a**) Expression of cathepsin B in a panel of melanoma cell lines and a melanocyte line. A total of 25 μg of total protein of whole-cell lysates from a melanocyte line and a panel of melanoma cell lines as indicated was subjected to western blot analysis of cathepsin B and GAPDH (as a loading control). The data shown are representative of three individual experiments. (**b**) The cathepsin B specific inhibitor L-3-*trans*-(propylcarbamoyl)oxirane-2-carbonyl]-L-isoleucyl-L-proline methyl ester (CA074Me) partially blocks TRAIL-induced apoptosis in ME4405 and Mel-AT, but not in the other melanoma cell lines. Upper panel: Cells were treated with CA074Me (10 μM) for 3 h before the addition of TRAIL (200 ng/ml) for a further 24 h. Apoptosis was measured by the propidium iodide method using flow cytometry. The data shown are the mean ± S.E. of three individual experiments. Lower panel: MM200 and ME4405 cells were treated with CA074Me (10 μM) for 3 h before the addition of TRAIL (200 ng/ml) for a further 3 h. In all, 25 μg of total protein of whole cell lysates was subjected to Western blot analysis of caspase-3 and GAPDH (as a loading control). The data shown are representative of three individual experiments. (**c**) CA074Me does not inhibit TRAIL-induced apoptosis in Mel-RM and Mel-FH cells deficient in cystatin B. Left panel: representative western blot graphs showing reduced cystatin B expression levels in Mel-RM and Mel-FH cells with cystatin B stably knocked down by short hairpin RNA (shRNA). Western blot analysis of GAPDH levels was included as a loading control. Right panel: Mel-RM and Mel-FH cells with cystatin B stably knocked down by shRNA were treated with CA074Me (10 μM) for 3 h before the addition of TRAIL (200 ng/ml) for a further 24 h. Apoptosis was measured by the propidium iodide method using flow cytometry. The data shown are the mean ± S.E. of three individual experiments. (**d**) Small interfering RNA (siRNA) knockdown of cathepsin B blocks TRAIL-induced apoptosis in ME4405, but not in MM200 cells. Left panel: ME4405 and MM200 cells were transfected with the control or cathepsin B siRNA. Twenty-four hours later, 25 μg of total protein of whole-cell lysates was subjected to western blot analysis of cathepsin B and GAPDH (as a loading control). The data shown are representative of three individual experiments. Right panel: ME4405 and MM200 cells were transfected with the control or cathepsin B siRNA. Twenty-four hours later, cells were treated with TRAIL (200 ng/ml) for a further 24 h. Apoptosis was measured by the propidium iodide method using flow cytometry. The data shown are the mean ± S.E. of three individual experiments

levels of FLIP expression and TRAIL-induced apoptosis in melanoma cells.[23] Nevertheless, in this study, inhibition of FLIP enhanced, whereas overexpression of $FLIP_L$ or $FLIP_S$ blocked, TRAIL-induced apoptosis in melanoma cells (Figure 4a and b), indicating that FLIP indeed has a part in regulation of sensitivity of melanoma cells to apoptosis-induced by TRAIL.[4,24] As with FLIP, the levels of cystatin B expression did not appear to correlate in general with the sensitivity of melanoma cells to TRAIL-induced apoptosis (Supplementary Figure 6). These observations reflect the complexity of regulation of TRAIL-induced apoptosis, and suggest that other mechanisms besides cystatin B and FLIP may operate cooperatively to protect melanoma cells from apoptosis induced by TRAIL.[4]



Although expression of FLIP can be regulated at multiple levels,[6,21,25] proteasomal degradation mediated by the ubiquitin-proteasome pathway is one of the most important mechanisms.[26,27] Consistent with this, inhibition of proteasomes readily increased the $FLIP_L$ protein levels in this study (Figure 5a). Significantly, the half-life time of $FLIP_L$ in melanoma cells deficient in cystatin B was shortened, whereas it was prolonged in those overexpressing cystatin B (Figure 5b and Supplementary Figure 5A and B). Moreover, overexpression of $FLIP_L$ blocked the enhancement of TRAIL-induced activation of caspases and apoptosis in cystatin B-deficient melanoma cells (Figure 5c and d). These results indicate that cystatin B impinges on turnover of $FLIP_L$ by stabilizing the protein in melanoma cells.

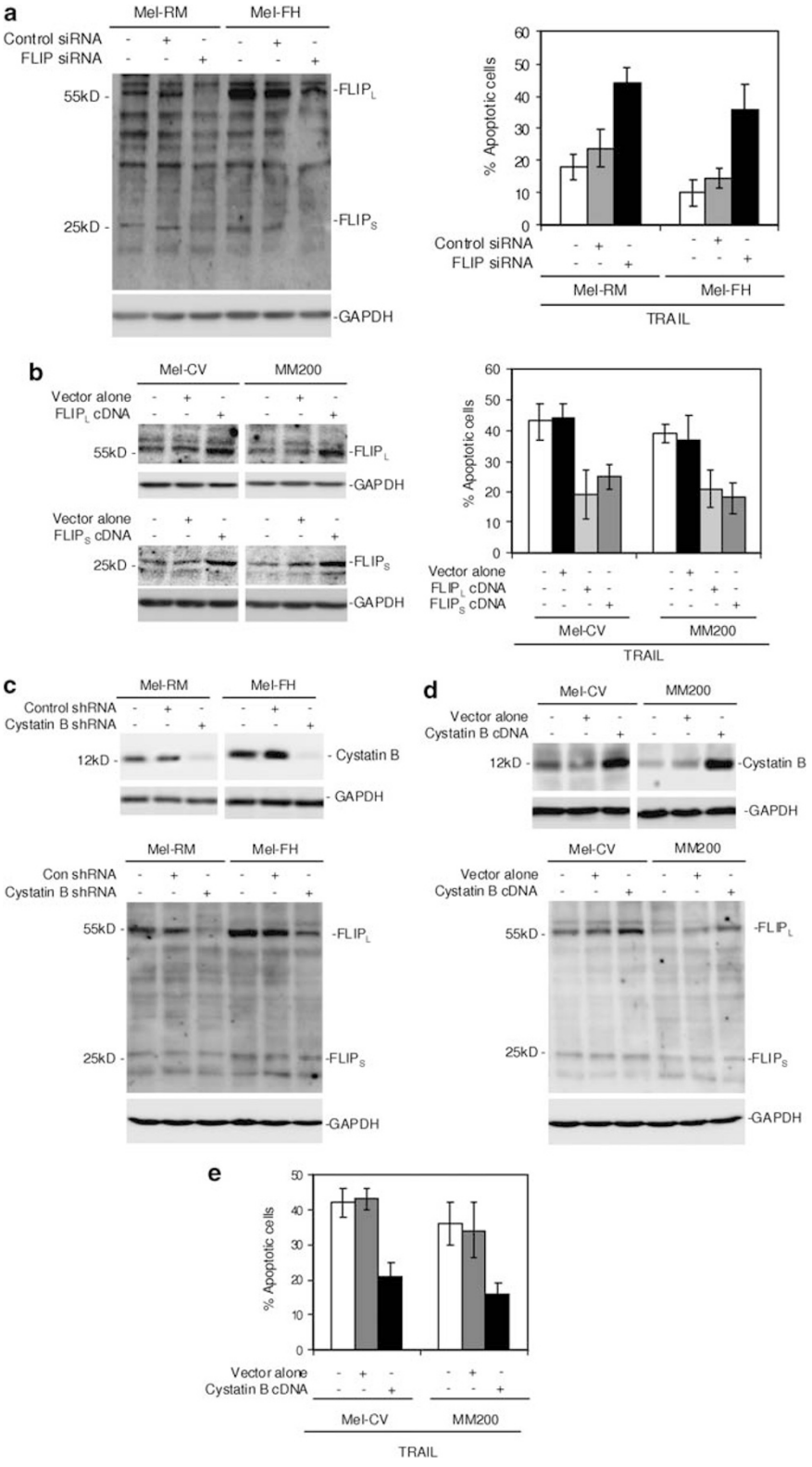
Proteins modified by polyubiquitin chains are recognized and degraded by the proteasome.[28] The specificity of the uniquitin-proteasome pathway is predominantly determined by the E3 ubiquitin ligase.[28] In particular, the member of the homologous to the E6-AP carboxyl terminus (HECT)-containing E3 ligase family, Itch, is known to interact with $FLIP_L$ and mediate its degradation.[21] We confirmed in this study that Itch interacted with $FLIP_L$, but the amount of Itch associated with $FLIP_L$ reduced in melanoma cells overexpressing cystatin B, which was associated with decreased ubiquitination of $FLIP_L$ (Figure 6a–c). Moreover, inhibition of Itch in melanoma cells deficient in cystatin B not only increased the levels of $FLIP_L$ but also reduced the increased sensitivity to TRAIL-induced apoptosis (Figure 6d and e). In contrast, inhibition of Itch in melanoma cells overexpressing cystatin B did not provide further protection against apoptosis induced by TRAIL (Figure 6f). Collectively, these results suggest a role for cystatin B in regulation of the interaction of Itch with $FLIP_L$, which is, at least in part, responsible for sensitization of melanoma cells to TRAIL-induced apoptosis by inhibition of cystatin B in melanoma cells.

How cystatin B inhibits the Itch-$FLIP_L$ interaction remains unknown. One possibility is that cystatin B may bind to Itch and/or $FLIP_L$, thereby blocking their interactions. However, neither Itch nor $FLIP_L$ could be pulled down from whole-cell lysates with GST-cystatin B (Figure 6g), nor could the two proteins be co-precipitated (data not shown), suggesting that cystatin B may not physically interact with Itch or $FLIP_L$ in melanoma cells. Itch-mediated proteasomal degradation of $FLIP_L$ is known to be regulated by JNK-mediated Itch phosphorylation.[21] Activation of Akt, PKC, and casein kinase (CK2) has also been shown to protect $FLIP_L$ from proteasomal degradation.[29–31] In addition, p53 has been reported to form a complex with Itch and $FLIP_L$ upon treatment with cisplatin that facilitated the downregulation of $FLIP_L$ in ovarian cancer cells.[32] It is conceivable that cystatin B may interfere with the Itch–$FLIP_L$ interaction by impinging on one or more of these mechanisms. Potential regulation of Itch-mediated degradation of $FLIP_L$ by multiple mechanisms may also account for the observation that there was no general correlation between the endogenous levels of $FLIP_L$ and cystatin B in melanoma cells (Figures 1a, 4a and d). For example, similar levels of cystatin B were detected in both Mel-FH and Mel-RM cells, but Mel-FH cells expressed higher levels of $FLIP_L$ than Mel-RM cells. Regulation of $FLIP_L$ at levels besides post-translational degradation may also have a part in the lack of correlation between the expression of $FLIP_L$ and cystatin B.[6,21,25]

There is increasing evidence that cystatin B expression is elevated in cancer cells, which may serve as a biomarker for disease progression and prognosis of patients.[14,16,17] However, the current understanding of the mechanism(s) of cystatin B action under physiological and pathological conditions remains largely confined to its ability to inhibit cysteine cathepsins.[33] With regard to regulation of apoptosis, cystatin B-deficient mice are known to exhibit increased apoptosis of cerebella granule cells associated with increased expression of apoptosis genes, many of which are not the genes encoding cysteine cathepsins.[34] Moreover, sensitization of cystatin B-deficient thymocytes to staurosporin-induced apoptosis has been shown to be independent of cysteine cathepsins.[35] To our knowledge, this study is the first to show that cystatin B protects melanoma cells from TRAIL-induced apoptosis by inhibiting the interaction between Itch and $FLIP_L$. Nonetheless, we do not rule out other mechanisms by which cystatin B may protect melanoma cells from apoptosis, such as inhibition of oxidative stress.[36] Cystatin B deficiency has been reported to sensitize neurons to apoptosis mediated by oxidative stress.[36] The latter is known to enhance TRAIL-induced apoptosis in various types of cells.[37]

Figure 3 Inhibition of cystatin B enhances TNF-related apoptosis-inducing ligand (TRAIL)-induced mitochondrial apoptotic events and activation of caspase-8. (**a**) Cystatin B deficiency enhances TRAIL-induced reduction in mitochondrial membrane potential ($\Delta\Psi$m). Mel-RM and Mel-FH cells with cystatin B stably knocked down by Short hairpin RNA (shRNA) as shown in B treated with TRAIL (200 ng/ml) for 3 h were subjected to measurement of $\Delta\Psi$m by JC-1 staining in flow cytometry. The number in each left bottom quadrant represents the percentage of cells with reduction in $\Delta\Psi$m. The data shown are representative of three individual experiments. (**b**) Cystatin B deficiency increases TRAIL-induced mitochondrial release of cytochrome C into the cytosol. Left panel: representative western blot graphs showing reduced cystatin B expression levels in Mel-RM and Mel-FH cells with cystatin B stably knocked down by shRNA. Western blot analysis of GAPDH levels was included as a loading control. Right panel: 25 μg of total protein of cytosolic fractions from Mel-RM and Mel-FH cells with cystatin B stably knocked down by shRNA treated with TRAIL (200 ng/ml) for 3 h was subjected to Western blot analysis of cytochrome C. Western blot analysis of β-actin levels was included as a loading control. The data shown are representative of three individual experiments. (**c**) Cystatin B deficiency enhances TRAIL-induced activation of caspase-8 and -9. In total 25 μg of whole-cell lyastes from Mel-RM and Mel-FH cells with cystatin B stably knocked down by shRNA as shown in B treated with TRAIL (200 ng/ml) for 3 h were subjected to western blot analysis of caspase-8, casapse-9, and GAPDH (as a loading control). The data shown are representative of three individual experiments. (**d**) Inhibition of caspase-8 by the selective inhibitor z-IETD-fmk blocked TRAIL-induced apoptosis in cystatin B-deficient Mel-RM and Mel-FH cells. Mel-RM and Mel-FH cells with cystatin B stably knocked down by shRNA as shown in B were treated with z-IETD-fmk (20 μM) for 1 h before the addition of TRAIL (200 ng/ml) for a further 24 h. Apoptosis was measured by the propidium iodide method using flow cytometry. The data shown are the mean $\pm$ S.E. of three individual experiments. (**e**) Cystatin B deficiency enhances TRAIL-induced recruitment of caspase-8 to Fas-associated death domain (FADD). Mel-RM and Mel-FH cells with cystatin B stably knocked down by shRNA as shown in B were treated with TRAIL (200 ng/ml) for 30 min. Whole-cell lysates were subjected to immunoprecipitation with an antibody against FADD. A total of 30 μg of total protein of the resulting precipitates was subjected to SDS-PAGE and probed with antibodies against caspase-8, FLICE-like inhibitory protein (FLIP), and FADD. The data shown are representative of three individual experiments

In conclusion, we have shown in this study that cystatin B is an endogenous inhibitor of TRAIL-induced apoptosis of melanoma cells. This is, at least in part, because of the inhibition of Itch-mediated proteasomal degradation of $FLIP_L$. These findings appear to be of practical significance, in that they provide a molecular basis for targeting



cystatin B to enhance therapeutic efficacy of TRAIL in human melanoma.

Materials and Methods

Cell lines. Human melanoma cell lines Mel-RM, MM200, IgR3, Mel-CV, Mel-FH, Mel-AT, Sk-Mel-28, Sk-Mel-110, ME1007, and ME4405, have been described previously.[23] They were cultured in DMEM containing 5% FCS (Commonwealth Serum Laboratories, Melbourne, Vic, Australia). The cultured human melanocyte line HEMn-MP was purchased from Banksia Scientific (Bulimba, Qld, Australia) and the cells were cultured in medium supplied by Clonetics (Edward Kellar, Vic, Australia).

Antibodies (Abs), recombinant proteins, and other reagents. The mouse monoclonal Ab (MAb) against Cystatin B was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The rabbit polyclonal Abs against caspase-3, caspase-9, and the mouse MAbs against caspase-8, and FADD were from Stressgen (Victoria, BC, Canada). The mouse MAb against cathepsin B and the rabbit polyclonal Ab against cathepsin L were purchased from Calbiochem (La Jolla, CA, USA). The mouse MAbs against Itch, cytochrome C, and PARP were from Pharmingen (Bioclone, Marrickville, NSW, Australia). The rabbit polyclonal anti-Bax against amino acids 1 through 20 was purchased from Upstate Biotechnology (Lake Placid, NY, USA). The mouse MAb against FLIP and the rat MAb against FLIP were from Alex Biochemicals (San Diego, CA, USA). Rat IgG, mouse IgG, and rabbit IgG were from Santa Cruz Biotechnology. Recombinant human TRAIL was supplied by Genentech (San Francisco, CA, USA). The preparation was supplied as a leucine zipper fusion protein, which required no further cross-linking for maximal activity. The cell-permeable cathepsin B inhibitor L-3-*trans*-[(propylcarbamoyl)oxirane-2-carbonyl]-L-isoleucyl-L-proline methyl ester (CA074Me), the cathepsin L inhibitor Z-Phe-Phe-CH_2F (z-FF-fmk), the general caspase inhibitor Z-Val-Ala-Asp(OMe)-CH_2F (z-VAD-fmk), the caspase-8-specific inhibitor Z-Ile-Glu(Ome)-Thr-Asp(Ome)-CH_2F (z-IETD-fmk), the proteasome inhibitor carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (MG132), and cycloheximide were purchased from Calbiochem.

Apoptosis. Quantitation of apoptotic cells by measurement of sub-G1 DNA content using the propidium iodide (PI) method was carried out as described elsewhere.[23] In brief, melanoma cells were adhered overnight in 24-well plates (Falcon 3047; Becton Dickinson, Lane Cove, NSW, Australia) at a concentration of 1×10^5 cells per well. Cells were treated as desired. Floating and adherent cells were then harvested and incubated overnight at 4 °C in the dark with 750 μl of a hypotonic buffer (50 μg/ml PI in 0.1% sodium citrate plus 0.1% Triton X-100) before flow cytometric analysis using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA).

Flow cytometry. Immunostaining on intact and permeabilized cells was carried out as described previously.[23] Analysis was carried out using a FACScan flow cytometer (Becton Dickinson).

$\Delta\Psi$m. Melanoma cells were seeded at 1×10^5 cells per well in 24-well plates and allowed to reach exponential growth for 24 h before treatment. Changes in $\Delta\Psi$m were studied by staining the cells with the cationic dye, JC-1, according to the manufacturer's instructions (Molecular Probes, Eugene, OR, USA) as described previously.[19]

Western blot analysis. Western blot analysis was carried out as described previously.[19] Labeled bands were detected by Immun-Star HRP chemiluminescent kit, and images were captured and the intensity of the bands was quantitated with the Bio-Rad VersaDoc image system (Bio-Rad, Regents Park, NSW, Australia).

Immunoprecipitation. Methods used were as described previously with minor modification.[38] Briefly, 100 μl of lysates were pre-cleared by incubation with 20 μl of a mixture of protein A and protein G sepharose packed beads (Santa Cruz Biotech, Santa Cruz, CA, USA) in a rotator at 4 °C for 2 h and then with 20 μl of freshly packed beads in a rotator at 4 °C overnight. In all, 10 μg of the designed antibody or corresponding control immunoglobulin was then added to the lysate and rotated at 4 °C for 2 h. The beads were then pelleted by centrifugation and washed five times with ice-cold lysis buffer before elution of the proteins from the beads in lysis buffer at room temperature for 1 h. A total of 25–30 μg of total protein of the resulting immunoprecipitates was then subjected to SDS-PAGE and western blot analysis.

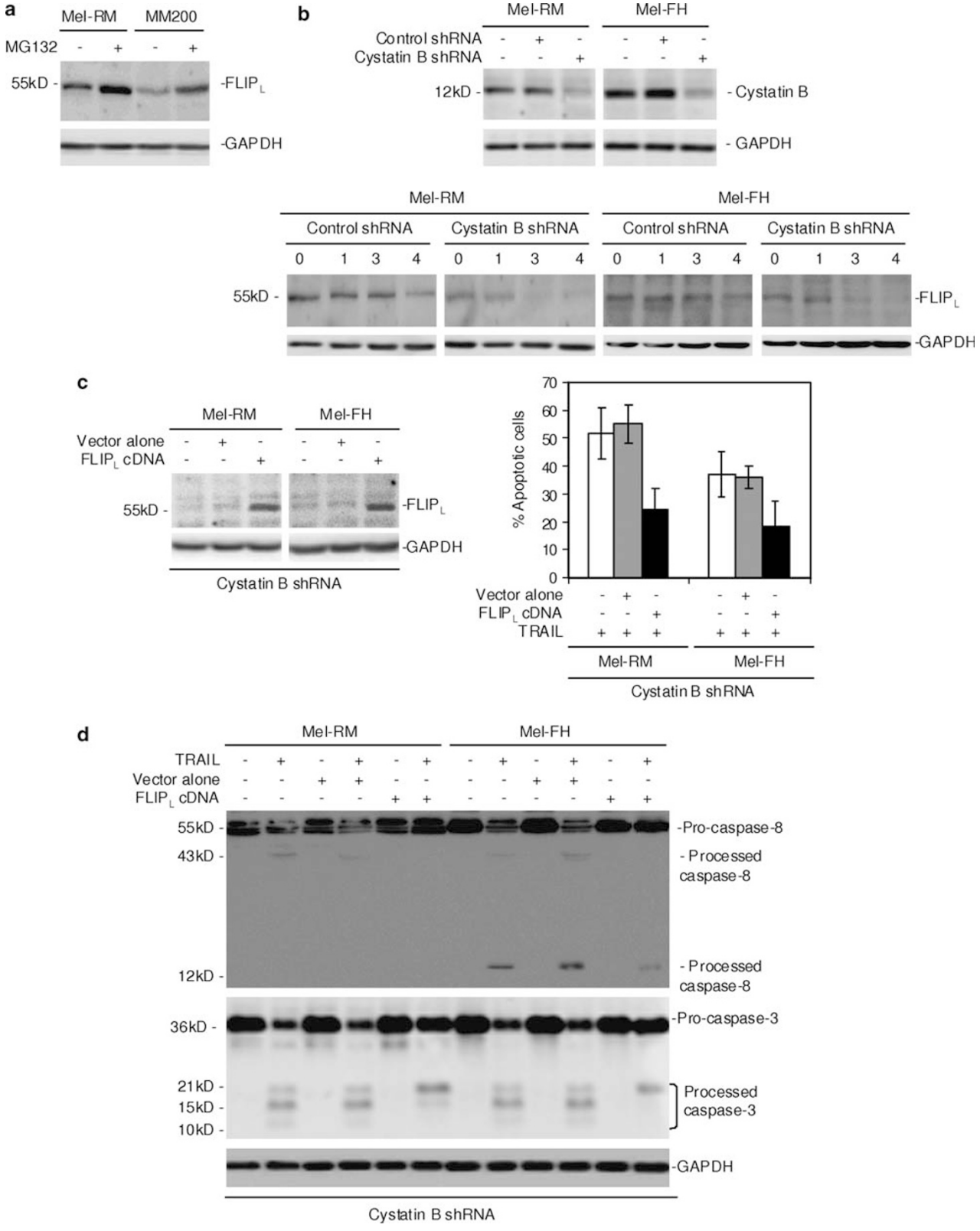
Preparation of mitochondrial and cytosolic fractions. Methods used for subcellular fractionation were similar to those described previously.[23]

GST-pulldown assays. Using a plasmid vector containing cystatin B as template, a PCR product was amplified with 5′-cgtcgGGATCCagatgatgtgcggggcgc-3′ and 5′-cGAATTCagaaataggtcagctcatcatgc-3′ oligonucleotides to incorporate *Bam*HI and *Eco*RI restriction sites. The product was subcloned into the pGEX-3 vector (GE Healthcare, Sydney, NSW, Australia) and the DNA sequence verified by automated sequencing. BL21 (DE3) cells (Stratagene, Melbourne, VIC., Australia) were then transformed with pGEX-3X-cystatin B or with pGEX-3X to express GST alone before preparation of recombinant proteins adsorbed to glutathione sepharose 4B (GE Healthcare) as previously described. Whole cell lysates were incubated with GST or GST-cystatin B at 4 °C for 2 h before washing the beads four times in lysis buffer. Samples were then eluted with SDS-PAGE sample buffer and analyzed by western blotting.

Plasmid vector and transfection. Cystatin B cDNA cloned into the pcDNA3.1 vector was kindly provided by Dr. D-G Kim (Korea Research Institute of

Figure 4 Sensitization of melanoma cells to TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis by inhibition of cystatin B is associated with down-regulation of FLICE-like inhibitory protein ($FLIP_L$). (**a**) Inhibition of FLIP sensitizes melanoma cells to TRAIL-induced apoptosis. Left panel: Mel-RM and Mel-FH cells were transfected with the control or FLIP siRNA. Twenty-four hours later, 30 μg of total protein of whole-cell lysates was subjected to western blot analysis of FLIP and GAPDH (as a loading control). The data shown are representative of three individual experiments. Right panel: Mel-RM and Mel-FH cells were transfected with the control or FLIP siRNA. Twenty-four hours later, cells were treated with TRAIL (200 ng/ml) for a further 24 h. Apoptosis was measured by the propidium iodide method using flow cytometry. The data shown are the mean ± S.E. of three individual experiments. (**b**) Overexpression of FLIP inhibits melanoma cells from TRAIL-induced apoptosis. Left panel: 30 μg of total protein of whole-cell lysates from Mel-CV and MM200 cells stably transfected with the vector alone or cDNA encoding $FLIP_L$ or $FLIP_S$ was subjected to western blot analysis of FLIP and GAPDH (as a loading control). The data shown are representative of three individual experiments. Right panel: Mel-CV and MM200 cells stably transfected with the vector alone or the cDNA encoding $FLIP_L$ or $FLIP_S$ were treated with TRAIL (200 ng/ml) for 24 h. Apoptosis was measured by the propidium iodide method using flow cytometry. The data shown are the mean ± S.E. of three individual experiments. (**c**) $FLIP_L$ expression is downregulated in melanoma cells deficient in cystatin B. Upper panel: representative western blot graphs showing reduced cystatin B expression levels in Mel-RM and Mel-FH cells with cystatin B stably knocked down by short hairpin RNA (shRNA). Western blot analysis of GAPDH levels was included as a loading control. Lower panel: 30 μg of total protein of whole-cell lysates from Mel-RM and Mel-FH cells with cystatin B stably knocked down by shRNA was subjected to western blot analysis of FLIP and GAPDH (as a loading control). The data shown are representative of three individual experiments. (**d**) $FLIP_L$ expression is increased in melanoma cells over-expressing cystatin B. Upper panel: 25 μg of total protein of whole-cell lysates from Mel-CV and MM200 cells stably transfected with the vector alone or cDNA encoding cystatin B was subjected to western blot analysis of cystatin B and GAPDH (as a loading control). The data shown are representative of three individual western blot analyses. Lower panel: 30 μg of total protein of whole-cell lysates from Mel-CV and MM200 cells stably transfected with the vector alone or cDNA encoding cystatin B were subjected to western blot analysis of FLIP and GAPDH (as a loading control). The data shown are representative of three individual western blot analyses. (**e**) Overexpression of cystatin B inhibits TRAIL-induced apoptosis of melanoma cells. Mel-CV and MM200 cells stably transfected with the vector alone or cDNA encoding cystatin B as shown in **d** were treated with TRAIL (200 ng/ml) for 24 h. Apoptosis was measured by the propidium iodide method using flow cytometry. The data shown are the mean ± S.E. of three individual experiments

Bioscience and Biotechnology, Daejeon, Republic of Korea) and described elsewhere.[16] $FLIP_L$ cDNA and $FLIP_S$ cDNA cloned into the pCR vector were kindly provided by Dr. H Nakano (Juntendo University School of Medicine, Tokyo, Japan).[39] Melanoma cells were seeded at 1×10^5 cells per well in 24-well plates 24 h before transfection. Cells were transfected with 0.8 μg plasmid as well as the empty vector (Sigma-Aldrich, Castle Hill, NSW, Australia) in Opti-MEM medium (Invitrogen, Carlsbad, CA, USA) with Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol. Six hours after transfection,



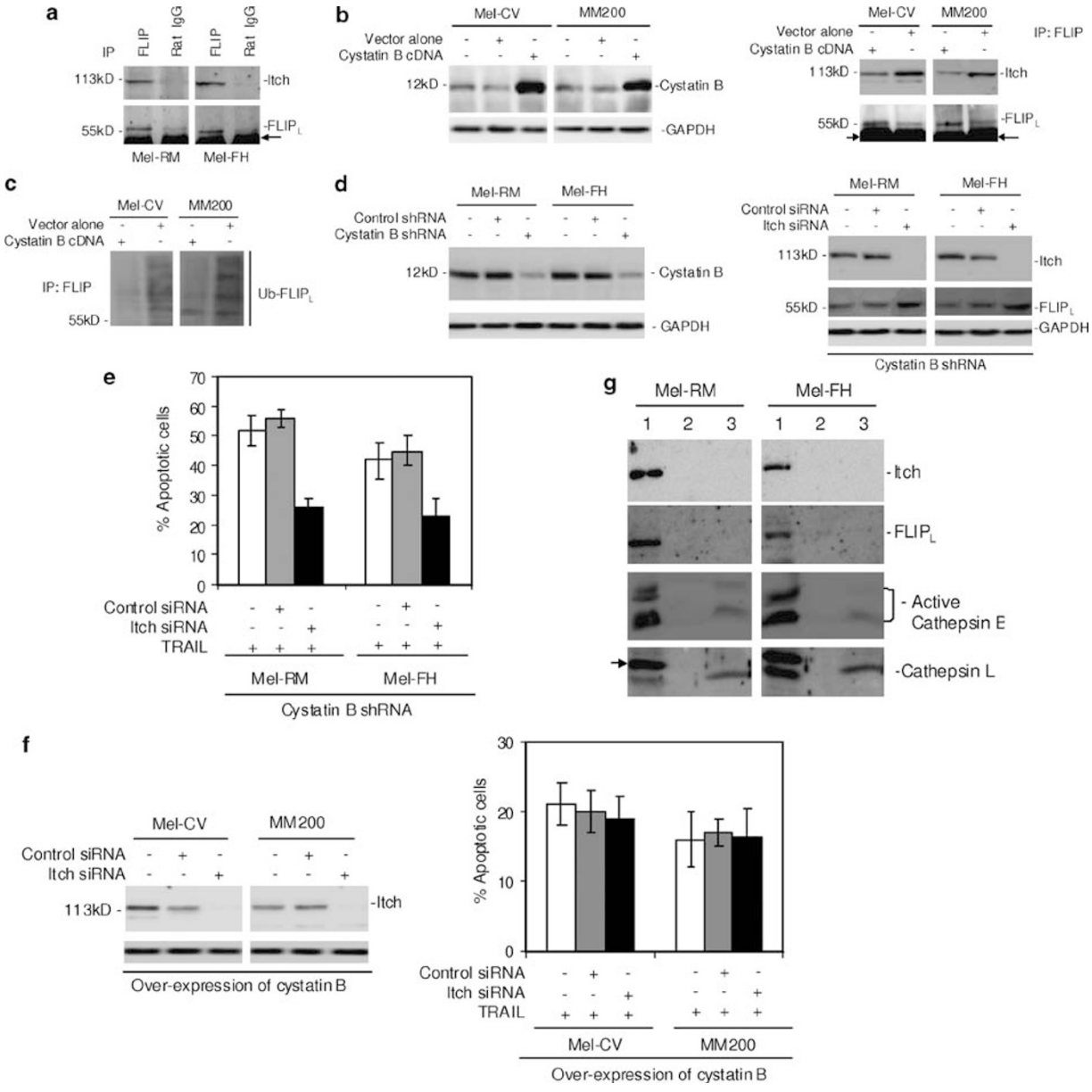


Figure 6 For caption see next page

Figure 5 Cystatin B stabilizes FLICE-like inhibitory protein (FLIP)$_L$ in melanoma cells. (**a**) Inhibition of proteasomal degradation increases the levels of FLIP$_L$. In all, 30 μg of total protein of whole cell lysates from Mel-RM and MM200 cells with or without treatment with MG132 (10 μM) for 2 h were subjected to western blot analysis of FLIP and GAPDH (as a loading control). The data shown are representative of three individual western blot analyses. (**b**) Deficiency in cystatin B reduces the half-life time of FLIP$_L$. Upper panel: representative western blot graphs showing reduced cystatin B expression levels in Mel-RM and Mel-FH cells with cystatin B stably knocked down by short hairpin RNA (shRNA). Western blot analysis of GAPDH levels was included as a loading control. Lower panel: Mel-RM and Mel-FH cells with cystatin B stably knocked down by shRNA were treated with cycloheximide (10 μg/ml) for indicated periods. A total of 30 μg of total protein of whole-cell lysates was subjected to western blot analysis of FLIP and GAPDH (as a loading control). The data shown are representative of three individual experiments. (**c**) Overexpression of FLIP$_L$ inhibited sensitization of melanoma cells to Tumor necrosis factor (TNF)-related apoptosis-inducing ligandTRAIL-induced apoptosis by inhibition of cystatin B. Left panel: Mel-RM and Mel-FH cells with cystatin B stably knocked down by shRNA as shown in B were transiently transfected with the vector alone or cDNA encoding FLIP$_L$. Twenty-four hours later, 30 μg of total protein of whole-cell lysates was subjected to western blot analysis of FLIP and GAPDH (as a loading control). The data shown are representative of three individual expreiments. Right panel: Mel-RM and Mel-FH cells with cystatin B stably knocked down by shRNA as shown in B were transiently transfected with the vector alone or cDNA encoding FLIP$_L$. Twenty-four hours later, cells were treated with TRAIL (200 ng/ml) for 24 h. Apoptosis was measured by the propidium iodide method using flow cytometry. The data shown are the mean ± S.E. of three individual experiments. (**d**) Overexpression of FLIP$_L$ inhibited TRAIL-induced activation of caspase-8 and -3 in melanoma cells deficient in cystatin B. Mel-RM and Mel-FH cells with cystain B stably knocked down by shRNA as shown in B were transiently transfected with cDNA encoding FLIP$_L$ as shown in C. Cells were treated with TRAIL (200 ng/ml) for 3 h. In total 30 μg of total protein of whole-cell lysates was subjected to Western blot analysis of casapse-8, caspase-3, and GAPDH (as a loading control). The data shown are representative of three individual experiments

Figure 6 Cystatin B prevents FLICE-like inhibitory protein (FLIP)$_L$ from degradation by Itch. (**a**) Itch binds to FLIP$_L$ in melanoma cells. Whole-cell lysates from Mel-RM and Mel-FH were subjected to immunoprecipitation using a rat antibody against FLIP. Purified rat IgG was used as a control. In all, 30 μg of total protein of resulting precipitates were subjected to SDS-PAGE and probed with antibodies against Itch and FLIP. The arrow-head points to bands of immunoglobulin heavy chain. The data shown are representative of three individual experiments. (**b**) Overexpression of cystatin B reduces the amount of Itch associated with FLIP$_L$. Left panel: representative western blot graphs showing cystain B was overexpressed in Mel-CV and MM200 cells stably transfected with cDNA encoding cystatin B but not in those transfected with the vector alone. Western blot analysis of GAPDH levels was included as a loading control. Right panel: whole-cell lysates from Mel-CV and MM200 cells overexpressing cystatin B were subjected to immunoprecipitation using a rat antibody against FLIP. A total of 30 μg of total protein of the resulting precipitates was subjected to SDS-PAGE and probed with antibodies against Itch and FLIP. The arrow-head points to bands of immunoglobulin heavy chain. The data shown are representative of three individual experiments. (**c**) Overexpression of cystatin B-blocked ubiquitination of FLIP$_L$. Whole-cell lysates from Mel-CV and MM200 cells overexpressing cystatin B as shown in B were subjected to immunoprecipitation using a rat antibody against FLIP. A total of 30 μg of total protein of the resulting precipitates were subjected to SDS-PAGE and probed with an antibody against ubiquitin. The data shown are representative of three individual experiments. (**d**) Inhibition of Itch increased FLIP$_L$ expression in melanoma cells deficient in cystatin B. Left panel: representative western blot graphs showing reduced cystatin B expression levels in Mel-RM and Mel-FH cells with cystatin B stably knocked down by short hairpin RNA (shRNA). Western blot analysis of GAPDH was included as a loading control. Right panel: Mel-RM and Mel-FH cells with cystatin B stably knocked down by shRNA were transfected with the control or Itch siRNA. Twenty-four hours later, 30 μg of total protein of whole-cell lysates was subjected to western blot analysis of Itch, FLIP$_L$, and GAPDH (as a loading control). The data shown are representative of three individual experiments. (**e**) Inhibition of Itch blocked TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in melanoma cells deficient in cystatin B. Mel-RM and Mel-FH cells with cystatin B stably knocked down by shRNA were transfected with the control or Itch siRNA as shown in **d**. Twenty-four hours later, cells were treated with TRAIL (200 ng/ml) for 24 h. Apoptosis was measured by the propidium iodide method using flow cytometry. The data shown are the mean ± S.E. of three individual experiments. (**f**) Inhibition of Itch could not provide further protection against TRAIL-induced apoptosis in melanoma cells overexpressing cystatin B. Left panel: Mel-CV and MM200 cells stably tranfected with cDNA encoding cystatin B as shown in **b** were transfected with the control or Itch siRNA. Twenty-fours later, 30 μg of total protein of whole cell lysates was subjected to western blot analysis of Itch and GAPDH (as a loading control). The data shown are representative of three individual experiments. Right panel: Mel-CV and MM200 cells stably tranfected with cDNA encoding cystatin B as shown in B were transfected with the control or Itch siRNA. Twenty-fours later, cells were treated with TRAIL (200 ng/ml) for a further 24 h. Apoptosis was measured by the propidium iodide method using flow cytometry. The data shown are the mean ± S.E. of three individual experiments. (**g**) Cystatin B was not physically associated with Itch or FLIP$_L$. Whole-cell lysates from Mel-RM and Mel-FH cells were subjected to GST-pull down using either GST-cystatin B (line 3) or GST (line 2) as bait. In all, 30 μg of total proteins pulled down was subjected to SDS-PAGE and probed with antibodies against Itch and FLIP. Whole-cell lysates were included as a control (line 1). Analysis for cathepsin B and L that are known to interact with cystatin B was also included as an additional control. The arrow-head points to a non-specific band generated with the antibody against cathepsin L. The data shown are representative of three individual experiments

the cells were switched into antibiotic-free medium containing 5% FCS for a further 24 h. Cells were then passaged at 1 : 10 into fresh medium for a further 24 h followed by G418 (Sigma-Aldrich) selection.

Real-time PCR. Real-time RT-PCR was carried out using the ABI Prism 7700 sequence detection system (PE Applied Biosystems, Mulgrave, VIC., Australia) as described previously.[40] For FLIP, assay-on demand for FLIP (Assay ID: IS01117851-ml) was used according to the manufacturer's protocol (Applied Biosystems, Foster City, CA, USA). Analysis of cDNA for GAPDH was included as a control. After incubation at 50 °C for 2 min followed by 95 °C for 10 min, the reaction was carried out for 40 cycles of the following: 95 °C for 15 s and 60 °C for 1 min. The threshold cycle value (C_t) was normalized against GAPDH cycle numbers. The relative abundance of mRNA expression of a control sample was arbitrarily designated as 1, and the values of the relative abundance of mRNA of other samples were calculated accordingly.

siRNA. The siRNA constructs used were obtained as the siGENOME SMARTpool reagents (Dharmacon, Lafayette, CO, USA). The siGENOME SMARTpool Itch (M-007196-01), the siGENOME SMARTpool FLIP (M-003772-06), the siGENOME SMARTpool cystatin B (M-017240-00), the siGENOME SMARTpool cathepsin B (M-004266-03), and the non-targeting siRNA control, SiConTRolNon-targeting SiRNA pool (D-001206-13-20) were purchased from Dharmacon. Transfection of siRNA pools was carried out as described previously.[40]

shRNA knockdown. Melanoma cell lines were seeded at 1×10^4 per well in 96-well plates and left to attach overnight. Sigma MISSION Lentiviral Transduction Particles for shRNA-mediated knockdown of cystatin B (SHVRS-NM-000100) were applied to ~70% confluent cells in the presence of polybrene (4 or 8 μg/ml) at MOIs of 0.5, 1, or 5 in 100 μl DMEM. After 16–24 h, the culture medium was replaced and cells were left another 24 h. Cells were selected with 2 μg/ml puromycin for 3 days until mock-transduced controls (polybrene only) were completely dead. For each transduced melanoma cell line, up to four wells of cells per lentiviral clone were tested for knockdown by western blot analysis. Cells with lowest cystatin B levels were expanded for experimental use.

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (http://www.nature.com/cdd)