Combining radiotherapy with death ligands in cancer treatment: feasibility and molecular mechanisms
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TRAIL-receptor-1 and -2 plasma membrane levels are differentially regulated by dynamin-dependent endocytosis and MARCH family ubiquitin ligases

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TRAIL death receptors can signal for apoptosis, but can also activate the anti-apoptotic NF-κB pathway. It is emerging that the subcellular localization of death receptors dictates the nature of their signaling output. For this reason, we examined the regulation of TRAIL receptor trafficking in relation to apoptosis-induction, using breast-, cervix- and melanoma tumor cell lines as model systems. A large pool of TRAIL receptor (R)1 and -R2, resided at steady state in late endosomal/lysosomal compartments. This pool was most likely targeted for degradation, since we found no evidence for sorting of TRAIL receptors from the lysosomal compartments to the plasma membrane. Blocking receptor internalization by dominant negative Dynamin-1 did not inhibit TRAIL-induced apoptosis, suggesting that apoptosis signaling took place at the cell surface and not in endosomes. Dominant negative Dynamin-1 enhanced TRAIL-R1, but not TRAIL-R2 cell surface expression, indicating a differential regulation of both receptors by dynamin-dependent endocytosis at steady state. In conjunction with this, members of the MARCH ubiquitin ligase family specifically reduced cell surface expression of TRAIL-R1, but not TRAIL-R2. This occurred by targeting of the unique lysine residue 273 in TRAIL-R1, as its mutation into alanine abrogated the impact of the MARCH proteins on TRAIL-R1 downregulation. We conclude that in the cell lines studied, (dynamin-mediated) TRAIL-R1 and -R2 endocytosis is not required for apoptosis induction, suggesting that apoptosis signaling occurred from TRAIL receptors at the plasma membrane. In addition, TRAIL-R1 cell surface expression at steady state is selectively regulated by MARCH ubiquitin ligases, which target lysine 273 and sort TRAIL-R1 into a dynamin-dependent endocytic route that most likely attenuates pro-apoptotic signaling.

Introduction

Pre-clinical studies indicated that triggering of tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) death receptors by recombinant ligand or agonistic antibodies is a promising anti-cancer regimen, since TRAIL induced apoptosis in a large panel of long-term established tumor cell lines, but was not toxic to normal tissue [1, 2]. Currently, TRAIL receptor agonists have been pursued in several phase I and II clinical trials (reviewed in [3]). Death receptors (TRAIL-R1/-R2, CD95/Fas, TNF receptor (R)-1) activate the extrinsic pathway for apoptosis induction, in which ligand-induced receptor clustering leads to recruitment of components of the death-inducing signaling complex (DISC) to the death receptor tail. Within the DISC, inducer Caspase-8/-10 are activated, processed and released into the cytosol, where – in the direct pathway for apoptosis induction – they find effector caspases as their targets [4, 5]. In addition, Caspase-8/10 activate the mitochondrial pathway for effector caspase activation by cleaving and activating BH3-only protein Bid [6]. The efficacy of death receptors to induce apoptosis independent of the mitochondria depends on the cell type. Cells have been classified as Type I or Type II by their ability to induce apoptosis via CD95 [7-9] or TRAIL death receptors [10, 11] in the presence of Bcl-2 overexpression. In Type II cells, the mitochondrial pathway is required for apoptosis induction, whereas in Type I cells it is not. Death receptors can in addition activate alternative signaling pathways, including the NF-κB pathway, which inhibits apoptosis by transcriptionally upregulating inhibitory Bcl-2 family members, inhibitor of apoptosis proteins (IAPs) and c-FLIP [12]. Apoptosis signaling by TNFR-1 [13, 14] and CD95 [15] in Type I cells was found to be initiated from receptor complexes in endocytic compartments, whereas receptors on the cell surface activated the NF-κB survival pathway. However, for TRAIL-R1/-R2 this seemed not to be the case. Although these receptors were internalized after ligand binding in BJAB Type I cells studied, via clathrin dependent and independent routes, internalization was not required for pro-apoptotic signaling [16, 17].

A number of immunohistological studies indicate that, at the steady state, a large proportion of death receptors resides intracellularly [18-22]. For TNFR-1, TRAIL-receptors and CD95, these compartments were partially defined as the trans-Golgi network (TGN) [20, 23, 24], but further specifications are lacking. Given that different signaling complexes can associate with death receptors at the plasma membrane or in endosomes [13-15], it is possible that plasma membrane- and intracellular death receptor pools fulfill different functions. There is evidence that the pool of intracellular (TGN) localized TRAIL receptors communicates with, and is sorted to the cell surface, thereby actively controlling the pool of membrane localized receptors. Triggering of cell surface-localized receptors induced their endocytosis, but overall, no overt changes in cell surface expression were seen.
upon receptor stimulation [20]. TRAIL receptors can be deposited in the TGN directly via the biosynthetic route or after retrograde transport of receptor complexes that are endocytosed from the plasma membrane. Upon their synthesis, Type I transmembrane receptors (such as TRAIL-R1/-R2) are imported into the ER via the signal recognition particle and subsequently transported through the Golgi to the cell surface. It is known that protein sorting can occur at the level of the TGN. From the TGN, there is a default route to the plasma membrane, but also a retrograde route to the ER [25] and one to the endosomal system (e.g. [26, 27]).

In addition, TRAIL receptors (e.g. [16, 17]) and many other transmembrane receptors, including G protein-coupled receptors and receptor tyrosine kinases [28] undergo both ligand-induced and ligand-independent internalization from the plasma membrane. Internalization of membrane receptors serves at least two functions (1) attenuation of signaling of the activated receptor and (2) placing the activated cell surface receptor in the appropriate cellular location to interact with downstream signaling molecules [29]. For CD95, it has been described that upon ligand binding, activated receptors are targeted to clathrin-coated membrane invaginations. GTP hydrolysis by dynamin drives the scission of these ‘pits’ to release endocytic transport vesicles from the plasma membrane, after which fusion with early endosomes occurs. During progression along the endocytic pathway, endosomal cargo is sorted to the appropriate subcellular destination. Among these fates are retention in the endosome, recycling back to the plasma membrane, delivery to a lysosomal degradation pathway and retrograde transport to the Golgi or TGN [29].

One of the mechanisms by which transmembrane receptors are sorted from the plasma membrane into the endocytic pathway, is through ubiquitination. In addition to the central role that (poly) ubiquitination plays in cellular homeostasis by targeting proteins to proteosomal degradation [30], ubiquitination also regulates the sorting of proteins along the endocytic route to lysosomes (reviewed in [31]). Proteins are ubiquitinated by ubiquitin ligases (E3s) that simultaneously interact with the substrate and a ubiquitin conjugating enzyme (E2). The E2 receives activated ubiquitin from the ubiquitin activating enzyme (E1) (reviewed in [32, 33]). Recently, it has been reported that a number of transmembrane glycoproteins are targeted for ubiquitination and subsequent sorting to lysosomal membranes by membrane-associated RING-CH (MARCH) E3s [34]. Viral K3s of the MARCH family, which are supposedly derived from their mammalian homologues [34] can also internalize a number of transmembrane glycoproteins (e.g. [35]). It was proposed that the RING-CH proteins mediate the ubiquitination of the cytosolic tails of the target transmembrane proteins [36-38]. In addition, the E3 Receptor Internalization and Degradation (RID), encoded by the adenovirus type 5 induces internalization of both TRAIL-R1 and CD95 from the cell surface and directs their transport to lysosomal structures [39, 40].

Although TRAIL is a promising anti-cancer regimen, many tumor cells are resistant to TRAIL-induced apoptosis (e.g. [41]). Collective evidence indicates that death receptor trafficking is highly regulated and intimately related to pro-apoptotic signaling. To better understand what determines TRAIL-sensitivity of tumor cells, we examined the regulation of TRAIL receptor cell surface expression by components of the endocytic machinery and their impact on apoptosis induction.

Results

A large pool of exogenous TRAIL-receptors resides in late endosomal/lysosomal compartments

To facilitate the study of TRAIL receptor trafficking, TRAIL-R1 and TRAIL-R2 were fused to mRFP. The receptor chimeras were stably expressed in MCF7-C3 cells by retroviral transduction. Plasma membrane expression of TRAIL-R1 and TRAIL-R2 in transduced cells was about 10-fold higher than in non-transduced cells, according to flow cytometry using fluorescent mAbs specific for each receptor (Fig. 1A). This indicates that transport of TRAIL receptors to the plasma membrane was not impeded by the fusion of mRFP. It is also apparent that, even after selection, a large proportion of cells reverted to endogenous levels of TRAIL-R1 and TRAIL-R2 expression (Fig. 1A), indicating that cells are selected against high TRAIL receptor membrane expression. This is consistent with the ability of TRAIL-R1 or TRAIL-R2 to induce ligand-independent clustering of death receptors and apoptosis upon overexpression [42, 43].

To investigate the localization of TRAIL receptors in the endosomal pathway at steady state, transduced cells were fixed and stained with antibodies to early endosome antigen-1 (EEA-1) or the late endosomal/lysosomal marker CD63 and examined by CLSM. As determined by pixel analysis, the TRAIL receptors only marginally co-localized with EEA-1 (Fig. 1B), but strongly co-localized with CD63 (Fig. 1C). TRAIL receptors did not significantly co-localize with the trans-Golgi network markers TGN46 or TGN97 (data not shown). Myc-tagged TRAIL-R2, retrovirally expressed in MCF7-C3 cells also substantially co-localized with CD63 and not with EEA-1 (Fig. S1A), indicating that the localization of TRAIL receptors in late endosomal/lysosomal compartments is not a resultant of mRFP tagging. In addition, TRAIL-R1.mRFP or TRAIL-R2.mRFP similarly co-localized with CD63 upon stable expression in MelJuSo cells (Fig. S1B), and not with EEA-1 or TGN markers (data not shown), indicating that their localization to late endosomal compartments was not cell type specific. As antibodies directed against
Regulation of TRAIL receptor membrane levels

Figure 1. A large pool of exogenous TRAIL receptors resides in late endosomal/lysosomal compartments. (A) Cell surface expression of TRAIL-R1 and -R2 in MCF7-C3 cells stably transduced to express TRAIL-R1.mRFP (left) or TRAIL-R2.mRFP (right). TRAIL-R1 or TRAIL-R2 was detected by specific mAbs, followed by fluorochrome-conjugated secondary antibody. Filled gray histogram: expression of endogenous TRAIL receptors on control MCF7-C3 cells; open black histogram: expression of TRAIL receptors on transduced MCF7-C3 cells; open gray histogram: secondary antibody only. Values indicate the corresponding mean fluorescence intensity (MFI).

(B) CLSM of TRAIL-R1/-R2.mRFP transduced cells after staining with anti-EEA-1 mAb followed by Alexa Fluor 488 goat anti-mouse Ig. (C) CLSM of TRAIL-R1/-R2.mRFP transduced cells after staining with FITC-conjugated anti-CD63 mAb. Pixel analysis in B,C indicates colocalization. Scale bar: 10 μM.

(D) Immuno-EM of TRAIL-R1.GFP transduced (left) and TRAIL-R2.GFP transfected (right) MCF7-C3 cells. GFP (TRAIL receptors) and CD63 were detected by specific antibodies and decorated with Protein A-conjugated large (15 nm) and small (10 nm) gold particles respectively. PM, plasma membrane. Arrows point to vesicular structures, in which CD63 and TRAIL receptors colocalize. (Scale bar: 150 nm)
GFP (but not mRFP) have been successfully used in immuno-EM, we examined subcellular distribution of GFP-tagged TRAIL-R1/-R2 in MCF7-C3 cells by immuno-EM. Here, we also observed partial co-localization of GFP-tagged TRAIL-R1 and TRAIL-R2 with CD63 in vesicular structures (Fig. 1 D). Collectively, these findings indicate that a large pool of intracellular TRAIL receptors resides in late endocytic structures.

The late endosomal/lysosomal pool of TRAIL receptors is not sorted to the cell surface

Plasma membrane glycoproteins, such as the Transferrin receptor readily enter the endocytic pathway via clathrin-coated pits. After internalization, Transferrin receptor recycles to the plasma membrane [44]. To investigate whether the pool of late endosomal/lysosomal TRAIL receptors is also sorted to the plasma membrane, we used Rab-interacting lysosomal protein (RILP) overexpression to disrupt lysosomal transport and monitored its impact on TRAIL receptor surface levels. RILP directs the movement of late-endosomes/lysosomes toward the minus-end of microtubules by recruiting the dynein-dynactin motor [45, 46], and its overexpression leads to clustering of these vesicles around the microtubule-organizing center (MTOC). Therefore, potential retrograde transport of TRAIL receptors from the late endosomal/lysosomal compartments to the plasma membrane will be disrupted. Wild-type MCF7-C3 cells or MCF7-C3 cells stably expressing TRAIL-R1.mRFP or TRAIL-R2.mRFP were transiently transfected with GFP-RILP. In these cells, the majority of intracellular TRAIL receptors colocalized with RILP perinuclearly, at a location that is consistent with RILP enforcing the dynein motor-driven transport of lysosomes towards the MTOC (Fig. 2 A). Cell surface levels of TRAIL receptors, both in wild-type MCF7-C3 cells and cells stably expressing TRAIL-R1/-R2.mRFP were not affected by RILP overexpression (Fig. 2 B). This indicates that the late endosomal/lysosomal pool of TRAIL receptors was not sorted to the plasma membrane, since in that case, a depletion of TRAIL receptors from the cell surface would be expected in cells that overexpressed RILP. In addition, RILP overexpression did not affect sensitivity to TRAIL-

![Figure 2](image-url)

*Figure 2. The late endosomal / lysosomal pool of TRAIL receptors is not sorted to the cell surface. (A) MCF7-C3 cells expressing TRAIL-R1.mRFP (top) or TRAIL-R2.mRFP (bottom) were transiently transfected with RILP-GFP, fixed, counterstained with TO-PRO-3 and analyzed by CLSM. (Scale bar: 10 μM). Pixel analysis indicates colocalization. (B) The same cells as in (A) as well as control MCF7-C3 cells transfected with RILP-GFP were analyzed for TRAIL-R1 (left) and TRAIL-R2 (right) membrane expression by flow cytometry. (C) MCF7-C3 cells expressing GFP-RILP were stimulated with IZ-TRAIL for 4 h. Apoptosis induction (Caspase-3 activation) in the RILP-transfected GFP positive population (RILP) and in the untransfected GFP negative population (Control) was analyzed by flow cytometry. Values indicate mean ±SD of 3 independent experiments.*
induced apoptosis (Fig. 2 C). Collectively, these results indicate that the late endosomal/lysosomal pool of TRAIL receptors is not sorted to the cell surface, suggesting that it is an end-stage pool, targeted for degradation.

**Blocking dynamin-mediated endocytosis does not affect apoptosis-induction by TRAIL, but differentially impacts on TRAIL-R1 versus TRAIL-R2 membrane levels**

MCF7-C3 cells are Type I cells for which it has been described that internalization of CD95 and TNFR-1 is essential for apoptosis signaling [14, 15]. We therefore examined whether TRAIL receptor internalization upon ligand binding was required for apoptosis signaling. To block internalization (both by clathrin-dependent and –independent mechanisms), we overexpressed a GTPase defective mutant of Dynamin-1 (K44A). In these cells, coated pits do not become constricted and coated vesicles do not bud off into the cytoplasm [47, 48]. Overexpression of K44A Dynamin-1 abrogated the ability of cells to internalize FITC-conjugated Transferrin (Fig. 3 A), thus validating the effect of this mutant. However, its overexpression did not impede TRAIL-induced apoptosis (Fig. 3 B).

Interestingly, when TRAIL receptor expression was analyzed in cells transfected with wild-type or K44A Dynamin-1, a differential impact of K44A Dynamin-1 on TRAIL-R1 versus TRAIL-R2 levels was observed. Membrane expression of TRAIL-R2 was not affected by either wild-type or K44A Dynamin-1. However, blocking internalization by K44A Dynamin-1 led to increased TRAIL-R1 surface levels in GFPhigh cells that presumably also expressed high levels of K44A Dynamin-1 (Fig. 3 C).

These data indicate that dynamin-dependent endocytosis is not required for TRAIL-induced apoptosis, which is in agreement with a previously published report using Type I BJAB cells [17]. This finding further supports the concept that the pool of TRAIL receptors that resides in late endosomes/lysosomes is not involved in apoptosis signaling. We also conclude that endocytosis of TRAIL-R1 and TRAIL-R2 from the plasma membrane is differentially regulated. Whereas TRAIL-R1 membrane levels were affected by inhibiting dynamin-dependent endocytosis, this was not observed for TRAIL-R2 in the time-frame analyzed.

![Figure 3](image-url)

**Figure 3.** Blocking dynamin-mediated endocytosis does not affect apoptosis-induction by TRAIL, but differentially impacts on TRAIL-R1 versus TRAIL-R2 membrane levels. (A) CLSM of MCF7-C3 cells transfected with K44A Dynamin-1, analyzed 10-20 min after incubation with FITC-conjugated Transferrin. (Scale bar: 50 μM). Transferrin uptake was quantified (right panel) in MCF7-C3 cells transfected with wild-type (WT, n=50) or K44A Dynamin-1 (K44A, n=100) and are representative of multiple experiments. K44A Dynamin-1 cells were further classified as ‘low’ or ‘high’ Dynamin-1 expressors based on the intensity of Dynamin-1 staining (dim or bright respectively). (B) MCF7-C3 cells were co-transfected with Dynamin-1 (WT or K44A) and GFP (3:1 ratio), or GFP only as a control (Control) for 24 hours. Next, cells were stimulated with 50 ng/ml IZ-TRAIL and analyzed for active Caspase-3 by flow cytometry at the indicated periods of time. Apoptosis induction per sample was analyzed for both nontransfected, GFP negative (-) and transfected - GFP positive - cell populations. (C) The same cells as in (B) were stained for membrane TRAIL-R1 (middle columns) and TRAIL-R2 (right columns) and expression of TRAIL receptors in live cells was subsequently evaluated by flow cytometry. Values indicate mean fluorescence intensity of TRAIL receptor expression in the corresponding GFP positive (GFP and Dynamin-1 transfected) and GFP negative (untransfected) cell populations.
**MARCH proteins downregulate surface levels of (selectively) TRAIL-R1**

TRAIL-R1 and TRAIL-R2 have 58% overall identity at the protein level [43], which leaves room for differential regulation of receptor trafficking by post-translational processes. In fact, it has been described that cell surface expression of TRAIL-R1 and not TRAIL-R2 is inhibited by downregulation of components of the signal recognition particle [49]. Alignment of the membrane-proximal intracellular regions of both receptors revealed a single lysine residue (K273) in TRAIL-R1 that was not conserved in TRAIL-R2 (Fig. 4A). This made TRAIL-R1 a potential target for the recently identified MARCH family of ubiquitin ligases. It was demonstrated that members of this family regulate internalization and transport of a variety of transmembrane receptors to lysosomes, presumably for degradation. Lysine residues that served as MARCH targets were localized in the membrane-proximal region of the cytoplasmic tail of these receptors [34].

We used a flow cytometric assay, as described previously [34] to assess whether cell surface expression of TRAIL-R1 and/or TRAIL-R2 was regulated by MARCH proteins. GFP-tagged MARCH 1, 2, 4, 8 or 9 were transiently expressed in MCF7-C3 cells (Fig. 4 B,C), melanoma cell line MelJuSo (Fig. 4 C) or cervix carcinoma cell line HeLa (Fig. S3). The impact of MARCH proteins on TRAIL-R1/-R2 cell surface expression was assessed by detection of both TRAIL receptors with specific mAbs as assessing the mean fluorescence intensity of TRAIL-R1/-R2 in MARCH expressing (GFP positive) cells versus control (GFP negative) cells in the same population. All MARCH proteins tested reduced cell surface expression of TRAIL-R1 to a significant extent, but MARCH 1 and MARCH 9 had the most pronounced effect in both MCF7-C3 and MelJuSo cells (Fig. 4C). In contrast, the MARCH proteins had a modest and mostly not significant effect on TRAIL-R2 cell surface expression in the three cell lines.

**Figure 4. MARCH proteins downregulate surface levels of (selectively) TRAIL-R1.** (A) Comparison of the membrane-proximal cytoplasmic tail of human TRAIL-R1 and TRAIL-R2. The last 4 C-terminal transmembrane amino acids are indicated in italics and lysine residues (potential MARCH targets) are indicated in bold. The areas of homology are aligned. (B) MCF7-C3 cells were transfected with GFP-tagged MARCH 1, 2, 4, 8, 9 or GFP as a control (Control). Cell surface levels of TRAIL-R1 (top) and TRAIL-R2 (bottom) were determined by flow cytometry. Values indicate mean fluorescence intensity of TRAIL receptors in the corresponding GFP positive and GFP negative cell populations. (C) Quantification of TRAIL-R1 (open bars) and TRAIL-R2 (filled bars) expression in MCF7-C3 cells (left panel) and MelJuSo cells (right panel) transfected with GFP-tagged MARCH 1, 2, 4, 8, 9 or GFP as a control (-). For analysis, fluorescence intensity of 2nd Ab in the GFP positive and negative cell populations was subtracted from fluorescence intensity of TRAIL-R staining and normalized to TRAIL-R expression in the GFP negative population. Values represent mean ±SD of 2 (MelJuSo) or at least 3 (MCF7-C3) independent experiments. Statistical differences (*P<0.05, **P<0.01, ***P<0.001) indicated for TRAIL-R1 expression between GFP control transfected cells and cells transfected with the respective MARCH constructs. Statistical differences (##P<0.05) indicated for TRAIL-R2 expression between GFP control transfected cells and cells transfected with the respective MARCH constructs.
tested (Fig. 4 B,C and results not shown). We conclude that MARCH family members specifically target TRAIL-R1 – as opposed to TRAIL-R2 – and downregulate its plasma membrane levels at steady state.

**Downregulation of TRAIL-R1 by a number of MARCH proteins is reversed in cells overexpressing K44A Dynamin-1**

The MARCH proteins may downregulate TRAIL-R1 cell surface expression either by ubiquitination and subsequent internalization of TRAIL-R1 at the plasma membrane or by ubiquitination of TRAIL-R1 in the biosynthetic route prior to plasma membrane deposition. To discriminate between these possibilities, we tested to which extent K44A Dynamin-1 could prevent the downregulation of TRAIL-R1 by MARCH proteins. For this purpose, the various MARCH proteins were individually co-expressed with K44A Dynamin-1 in MCF7-C3 or HeLa cells and TRAIL-R1 plasma membrane expression was evaluated as described for Fig. 4. Downregulation of TRAIL-R1 by MARCH 1, MARCH 2, MARCH 4 and MARCH 8 could be prevented by the Dynamin-1 mutant to a significant degree (Fig. 5; Fig. S3). This strongly suggests that these MARCH proteins target plasma membrane-localized TRAIL-R1 and promote its endocytosis via a dynamin-dependent pathway. K44A Dynamin-1 could not fully rescue TRAIL-R1 from downregulation by MARCH 9 in MCF7-C3 or HeLa cells (Fig. 5; Fig. S3). Co-expression of K44A Dynamin-1 and the MARCH proteins did not affect TRAIL-R2 membrane levels in MCF7-C3 cells (data not shown).

We conclude that MARCH 1, 2, 4, 8 likely promote dynamin-dependent endocytosis of TRAIL-R1 from the plasma membrane. In addition, MARCH 9 in particular may downregulate TRAIL-R1 cell surface levels at steady state by having an additional impact on the trafficking of intracellularly-localized receptors.

**K273A mutation in the cytoplasmic tail of TRAIL-R1 impedes the ability of MARCH 1 and 9 to downregulate TRAIL-R1**

As mentioned above, TRAIL-R1 contains a unique membrane-proximal lysine residue (K273) in its cytoplasmic tail that is a potential target site for MARCH-mediated ubiquitination. To test its involvement in TRAIL-R1 downregulation by the MARCH proteins, this residue was mutated into alanine. Subsequently, MCF7-C3 cells were transduced to stably express either wild-type TRAIL-R1.mRFP or K273A TRAIL-R1.mRFP. Flow cytometry revealed a clear population of cells expressing the introduced TRAIL-R1 at the plasma membrane. Fluorescence intensities of exogenous wild-type and K273A TRAIL-R1 were comparable, indicating that TRAIL-R1 transport to the cell surface in steady state
was not affected by the lysine mutation. As observed earlier for the wild-type TRAIL-R1 and TRAIL-R2 (Fig. 1A), cells were selected over time against high levels of exogenous mutant TRAIL-R1 expression. MCF7-C3 cells expressing wild-type or K273A mutant TRAIL-R1.mRFP were transiently transfected with GFP empty vector as a control or GFP-tagged MARCH 1 and MARCH 9, which were most efficient in TRAIL-R1 downregulation. Because the flow cytometry lasers were not able to efficiently excite mRFP in transduced cells, we could not evaluate the differential impact of the MARCH proteins on the mRFP positive and negative cell populations. We therefore monitored within the GFP positive MARCH-expressing cell population the number of cells expressing high (exogenous) levels versus low (endogenous) levels of TRAIL-R1. The quadrants were set (with the aid of density lines) such that the pool of endogenous and exogenous TRAIL-R1 expressing cells in the GFP negative cell population were appropriately separated (Fig. 6B). As shown in Fig. 6B and quantified from 4 independent experiments in Fig. 6C, MARCH 1 and MARCH 9 induced a significant redistribution of wild-type exogenous TRAIL-R1 to the endogenous TRAIL-R1 gate as compared to the GFP
endocytosis terminates apoptosis signaling and collectively, these data indicate that receptor surface expression or apoptosis signaling (results not shown). Active Rab6A also did not affect TRAIL-R1/-R2 cell overexpression of dominant negative or constitutively retrograde transport from endosomes to the TGN by CD63. However, inhibition of transport sorting to the cell surface. Interference with potential localization to initiate apoptosis signaling. It follows that inhibition of dynamin-dependent endocytosis in the cell lines studied here did not impede apoptosis signalling by TRAIL-R1/-R2. This indicates that in these cells, TRAIL receptors do not require an endosomal localization to initiate apoptosis signaling. It follows from these data that the late endosomal/lysosomal pool of TRAIL receptors is unlikely to be functional in apoptosis signaling.

We addressed the possibility that the late endosomal/lysosomal pool of TRAIL-R1/-R2 was connected to the functional receptor pool at the cell surface, by recycling like CD63. However, inhibition of transport of the compartments back to the cell surface by transient overexpression of RILP did not affect TRAIL receptor plasma membrane levels in the time-frame of the experiment. In addition, downregulation of RILP expression by RNAi similarly did not alter cell surface expression of TRAIL-R1/-R2 (results not shown). This suggests that the lysosomal receptor pool is not sorted to the cell surface. Interference with potential retrograde transport from endosomes to the TGN by overexpression of dominant negative or constitutively active Rab6A also did not affect TRAIL-R1/-R2 cell surface expression or apoptosis signaling (results not shown).

Collectively, these data indicate that receptor endocytosis terminates apoptosis signaling and suggest that the late endosomal/lysosomal pool of TRAIL receptors is targeted for degradation.

Using dominant negative Dynamin-1, we found that the turnover of TRAIL-R1 and TRAIL-R2 at the plasma membrane at steady state (in absence of TRAIL binding) is differentially regulated; plasma membrane levels of TRAIL-R1, but not TRAIL-R2 increased upon transient overexpression of K44A Dynamin-1. This finding does not rule out that TRAIL-R2 is subject to dynamin-dependent endocytosis, but indicates a difference between the two receptors with regards to the dynamics of this process. It is also possible that ligand-induced internalization of TRAIL receptors and their internalization at steady state depend on different mechanisms. Therefore, homeostatic regulation of TRAIL-R2 membrane levels may not involve dynamin-dependent mechanisms, whereas this is not necessarily the case for ligand-induced internalization. TRAIL internalization in BJAB cells still occurred in cells that overexpressed K44A Dynamin [17], indicating that TRAIL and its receptors can be internalized via dynamin-independent routes as well.

All MARCH family ubiquitin ligases tested here reduced plasma membrane expression of TRAIL-R1, while having little or no effect on TRAIL-R2, in line with a differential mechanism of receptor turnover at the plasma membrane. By combined expression of MARCH 1, 2, 4, 8, 9 proteins and dominant negative Dynamin-1, we provided strong evidence that most MARCH proteins, with the exception of MARCH 9, primarily targeted plasma membrane localized TRAIL-R1 for endocytosis. MARCH proteins have been shown to reside in differential subcellular (membrane-associated) compartments. Only MARCH 9 was found in compartments that co-stained with the TGN marker AP-1 [34], suggesting that TRAIL-R1 could be targeted by MARCH 9 in the TGN for ubiquitination and lysosomal degradation, thereby preventing anterograde transport to the cell surface.

It has been appreciated by a number of studies that plasma membrane expression of TRAIL-R1 and TRAIL-R2 is differentially regulated. A genetic screen identified components of the signal recognition particle as regulators of TRAIL-R1, but not TRAIL-R2 plasma membrane deposition, but the underlying mechanism is thus far unclear [49]. In addition, the adenovirus E3-10.4K/14.5K complex [51] and the viral E3 RID [40] downregulated CD95 surface levels and conferred resistance to CD95-mediated apoptosis. In case of RID, CD95 was targeted for lysosomal degradation. RID also downregulated TRAIL-R1 cell surface levels by targeting targeted TRAIL-R1 for lysosomal degradation [39]. To date, ability of viral E3s to downregulate TRAIL-R2 have not been reported. Analogous to the effect of TRAIL-R1 downregulation by these viral E3s, it is likely that the MARCH proteins here studied downregulate surface TRAIL-R1, by directing their sorting into late endosomal/lysosomal compartments as observed in
transport of TRAIL-R2 to lysosomes requires further investigation. It is likely that other mediators than MARCH protein family members play a role, since their impact on TRAIL-R2 membrane downregulation was much less pronounced (Fig. 4 B,C) in the timeframe analyzed. The cytoplasmic tail of TRAIL-R2 (Fig. 4 A) contains a number of lysine residues, as well as a dileucine sequence that could potentially serve as internalization motifs without requiring ubiquitination. The YxxL internalization motif in the death domain of CD95 (aa291-294 [15]), can also be found (albeit at a different position in the death domain) in TRAIL-R1, -R2 and TNFR-1. The YxxW internalization motif of TNFR-1 in its membrane-proximal cytoplasmic tail (aa207-210 [14]) is not present in TRAIL-R1, -R2 or CD95. Lysine 273 in TRAIL-R1 was identified as a potential target site for MARCH-mediated ubiquitination, because its mutation impaired the ability of MARCH 1 and MARCH 9 to downregulate TRAIL-R1 cell surface levels (Fig. 6 B,C). Co-expression of TRAIL-R1 with MARCH proteins in the presence of wild-type ubiquitin and ubiquitin mutant molecules should point out whether TRAIL-R1 is indeed ubiquitinated and whether mono- or poly ubiquitination is required for TRAIL-R1 endocytosis. In addition, co-localization of the different MARCH proteins and WT versus K273A TRAIL-R1 will provide additional evidence for the sub-cellular localization of TRAIL-R1 and MARCH protein interaction. In this study, MARCH-mediated TRAIL-R1 downregulation involved deliberate expression of exogenous MARCH proteins. A number of MARCH proteins, in particular MARCH 9 are ubiquitously expressed [34]. In addition MARCH 1 mRNA was found to be overexpressed in breast tumors (www.oncomine.org). Transcripts of MARCH 2, 3, 5, 6, 7 and 9 were found in MCF7-C3 cells (results not shown). Deliberate downregulation of endogenous MARCH proteins in MCF7-C3 cells by RNA interference should reveal which MARCH family members predominantly regulate TRAIL-R1 cell surface expression under physiological conditions. Since TRAIL receptor internalization was not required for Caspase-3 activation (Fig. 3), most likely TRAIL-induced apoptosis signaling occurs at the plasma membrane. Therefore, it is conceivable that TRAIL-R1 downregulation from the cell surface by MARCH proteins may affect sensitivity to TRAIL receptor agonists. The extent to which this can be revealed will depend on expression and functionality of TRAIL-R2 and on the agonistic agent employed. Recombinant human TRAIL can signal for apoptosis through both receptors, but the reliance on either receptor to induce apoptosis varies between cell types (e.g. [52-54]). In addition, receptor-selective antibodies or recombinant human (rh) TRAIL variants display differential agonistic activities, again depending on the cell type. Therefore, the impact on TRAIL-induced apoptosis by MARCH proteins should either be evaluated in a TRAIL-R2 null cell system, or by specifically activating TRAIL-R1 using for instance anti-TRAIL-R1 agonistic antibodies. In this study, we identified a novel mode of TRAIL-R1 regulation by members of the MARCH E3 ubiquitin ligase family. MARCH 1, 2, 4, 8, and 9 could all specifically downregulate TRAIL-R1 levels, leaving TRAIL-R2 cell surface levels largely unaffected. Lysine 273 in TRAIL-R1 was identified as the potential MARCH ubiquitination site, as its mutation into alanine abrogated MARCH-mediated TRAIL-R1 downregulation to a large extent. Future studies will have to identify the nature and subcellular location of TRAIL-R1 ubiquitination. In addition, the physiological and functional role of the MARCH proteins in TRAIL-R1 downregulation and apoptosis sensitivity to TRAIL should be addressed. Once molecular diagnosis in treatment guidance for cancer patients is firmly in place, these results may provide a guideline for patient assignment in future clinical trials incorporating TRAIL receptor agonists.

Materials and Methods

Cells and reagents

Human breast carcinoma cells MCF-7 and derivatives, human cervix carcinoma line HeLa, human melanoma line MelJuSo and the HT1080 packaging cell line FLY [55] were cultured in Dulbecco’s modified Eagle medium (DMEM) with 8% fetal bovine serum (FBS) and antibiotics. Recombinant human isoleucine-zipped (iz) and FLAG-tagged TRAIL were kindly provided by H. Walczak (Imperial College London, UK). Monoclonal antibodies (mAbs) used were: anti-Myc (clone 9E10, Sigma-Aldrich); biotinylated anti-human TRAIL-R1 (clone DJR1) and biotinylated anti-human TRAIL-R2 (clone DJR2-4) from eBioscience; anti-early endosomal antigen (EEA)-1 (clone 14) and anti-Dynamin-1 (clone 41) from BD Transduction Laboratories; FITC-conjugated anti-CD63 (clone MEM-259, AbCam). Rabbit anti-active Caspase-3 antibody and Allophycocyanine (APC)-conjugated streptavidin were from BD Biosciences. Fluorescent secondary antibodies, conjugated to Alexa Fluor 488 (green), Alexa Fluor 568 (red), Alexa Fluor 633 (far red, shown as blue), and Alexa Fluor 647 (far red, FL-4 in flow cytometry) were from Molecular Probes.

Constructs

The retroviral vector LZRS-Caspase-3-MS-IRES-Zeo was generated by first introducing BamHI and EcoRI restriction sites by PCR using primers #1 and #2 using pCDNA3-Caspase-3 (kindly provided by H. Walczak) as template. TRAIL-R1 and -2 were fused to monomeric Red Fluorescent Protein (mRFP) to allow tracking in live cells. The retroviral vectors pMX-TRAIL-R1.mRFP and pMX-TRAIL-R2.mRFP were generated by first introducing EcoRI and BamHI restriction sites by PCR using pCDNA3-TRAIL-R1 and pCDNA3-TRAIL-R2 (kindly provided by H. Walczak) as template. Primers used were #3 and #4 for TRAIL-R1; #5 and #6 for TRAIL-R2. Next, PCR fragments were ligated into linearized pmRFP-N1 using EcoRI and BamHI restriction sites. Subsequently, TRAIL-R1.mRFP and TRAIL-R2.mRFP were excised and ligated into linearized pMX-Blasticidin (Bsd) using EcoRI and NotI restriction sites. For immuno-EM, TRAIL-R1 and -2 were fused to GFP in pEGFP-N1 and TRAIL-R2 was subcloned into pMX-I-Bsd by the same cloning strategy as described
above, pMX-TRAIL-R2.Myc was generated by first introducing a C-terminal myc tag and EcoRI and NotI restriction sites by PCR (primers #7 and #8) in pcDNA3-TRAIL-R2. Subsequently, the insert was ligated into linearized pMX-Bsd using EcoRI and NotI restriction sites. pEGFP-C1-RILP has been described previously [56]. pEGFP-N1-MARCH proteins were generated by first introducing EcorI and BamH1 restriction sites into the packaging sites by PCR using pHuD10-1-MARCH 1, pHuD10-1-MARCH 2, and pHuD10-1-MARCH 8 (kindly provided by E. Bartee), pOTB7-MARCH 4 and pOTB7-MARCH 9 (Genservice) as templates. Primers used were #9 and #10 for MARCH 1; #11 and #12 for MARCH 2; #13 and #14 for MARCH 4; #15 and #16 for MARCH 8; #17 and #18 for MARCH 9. Subsequently, the PCR fragments were ligated into pEGFP-N1 (Clontech) using EcoRI and BamH1 restriction sites. pcDNA3-Dynamin-1 (wild type and K44A mutant) constructs were generated by excision of Dynamin-1 cDNA from pMT2-Dynamin-1 (wild type and K44A mutant, described in [57]) and subsequent ligation into pcDNA3 using KpnI and XbaI restriction sites. The K273ATRAIL-R1 point mutant was generated in pcDNA3-TRAIL-R1 by PCR using the QuikChange site-directed mutagenesis kit according to the manufacturer’s instructions (Stratagene) using primers #19 and #20 and subsequent cloning into pMX-Bsd was performed as described. All constructs were verified by dideoxy nucleotide sequencing.  

### Table 1. Primers used for cloning and site-directed mutagenesis

<table>
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<tr>
<th>#</th>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
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<tr>
<td>1</td>
<td>Casp-3 FW</td>
<td>cgg gat cca tgg aga aca cctg aaa ac c</td>
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<tr>
<td>2</td>
<td>Casp-3 RV</td>
<td>gcc tta agt tag tga taa aag att gcc</td>
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<tr>
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<td>cct tgg aat tca tgg cgc cac cac cga gtc atc atc tag</td>
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<tr>
<td>4</td>
<td>TRAIL-R1 RV</td>
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</tr>
<tr>
<td>5</td>
<td>TRAIL-R2 FW</td>
<td>tgt gaa ttc gcc gcc acc atg cca cag cgg g</td>
</tr>
<tr>
<td>6</td>
<td>TRAIL-R2 RV</td>
<td>cca tct gac cgg ccc cag ctt cgc atg cgc ctc ttc tcc tcc g</td>
</tr>
<tr>
<td>7</td>
<td>TRAIL-R2.Myc FW</td>
<td>cct ggc aat tca tgg acc acc ggg gac aga acc ggc</td>
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<tr>
<td>8</td>
<td>TRAIL-R2.Myc RV</td>
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<tr>
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<tr>
<td>10</td>
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<td>aag gat ccc gga gtc ata cca ctt cag ggg cgc</td>
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<td>11</td>
<td>MARCH2 FW</td>
<td>tgt aat tgc cca cca tga cca cgg gtc act gc</td>
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<td>12</td>
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</tr>
<tr>
<td>16</td>
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<tr>
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</tr>
<tr>
<td>18</td>
<td>MARCH9 RV</td>
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</tr>
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<tr>
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</table>

### Transfection and retroviral transduction

Cells were transfected with FuGENE 6 according to the manufacturer’s instructions (Roche). Unless otherwise indicated, cells were manipulated 20-24 h after transfection. Production of Caspase-3 amphotropic retrovirus was done in the 293T human embryonic kidney cell-derived packaging cell line Phoenix-Ampho [58]. Briefly, 1.8 x 10^6 cells were seeded on a 10 cm dish and after 15 h transfected with 8 µg plasmid DNA. After 48 h, 5 x 10^6 MCF7 cells were transduced with virus-containing supernatant. Selection of transduced cells was initiated 4 days after transduction by the addition of 200 µg/ml Zeocin (Invitrogen).

Production of all other amphotropic retroviruses was done in the HT1080 packaging cell line FLY [55] and transduction of MCF7-C3 cells with freshly harvested virus supernatant was carried out as described [59]. Cells were selected after 3 days with 10 µg/ml Blasticidin (Sigma).

### CLSM

Cells (10^4 per sample) were grown and transected on glass coverslips in 6-well plates, washed in PBS and fixed with ice-cold (-20°C) methanol for 2 min. After rehydration in PBS, nonspecific binding sites were blocked for 30 min by using 1% Bovine Serum Albumin (BSA) in PBS. Incubations were performed with antibodies diluted in blocking buffer for 45-60 min, after which coverslips were washed and incubated for 30-45 min with the appropriate secondary antibodies diluted in blocking buffer. Next, coverslips were washed and mounted on glass slides using Vectashield (Vector Laboratories). All treatments were performed at room temperature. CLSM was performed using a TCS SP2 system using a 63x 1.32 NA oil immersion objective and confocal software (all obtained from Leica). Images were processed (cropping and level adjustment) using Photoshop software (Adobe). To minimize spectral leak-through, images were obtained by sequential scanning.

### Production and internalization of FITC-conjugated Transferrin

Transferrin was conjugated to FITC to analyze dynamin-mediated endocytosis. Briefly, 5 mg Transferrin (Sigma) was diluted in 12.5 ml 0.1 M NaHCO3, pH 9.5. 17.5 µl FITC (10 mg/ml in dimethylformamide) was incubated with Transferrin for 2 h (in dark, RT). FITC-conjugated Transferrin was separated from nonconjugated FITC molecules using a sephadex column (Sephadex G-25, Pharmacia), aliquotted and stored at -20°C until further use. To analyze Transferrin internalization by CLSM, transfected cells were incubated with 25 µg/ml FITC-conjugated Transferrin in binding medium (3% bovine serum albumin (BSA)/20 mM Heps, pH 7.2 in DMEM) and incubated at 37°C, 5% CO2 for 10-20 min. After incubation, coverslips were washed twice with PBS and further processed as described for analysis by CLSM. The percentage of Dynamin-1 transfected cells (wild-type or K44A mutant) that internalized FITC-conjugated Transferrin was calculated from 45 wild-type and 96 K44A mutant transfected cells, where the intensity of Dynamin-1 staining was also taken into account; Dynamin-1 bright cells were scored as K44Alow and Dynamin-1 dim cells were scored as K44Ahigh.
Immuno-EM

MCF7-C3 cells were processed for cryosectioning as described in [60]. Briefly, cells were fixed in 2% paraformaldehyde and 0.2% glutaraldehyde in 60 mM Pipes/25 mM Hepes/2 mM MgCl2/10 mM EGTA pH 6.9, and processed for cryosectioning. For immunolabeling, 50 nm sections were incubated for 10 min with 0.15 M glycine in PBS and for 10 min with 1% BSA in PBS to block free aldehyde groups and prevent aspecific antibody binding, respectively. Sections were incubated in PBS with 0.15 M glycine in PBS and for 10 min with 1% BSA for 1 h. Active Caspase-3 content and TRAIL-R1/-R2 cell surface expression on live cells was analyzed by flow cytometry as described [59, 61].

Apoptosis assays and flow cytometry

For apoptosis assays, cells were stimulated with the indicated doses of TRAIL in culture medium for the indicated periods of time at 37°C, 5% CO2. Active Caspase-3 content and TRAIL-R1/-R2 cell surface expression on live cells was analyzed by flow cytometry as described [59, 61].

Statistics

Statistical analysis was done with the unpaired Student’s t-tests and differences were considered significant for p < 0.05. Analyses were performed using GraphPad Prism version 4 for Windows (Graph Pad Software).

References

Supplementary Figure 1. TRAIL-R2.myc colocalizes with late endosomal / lysosomal compartments. (A) CLSM of TRAIL-R2.myc transduced MCF7-C3 cells after staining with anti-myc mAb followed by Alexa Fluor 568 goat anti-mouse Ig and FITC-conjugated anti-CD63 mAb. (B) CLSM of MelJuSo cells stably expressing TRAIL-R1.mRFP (top panels) or TRAIL-R2.mRFP (bottom panels). Cells were fixed, stained with FITC-conjugated anti-CD63 mAb and counterstained with TO-PRO-3. Pixel analysis indicates colocalization, Scale bars: 10 μM.
For calculating the impact of MARCH proteins on TRAIL-R downregulation, the mean fluorescence intensity (values indicated in the corresponding GFP positive and GFP negative gates) of the 2nd Ab control was subtracted from that of TRAIL-R expression in Fig. 4 B and Fig. 5 A.

Downregulation of TRAIL-R1 by a number of MARCH proteins is reversed in HeLa cells overexpressing K44A Dynamin-1. HeLa cells were transfected with GFP-tagged MARCH 1, 2, 4, 8, 9 or GFP as a control in the absence (top panels) or presence (bottom panels) of K44A Dynamin-1 (1:3 ratio). Cell surface levels of TRAIL-R1 were determined by flow cytometry and values indicate mean fluorescence of TRAIL-R1 in the GFP positive and GFP negative cell populations.