Trends box

- TRAIL-induced complexes I and II both act as cell death-inducing and gene-activatory signalling platforms
- The core components of TRAIL-induced signalling, TRAIL-R1/2, FADD, caspase-8, RIPK1 and $cFLIP_{L/S}$, are regulated by ubiquitination
- Ubiquitin writers, erasers and binders such as TRAF2, cIAP1/2, LUBAC, A20, TABs and NEMO are major regulatory components in TRAIL-induced signalling complexes
- Both, degradative (K48) and non-degradative (K63 and M1) poly-ubiquitination events control the TRAIL-induced signalling outcome
- Tight regulation of the function and expression of TRAIL-induced signalling complex components by ubiquitination is required to ensure appropriate activation of downstream signalling outputs
- Due to their decisive regulatory roles in mediating TRAIL signalling outputs in cancer cells, modulators of ubiquitination are promising therapeutic targets

Paving TRAIL's path with ubiquitin

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ABSTRACT

Despite its name, signalling induced by the tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is versatile. Apart from eliciting cell death by both apoptosis and necroptosis, TRAIL can also induce migration, proliferation and cytokine production, in cancerous and non-cancerous cells. Unravelling the mechanisms regulating the intricate balance between these different outputs could therefore facilitate our understanding of the role of TRAIL in tissue homeostasis, immunity and cancer. Ubiquitination and its reversal, deubiquitination, are crucial modulators of immune receptor signalling. This review discusses recent progress on the orchestration of TRAIL signalling outcomes by ubiquitination of various components of the signalling complexes, our understanding of the molecular switches that decide between cell death and gene activation and what remains to be discovered.

Ubiquitin: a central regulator of Death Receptor signalling

Death Receptors (DRs) are members of the Tumor Necrosis Factor (TNF)-Receptor Superfamily (TNFR-SF) characterised by the presence of a C-terminal intracellular domain of 60-80 amino acids called the Death Domain (DD). DRs can mediate a variety of signalling outcomes, spanning from induction of cell death to survival, proliferation, differentiation, migration as well as cytokine production and are thus major players of immunity and tissue homeostasis. In humans, eight members of the TNFR-SF form part of the DR family: TNFR1, CD95 (Fas/APO-1), DR3, TRAIL-R1 (DR4), TRAIL-R2 (DR5), DR6, EDAR and NGFR. This family can be further sub-divided depending on the main signalling outcome triggered by these receptors, (i.e. the death inducers, like TRAIL-R1/2 and CD95, versus the gene activators, like TNFR1). Among the ligands of the DR family, TRAIL, identified based on its homology with CD95L [1, 2], has been of particular interest due to its unique ability of killing cancer cells without causing overt toxicity when used as a systemic drug [3, 4]. On the basis of this discovery several TRAIL-R agonists (TRAs) have been tested in clinical trials. Unfortunately, however, these first-generation TRAs have all failed due to lack of efficacy [5]. On the contrary, the CD95L/CD95 and TRAIL/TRAIL-R systems were also recognised as potent mediators of non-apoptotic signalling shortly following their respective discoveries [6, 7]; until recently studies exploring this signalling arm remained scarce. An increasing number of studies now demonstrate CD95's pro-tumorigenic role in-vivo [8-12]. Similarly, important aspects of the biology of TRAIL in cancer have only recently been uncovered [13], e.g. the discovery of the pro-tumorigenic capacity of TRAIL to enhance migration, invasion and promote the production of tumor-supportive cytokines in resistant cancer cells (**BOX 1**) [12, 14-

17]. Thus, a deeper understanding of the regulation of the different outcomes of TRAIL-induced signalling is required in order to harness the biology of TRAIL for improved treatment of cancer and indeed other diseases, including auto-immune diseases [13, 18, 19].

Recently, various types of ubiquitination events (**BOX 2**) have emerged as crucial regulators of DR-mediated signalling. Ubiquitination involves the interplay of several actors ('readers', 'writers', 'erasers') which have most extensively been studied for the TNF/TNFR1 signalling system (**Figure 1**). TNF is a pro-inflammatory cytokine crucial in the response to infections, several auto-immune diseases as well as cancer-related inflammation [20]. Its signalling through TNFR1 involves the chronological and coordinated formation of two complexes, leading to different functional outcomes as first demonstrated by Micheau and Tschopp in 2003 [21]. Since then, we have learned a lot more about the formation of these complexes; in brief, binding of TNF to TNFR1 triggers the rapid formation of the TNFR1 signalling complex (TNFR1-SC; previously also referred to as TNF-RSC). Besides TNF and TNFR1, the TNFR1-SC contains TRADD, RIPK1, TRAF2, cIAP1/2, LUBAC and the IKK and TAB/TAK complexes. The latter two functional units trigger gene activation from this signalling complex. Importantly, recruitment of the TAB/TAK and IKK complexes to the TNFR1-SC relies on the recognition of K63- and M1-ubiquitin linkages by the ubiquitin binders, i.e. 'readers', TAB2/3 and NEMO. Whereas for the recruitment of the TAB/TAK complex only K63 chains are required, the recruitment of the IKK complex involves both, K63 and M1 linkages [22-24]. Ubiquitination events are also required for gene activation downstream of the TNFR1-SC. Indeed, association of the IKK complex with the TNFR1-SC activates IKKβ which in turn phosphorylates cytosolic IκB, leading to its proteasomal degradation and the release

of the NF-κB subunits p50 and p65. Activated IKKβ also mediates the *de novo* generation of p50. This occurs via the phosphorylation of p105 [25], leading to p105 ubiquitination by the E3-ligase complex KPC1 and subsequent partial proteasomal processing, resulting in the formation of p50 as a cleavage fragment of p105 [26]. The p50 and p65 NF-κB subunits then translocate to the nucleus, acting as dimers to promote transcription of genes mainly coding for cytokines and pro-survival proteins [26].

As crucial as it is to induce gene activation, it is equally important to be able to switch it off again. The reversal of ubiquitination, mediated by so-called deubiquitinases (DUBs), is central to this activity. An important DUB in this regard is CYLD which has recently been shown to cleave both, K63- and M1-linked ubiquitin chains in the TNFR1-SC, thereby destabilizing this complex [27, 28]. However, the extent to which CYLD activity exerts a negative regulatory effect on TNF-induced gene-activatory signalling appears to be cell type-dependent [27, 29-32]. Defective ubiquitination within the TNFR1-SC, due to absence of cIAP1/2 or LUBAC, destabilizes complex I, impairs gene-activatory signalling and leads to the formation of the cytoplasmic complex II. This complex is thought to form around de- or at least less ubiquitinated components of complex I, such as TRADD and RIPK1, to which additional components are recruited including FADD, $cFLIP_{L/S}$, caspase-8/10, RIPK3 and cytosolic RIPK1 [33-36]. Complex II acts as a Death-Inducing Signalling Complex (DISC), a term initially coined for the CD95-associated plasma membrane-bound complex; in the remainder of this review we will broaden the applicability of the term "DISC" to all complexes with death-inducing functionality.

Depending on the cellular context, but especially the relative expression of caspase-8, $cFLIP_L$ and $cFLIP_S$ isoforms, complex II can trigger different types of cell death.

Whilst $cFLIP_S$ completely prevents caspase-8 activation, the $cFLIP_L$/caspase-8 heterodimer is able to cleave RIPK1 and RIPK3 [37-39], two kinases required for necroptosis. Thereby, $cFLIP_S$ restricts apoptosis but promotes necroptosis whereas $cFLIP_L$ can limit necroptosis. As further explained in the next section, the expression level of $cFLIP_L$ can modulate its specific role in DR-induced cell death. Downstream of RIPK1 and RIPK3, the pseudo-kinase MLKL is also required for necroptosis. With respect to cell death signalling, ubiquitination and deubiquitination events are particularly crucial in regulating the transition from complex I to complex II and, thereby, exert an important role in orchestrating the TNF-induced signalling outcome **(Figure 2)**.

Whereas the role of ubiquitination as a master regulator of TNFR1 signalling has been established for years, the impact of this post-translational modification (PTM) on additional DR signalling pathways is just on the brink of being uncovered [32, 40, 41]. TRAIL/TRAIL-R-mediated signalling has recently received particular attention in this regard and will thus be the main focus of this review (**Key table 1**).

Regulation of TRAIL-induced cell death by ubiquitin

TRAIL binds to four different cell surface receptors referred to as TRAIL-R1 to TRAIL-R4. Only TRAIL-R1 (also known as death receptor 4; DR4) and TRAIL-R2 (DR5) contain a cytoplasmic DD capable of recruiting FADD, a requirement to mediate cell death induction. TRAIL-R3 is a GPI-anchored receptor and TRAIL-R4, whose cytoplasmic domain only contains a truncated DD, is not capable of inducing cell death but can induce the activation of NF-κB [42].

Upon binding to TRAIL-R1/2, TRAIL induces the formation of two complexes, the TRAIL-R-associated complex I, long referred to as the DISC, and a cytosolic

complex II devoid of TRAIL-Rs. Whilst the basic scheme thereby mirrors complex formation in TNF/TNF-R1 signalling, it was recently shown that, unlike in TNF signalling, both TRAIL-induced signalling complexes can serve as DISCs [43]. Notably, the finding that complexes I and II can both act as DISCs was initially reported for CD95 signalling [44, 45]. TRAIL can induce cell death via two different modalities: the well-defined, caspase-dependent process of apoptosis [1, 2] and a more recently discovered, caspase-independent process known as necroptosis. As in TNF/TNFR1 signalling, TRAIL-induced necroptosis requires the kinase activities of RIPK1 and RIPK3 [43, 44, 46-48]. Importantly, specific and distinct ubiquitination events modulate the function of several components of this pathway, thereby decisively influencing the ultimate outcome of TRAIL-induced signalling (**Figure 3**).

Regulation of TRAIL-induced death by ubiquitination of TRAIL-R1/2 and FADD

Degradative ubiquitination events regulate both TRAIL-R1 and TRAIL-R2. In particular, MARCH8 was suggested to mediate degradative ubiquitination of TRAIL-R1 on K273 [49]. However, further experimental evidence is necessary to ascertain the E3-ligase role of endogenous MARCH8 in directly ubiquitinating TRAIL-R1. Several studies point to differential roles of TRAIL-R1 and TRAIL-R2 in apoptotic and non-apoptotic signalling [16, 50-52] and it remains to be determined to which extent distinct PTMs and the resulting interactomes account for these functional differences.

Upon TRAIL stimulation, FADD directly binds to TRAIL-R1/2 by DD-mediated homotypic interactions and is required for the recruitment of all downstream components of complex I including RIPK1, caspase-8/10, cFLIP and LUBAC [43, 53-55]. Makorin Ring Finger Protein 1 (MKRN1) constitutively poly-ubiquitinates FADD

which drives its proteasomal degradation, thereby regulating FADD protein levels [56]. Accordingly, MKRN1 prevents apoptosis induction by TRAIL, CD95L and TNF *in vitro* and interferes with TRAIL-induced cell death in a breast cancer xenograft model *in vivo*. However, the lysine residue(s) targeted by MKRN1 on FADD remain(s) unknown. As FADD is required for all DD-dependent TRAIL- as well as CD95L-induced signalling outcomes [7, 14, 46, 57-60], MKRN1 also likely dampens cytokine production and necroptosis induced by these two DR ligands. By contrast, TNF-mediated gene induction is likely to be unaffected by MKRN1 due to the lack of a role for FADD therein, whilst this E3-ligase would promote TNF-induced necroptosis given the role of the FADD/caspase-8/cFLIP$_L$ complex in limiting this type of cell death [39, 61, 62].

Regulation of TRAIL-induced death by ubiquitination of caspase-8

Binding of FADD to trimerized TRAIL-R1 and TRAIL-R2 exposes the Death Effector Domain (DED) of FADD, leading to homotypic interaction with the DEDs of caspase-8 and cFLIP$_{L/S}$. DISC-recruited caspase-8 then nucleates the homo-oligomerization of caspase-8 as well as its hetero-oligomerization with caspase-10 and cFLIP$_{L/S,}$ forming structures coined DED-mediated filaments [63-67]. Recent studies have uncovered the roles of PTMs, in particular of ubiquitination, in modulating the activation of the initiator caspase-8.

Jin et al. reported that the E3-ligase Cullin-3 mediates K48/K63 ubiquitination of caspase-8 on K461 within the p10 subunit. This occurs upon TRAIL stimulation, in an RBX1-dependent manner within complex I [68]. The ubiquitin-binding protein p62 recognizes Cullin-3-ubiquitinated caspase-8, promoting caspase-8 oligomerization, activation and ensuing apoptosis. Notably, Cullin-3 also promotes TNF- and CD95L-

induced caspase-8 activation. Jin et al. also showed that overexpression of the DUB A20 reverses Cullin-3-mediated ubiquitination of caspase-8, thereby reducing caspase-8 activation [68].

Unlike Cullin-3-mediated ubiquitination of caspase-8, other ubiquitination events of caspase-8 appear to limit its activation. The E3-ligase HECTD3, for example, reduces caspase-8 activation upon TRAIL, TNF or anti-CD95 treatment; hence HECTD3 decreases TRAIL-induced apoptosis in MDA-MB-231 cells [69]. Upon overexpression, HECTD3 forms a complex with caspase-8 and catalyzes its K63-linked ubiquitination on K215, thereby preventing caspase cleavage and p18 subunit release. Since endogenous HECTD3 is not recruited to TRAIL complex I in a stimulation-dependent manner, the degree to which the proposed mechanism accounts for HECTD3's role in limiting cell death remains to be determined.

Subsequent to Cullin-3-mediated ubiquitination of caspase-8, TRAF2 is required for the K48-linked ubiquitination of caspase-8 on K224/229/231 within the p18 domain. This leads to the proteasomal degradation of caspase-8 and the termination of TRAIL-R- and CD95-mediated apoptotic signalling [70]. The possibility for TRAF2 to act as an E3-ligase has been contested from a structural point of view [71-73]. Intriguingly, Gonzalvez et al. provide in-vitro data on TRAF2 ubiquitinating the p18 subunit of caspase-8 and show that TRAF2's RING domain is required for limiting TRAIL-induced caspase-8 activation and cell death [70-73]. Independently of whether its E3-ligase activity is required or not, current accounts argue for an anti-apoptotic role of TRAF2, a finding that is in line with TRAF2's RING domain being required for prevention of TNF-induced apoptosis [74].

It was recently demonstrated that caspase-8 is also linearly ubiquitinated upon TRAIL stimulation [43]. LUBAC, which forms part of complexes I and II in TRAIL signalling, limits caspase-8 activation therein consequently inhibiting apoptosis. Intriguingly, LUBAC promotes recruitment of A20 to these complexes [43]. Thus, it would be interesting to define whether the putative eraser role of A20 towards K63-chains on caspase-8 influences HOIP's role in TRAIL signalling. HOIP-deficiency also restricts CD95L-induced cell death in Mouse Embryonic Fibroblasts (MEFs) and primary hepatocytes, whilst LUBAC has so far not been reported to form part of CD95 signalling complexes [75, 76]. In line with their roles as negative regulators of TRAIL-induced apoptosis in complex I and II, TRAF2, LUBAC and A20 as well as the accumulation of linear chains are relatively late events in comparison to FADD/caspase-8 association, indicative of a role in termination of caspase activation [43, 70, 77].

As is often the case in biology, whilst these recent discoveries bring answers to certain questions, they also pose new ones (see outstanding questions); e.g. it remains to be defined to which extent the different caspase-8 ubiquitination events are required for enabling and regulating caspase-8 oligomerization and activation. Furthermore, the impact of ubiquitination events on the stoichiometry of the caspase-8/caspase-10/cFLIP$_{L/S}$ hetero-oligomers would be interesting to address in a cellular context. It also remains to be determined how K63-linked ubiquitin modifications of caspase-8 by Cullin-3 versus HECTD3 differ from each other molecularly, so as to achieve the above-mentioned opposite outcomes with regards to caspase activation and cell death [68, 69]. Intriguingly, although caspase-10 is efficiently activated in complex I [78, 79] and possesses the target site K461, it does not appear to be ubiquitinated by Cullin-3 [68]. Contrary to other studies which mainly relied on

overexpression, Sprick et al. found that caspase-10 cannot compensate for caspase-8 loss in mediating TRAIL- or CD95L-induced apoptosis in caspase-8-deficient Jurkat cells [78-80]. Interestingly, a recent study by Horn et al. elegantly demonstrates that caspase-10 acts as a negative regulator of CD95L-induced apoptosis [81]. Defining whether the endogenous stability, oligomerization and/or activation of caspase-10 is influenced by ubiquitination could be informative to further understand its role in regulating the different outcomes of DR signalling.

Regulation of TRAIL-induced death by ubiquitination of cFLIP$_{L/S}$

The long and short isoforms of cFLIP are major regulators of TRAIL-induced signalling. Contrary to the cFLIP$_S$/caspase-8 heteromer, the cFLIP$_L$/caspase-8 heteromer is able to cleave RIPK1, RIPK3 and CYLD and can therefore restrict necroptosis [37-39, 82]. Importantly, the ratio of cFLIP$_L$, cFLIP$_S$ and caspase-8 regulates the degree of DED-mediated filament extension and, thereby, the extent of caspase-8 activation [67]. Indeed, cFLIP$_S$ prevents caspase-8 activity by abrogating DED-filament elongation thus preventing apoptosis. When expressed at low levels, cFLIP$_L$ promotes DED-filament elongation and would hence favour apoptosis. On the contrary, high expression of cFLIP$_L$ dampens caspase-8 oligomerisation which would account for its role in restricting apoptosis.

In accordance with these important, yet distinct roles in fine-tuning cell death signalling, the protein levels of cFLIP$_L$ and cFLIP$_S$ are tightly but differentially regulated by PTMs with ubiquitination featuring most prominently amongst them. Itch is a HECT-E3 ligase reported to specifically interact with cFLIP$_L$, mediating its K48-linked ubiquitination and proteasomal degradation upon TNF stimulation in a JNK-dependent manner [83]. Itch was also proposed to decrease the stability of cFLIP$_L$

and $cFLIP_s$ and to thereby promote TRAIL-induced apoptotic signalling [84, 85]. Several lysines are targeted to promote proteasomal degradation of $cFLIP_L$ and $cFLIP_S$, but the impact of their mutation on TRAIL-induced signalling remains to be assessed [86-88]. Conversely, the DUB USP8 interacts with $cFLIP_L$ via its caspase-like domain, leading to its deubiquitination, thus preventing the proteasomal degradation of $cFLIP_L$ [89]. In accord, USP8 limits TNF-, CD95L- and TRAIL-induced apoptosis, the latter also in-vivo [89]. USP8 might also modulate the stability of $cFLIP_L$ and $cFLIP_s$ indirectly by deubiquitinating Itch [90]. The involvement of USP8 in DR-induced necroptosis and gene-activatory signalling remains elusive. Whether and to which extent non-degradative ubiquitination of $cFLIP_L$ and/or $cFLIP_S$ also influences their respective functions in DR-signalling would also be interesting to explore.

As highlighted above, we are only beginning to grasp the complexity of the regulatory roles different E3s and DUBs exert on the function and stability of key components of the TRAIL signalling pathway. This points out a major research avenue that will likely lead to exciting fundamental, but potentially also therapeutically relevant findings with regards to TRAIL signalling which may extend to other immune receptor signalling systems (see Outstanding questions).

RIPK1 and RIPK3 are ubiquitinated during TRAIL-induced death

RIPK1 can be detected in both, complex I and II of TRAIL signalling to which it is recruited in a FADD/caspase-8-dependent manner. In both complexes RIPK1 is present as a heavily ubiquitinated component. Besides its role as a regulator of caspase-8 ubiquitination [68], A20 might limit TRAIL-induced apoptosis by K63-ubiquitinating RIPK1, as these chains were suggested to limit caspase-8 activation in

glioblastoma cells [91]. It is, however, puzzling that the K63-DUB A20 would also act as an E3 ligase that forms K63-linked chains. Hence, the mechanism by which caspase-8 recognizes, and is inhibited by, K63-decorated RIPK1 remains to be defined in more detail.

In addition to caspase-8, also RIPK1 is a LUBAC target upon TRAIL stimulation [43]. Similar to recent findings in TNFR1, IL1R and TLR1/2/3 signalling [92], the linear chains generated upon TRAIL stimulation are added on top of other chain types. As such, depletion of cIAP1/2 by a SMAC mimetic compound, which sensitizes cells to TRAIL- and CD95L-induced death, not only impairs recruitment of LUBAC and A20 to TRAIL complex I but also substantially reduces RIPK1 ubiquitination in this complex [43]. The latter event was previously also reported for complex I of CD95 signalling [44]. Thus, cIAP1/2 enable LUBAC recruitment and likely directly catalyse the formation of ubiquitin chains on RIPK1 which form the basis for linear chain addition, potentially in various DR signalling pathways. In the context of TNF signalling, LUBAC is currently thought to prevent death by limiting the formation of complex II through stabilisation of the TNFR1-SC [28, 75, 93-95]. Similarly, following TRAIL stimulation, RIPK1 also accumulates in the complex II that forms in cells which are deficient in HOIP or only deficient in its catalytic activity [43]. However, the model according to which ubiquitination events on RIPK1 solely prevent its transition from complex I to II in TNF signalling might well be too simplistic. Indeed, under specific necroptosis-triggering conditions RIPK1 is heavily ubiquitinated by M1 and K63-chains in the necrosome [96]. Herein, the K63 chains on RIPK1 appear to contribute to necroptosis induction whilst the role of the M1 chains remains enigmatic [97]. Intriguingly, HOIP's catalytic activity is not absolutely required for limiting RIPK1-kinase-dependent apoptosis upon TRAIL stimulation. Moreover, HOIP

prevents TRAIL-induced necroptosis and the association of heavily ubiquitinated RIPK3 and phosphorylated MLKL with a FADD/caspase-8/RIPK1-containing necroptosis-inducing complex; again independently of its activity [43]. The HOIP-dependent factors which modulate RIPK1 kinase-dependent apoptosis and necroptosis as well as RIPK3 and RIPK1 ubiquitination in complex II remain to be defined. Interestingly, depletion of cIAP1/2 or knock-down of TRAF2 also sensitize cells to TRAIL- and CD95L-induced necroptosis [44, 47]. The mechanism underlying the effect of TRAF2 or cIAP depletion in CD95 signalling would require an in-depth investigation. In TRAIL signalling, however, it is likely that part of this sensitization results from the absence of LUBAC recruitment to complex I and II.

The regulation of TRAIL-mediated non-death signalling by ubiquitin

TRAIL and CD95L can also promote cell survival, proliferation, migration, cytokine secretion and immuno-modulation [8-12, 14, 16, 59, 98-103]. Studies deciphering the involvement of ubiquitination in TRAIL- and CD95L-mediated non-apoptotic signalling remain scarce and have mainly focussed on cytokine production. TRAIL-induced cytokine production involves transcription factors such as NF-κB and members of the MAPK families such as JNK, ERK1/2 and p38 (**BOX 2**), which are known modulators of TNF-induced pro-inflammatory signalling [7]. Whilst MAPKs can participate in cytokine production, canonical activation of NF-κB appears to be the most prominent and consistently activated pathway driving TRAIL- and most likely also CD95L-induced cytokine production [14, 43, 59, 77, 100, 101, 104]. In accordance, TAK1 and IKKα/β, which are crucial in NF-κB activation, are required for TRAIL-induced cytokine production [14, 43, 77]. TRAIL can trigger NF-κB activation by binding to TRAIL-R1, TRAIL-R2 and TRAIL-R4 [7, 42]. Thereby, TRAIL signalling can promote the transcription of pro-inflammatory cytokines such as CCL2, IL-8,

CXCL1, CXCL5 and NAMPT and anti-apoptotic genes such as cFLIP and Mcl-1 [14, 105]. Hence, gene-activatory signalling can facilitate resistance to TRAIL-induced death in cancer cells [106].

In resistant cancer cells TRAIL and CD95L elicit the secretion of a similar repertoire of cytokines which, in the context of TRAIL-signalling, can modulate the immune-microenvironment to promote tumorigenesis [14, 59, 60]. TRAIL-induced gene-activatory signalling has long been associated with the cytosolic complex II, a complex which was recently dubbed the “FADDosome” [77, 99]. However, also the membrane-associated complex I of TRAIL signalling can induce gene activation [43]. Both complexes contain FADD, caspase-8, RIPK1 and the IKK complex, all of which are core factors for mediating TRAIL-induced gene activation [43, 77, 107].

FADD and Caspase-8 are essential for TRAIL-induced gene activation

In contrast to TNF signalling, the *bona fide* death ligands TRAIL and CD95L mediate gene-activatory signalling via FADD and caspase-8, which are also the core components facilitating death ligand-induced apoptosis [60]. The apical adaptor FADD is essential for the formation of both, complex I and II and is therefore also crucial for TRAIL- and CD95L-mediated gene-activatory signalling and cytokine production [14, 43, 59, 60, 99, 101, 108, 109]. Downstream of FADD, caspase-8 recruits several components in turn promoting TAB/TAK and IKK complex recruitment and activation [43, 77]. Therefore, contrary to the situation in TNF signalling, caspase-8 is required for TRAIL- and CD95L-induced gene activation and cytokine production [14, 43, 99, 101, 108, 110]. Intriguingly, the proteolytic activity of caspase-8 is dispensable herein and, if anything, limits rather than promotes cytokine production [14, 43, 60, 77, 101, 111]. This effect is likely due to caspase-8’s

ability to promote cell death and cleave RIPK1, a component of the TRAIL signalling complexes which induces cytokine production in certain cell types [14, 60, 112].

Apart from FADD, caspase-8 and RIPK1, the additional DED-containing proteins caspase-10 and cFLIP also influence TRAIL-induced gene activation [81, 109]. Unlike caspase-8, caspase-10 is not essential for TRAIL-induced cytokine production but contributes to it [77]. Although the underlying mechanism currently remains elusive for TRAIL signalling, with regard to CD95L-induced signalling it was recently shown that the activity of caspase-10 is dispensable for its function in gene activation [81].

Knockdown of $cFLIP_L$ and $cFLIP_S$ facilitates IKK recruitment to complex I of TRAIL signalling and elevates cytokine induction upon CD95L treatment [43, 60, 113]. However, owing to their differential abilities in enabling caspase-8 oligomerization and activity [67], the specific roles of $cFLIP_L$ versus $cFLIP_S$ in cytokine production could actually oppose each other, an aspect which remains to be resolved.

Regulation of TRAIL-mediated gene activation by E3 ligases

Apart from modulating apoptosis signalling, E3 ligases also regulate the gene-activatory outputs of TRAIL signalling as two core signalling components which are involved in TRAIL-induced gene activation, caspase-8 and RIPK1, are substantially targeted by ubiquitination [43, 77]. As explained above, the E3 ligases cIAP1/2 and HOIP are recruited to both TRAIL signalling complexes in a FADD/Caspase-8-dependent manner. Also, both TRAF2 and cIAP1/2 enhance TRAIL- and CD95L-mediated gene activation [43, 59, 77, 114]. As such, knockout of TRAF2 in cFLIP-expressing cells, as well as transient TRAF2 knockdown attenuated NF-κB activation, whilst overexpression enhanced cytokine induction [99, 101, 115]. In the

context of TNF signalling, TRAF2-cIAP1/2-mediated ubiquitination of RIPK1 facilitates NF-κB activation [116-119] wherein TRAF2's gene-activatory functions rely on its ability to recruit cIAP1/2 [74]. In line with a gene-activatory role for TRAF2 as a scaffold in TRAIL signalling, depletion of cIAP1/2 strongly decreased RIPK1 ubiquitination, IKK recruitment, NF-κB activation and blunted TRAIL-mediated cytokine secretion, whilst TRAF2 recruitment remained unaffected [14, 43]. Thus, TRAF2 likely promotes TRAIL-induced cytokine production by serving as the recruitment platform for cIAPs, as previously shown for TNF signalling [74]. Downstream of TRAF2, cIAP1/2 are also required for the recruitment of LUBAC to TRAIL complex I [43]. Although the molecular mechanism remains unexplored, HOIP-deficiency was demonstrated to decrease CD95L-induced NF-κB activation in primary murine hepatocytes [76].

LUBAC is decisive for TNFR1-induced gene-activatory signalling by mediating TNFR1-SC stabilization via linear ubiquitination of TRADD, RIPK1, NEMO and the TNFR1 [93, 94]. Yet, whilst FADD and caspase-8 are dispensable for recruitment of cIAPs and LUBAC to the TNFR1-SC, their recruitment to the TRAIL-R-SC requires FADD and caspase-8 [43, 120]. Within the TRAIL-induced complexes I and II, LUBAC promotes recruitment of the IKK complex and, thereby, mediates TRAIL-induced activation of NF-κB, as consistently found in various cancer cell lines and in several non-transformed cell types [43]. As LUBAC is required for TRAIL-induced secretion of cytokines and chemokines, this E3 ligase is likely of physiological relevance for TRAIL-mediated modulation of cancerous and non-cancerous tissue homeostasis [14].

In the TRAIL-induced signalling complexes HOIP linearly ubiquitinates caspase-8 and RIPK1, events which are likely required for IKK complex recruitment and

ensuing NF-κB activation [43]. It is important to note, however, that absence of HOIP neither completely abrogates TRAIL- nor TNF-induced NF-κB activation [28, 43, 94]. NEMO, the regulatory subunit of the IKK complex, contains a UBD in ABIN and NEMO (UBAN) domain, which has a significantly higher affinity for M1- than for K63-linked chains, as well as a zinc finger (ZF) which preferentially recognizes K63 chains. Together, this confers dual affinity for M1- and K63-linkages to NEMO [121, 122]. The coordinated formation and recognition of different linkages, formed by LUBAC, cIAP1/2 and possibly additional E3 ligases in turn enables the recruitment of the IKK complex to the TRAIL-R-SC, consequently activating the NF-κB pathway. Mechanistically, LUBAC might also promote TRAIL-induced cytokine production by limiting the activation of caspase-8 as explained above. Interestingly, HOIP itself is cleaved by caspase-8 upon TRAIL stimulation, although cleavage does not affect its role in TRAIL-induced apoptosis or gene activation, at least *in vitro* [43].

Regulation of TRAIL-mediated gene activation by deubiquitinases

Although the impact of deubiquitination events within TRAIL-induced signalling complexes on gene-activatory signalling output remains to be unravelled, certain functional insight has already been gained especially regarding the DUBs A20 and CYLD. Similar to its role in TNF signalling [28, 123], A20 limits TRAIL-induced IL-8 and IL-6 secretion [77] and its presence in both TRAIL signalling complexes was recently shown to be dependent on HOIP [43, 77]. This is likely due to a requirement of HOIP-catalysed M1 chains for A20 recruitment to these complexes, as shown for this DUB's recruitment to the TNFR1-SC [28, 124, 125]. Whether A20's effect on TRAIL signalling specifically requires its DUB activity or, as in TNF signalling, is owed to its function as a binder/occupier of linear ubiquitin linkages [28, 124, 125], remains to be established.

Similar to A20, CYLD is recruited to complexes I and II of TRAIL signalling in a HOIP-dependent manner [43]. Whilst not formally proven, it is again highly likely that, as in the case of the TNFR1- and NOD2-SCs, CYLD recruitment to these complexes also relies on its interaction with HOIP via SPATA2 [29, 126-128]. CYLD might limit TRAIL-induced NF-κB signalling as shown in overexpression systems [129, 130]. However, CYLD was only detected in the TRAIL-induced signalling complexes upon caspase inhibition. Accordingly, CYLD does not affect TRAIL-mediated cytokine production when caspases are active, possibly because caspase-8-mediated cleavage can inactivate CYLD [43, 77]. It remains to be determined whether conditions of caspase inhibition (e.g. viral infections) would render CYLD an efficient inhibitor of TRAIL-induced gene activation.

Concluding remarks and perspectives

The signalling pathways induced by TRAIL and TNF are initiated via their respective receptors and regulated via multiple common proteins, yet the two systems' respective primary signalling outcomes oppose each other as TRAIL's primary signalling output is cell death whereas that of TNF is gene activation. This feature was long thought to result from opposite bifurcations in the respective signalling pathways. Indeed, receptor-associated complexes were thought to drive cell death or gene activation from the respective complexes I of TRAIL and TNF signalling. Secondary cytoplasmic signalling complexes would in turn trigger the remaining signalling outcomes, i.e. cell death for complex II of TNF signalling and gene activation for complex II of TRAIL signalling. Recent findings, however, implicate ubiquitination in specifically modulating the formation and function of these different signalling complexes.

In the context of TNF signalling, these studies have highlighted the importance of ubiquitination events in controlling the transition from the gene-activatory TNFR1-SC to the death-inducing complex II. Importantly, in the case of TRAIL signalling, the study of signal modulation by ubiquitin has revealed that gene activation and cell death are not induced by spatially distinct signalling complexes, but that they are instead fine-tuned by ubiquitination events within TRAIL complexes I and II which can both function as DISCs and as gene-activatory platforms [43]. Hence, ubiquitination events control the delicate balance between apoptosis, necroptosis and cytokine production in distinct ways in TRAIL- versus TNF-induced signalling. This said, we are only beginning to understand the precise molecular events and mechanisms at heart of the distinct regulatory processes that are responsible for the differences in signalling outcome (see outstanding questions). The expanding availability of sophisticated tools in studying ubiquitination and deubiquitination events in ever more detail and, at the same time, on the proteomic scale [131-134], offers the unique opportunity to decipher the complex ubiquitin code that regulates TRAIL versus TNF signalling and identify the distinguishing hallmarks between them. Whilst historically mainly disregarded as druggable targets [135], specific therapeutic targeting of the proteins involved in modulating the ubiquitin code is now beginning to become reality [135, 136]. Since TRAIL/TRAIL-R signalling is implicated in tumor biology, inflammation and immunity, further understanding of the readers, writers and erasers of TRAIL's ubiquitin code will likely provide an opportunity for the identification of novel biomarkers and/or clinical targets for harnessing the TRAIL/TRAIL-R system therapeutically towards the treatment of various diseases including cancer but also inflammatory, auto-immune and infectious diseases.

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Figure legends

Figure 1. Ubiquitin chain types

Polyubiquitin chains consist of ubiquitin monomers which are joined via isopeptide or peptide bonds to form non-linear or linear chains, respectively. Conjugation occurs between the C-terminal carboxyl group of the incoming monomer and a specific ε-amino Lysine (K) or the N-terminal Methionine (Met 1) group of the proximal ubiquitin monomer; the latter linkage defines the structure of the ubiquitin chain and determines its respective functionality. Hybrid, also referred to as mixed ubiquitin chains, for example composed of both K63- and M1-linked chains, have also been identified and play major roles in regulating multiple signalling pathways [137].

Figure 2. Regulation of TNF signalling by ubiquitination

TNF binding triggers the oligomerization of TNFR1, enabling recruitment of the adaptor TRADD and the kinase RIPK1 by DD-mediated homotypic interactions. Next, TRADD recruits the RING-domain containing protein TRAF2, which recruits the E3-ligases cIAP1/2 that in turn ubiquitinate themselves and several other components of the TNFR1-SC. These ubiquitin chains provide a platform for recruitment of the TAB/TAK complex and the Linear Ubiquitin chain Assembly Complex (LUBAC). By targeting RIPK1, TRADD and TNFR1, LUBAC facilitates the recruitment and activation of the IKK complex. This initial complex, referred to as the TNFR1-SC or complex I of TNFR1 signalling, drives the activation of MAPKs (JNK, p38 and ERK) and the canonical NF-κB pathways. These pathways, with NF-κB at the forefront, in turn promote the transcription of pro-inflammatory cytokines and several cell death-inhibiting factors. A secondary cytoplasmic signalling complex is

also formed upon TNF stimulation. This complex II is composed of de-, or at least less ubiquitinated components of complex I, such as TRADD and RIPK1, to which additional components are recruited including FADD, $cFLIP_{L/S}$, caspase-8/10, RIPK3 and cytosolic RIPK1. Upon deficiency of crucial ubiquitin modulators, such as cIAPs or LUBAC, the stability of the TNFR1-SC can be compromised, resulting in dampening of gene-activatory signalling and enhanced complex II formation. Complex II can act as a DISC, from which both necroptotic and apoptotic signalling emerge depending in particular on the relative abundance of the different cFLIP isoforms. Therefore, complex II triggers a classical caspase-8-dependent apoptosis or a caspase-independent necroptosis, which relies on the activation of the kinases RIPK1, RIPK3 and the pseudo-kinase MLKL.

Figure 3. Regulation of TRAIL signalling by ubiquitination

Binding of TRAIL to TRAIL-R1/2 triggers the formation of the TRAIL-R-associated complex I. Within complex I, multiple ubiquitination events control the induction of apoptosis and gene-activatory signalling. For example, addition of K63-chains on caspase-8 by Cullin-3 promotes activation, whilst TRAF2-dependent K48-ubiquitination triggers the proteasomal degradation of this protease. Downstream of FADD, caspase-8 and cIAP1/2, LUBAC linearly ubiquitinates both caspase-8 and RIPK1 in complex I, thereby favouring A20 recruitment, limiting caspase-8 activation and promoting NF-κB activation which is the main driver of ensuing cytokine production. A second TRAIL-R-devoid, cytosolic complex, complex II, is detected upon TRAIL stimulation. The composition of complex II is very similar to that of complex I with caspase-8 and RIPK1 also being heavily ubiquitinated in complex II.

Similarly to complex I, these ubiquitination events dictate the prevailing signalling outcome. Under certain circumstances, e.g HOIP deficiency and caspase inhibition, RIPK3 and MLKL are recruited to this secondary complex and induce necroptosis. Whether RIPK3 and MLKL may also be recruited and activated within complex I remains to be investigated. cFLIP isoforms tightly and differentially regulate TRAIL signalling, likely owing to their different abilities to control DED-protein containing filament formation (not shown here). In addition to ubiquitination events within complex I and II, the basal levels of several core components of TRAIL signalling, e.g TRAILR1/2, FADD and $cFLIP_{L/S}$, are tightly regulated by degradative ubiquitination driven by several E3-ligases (not represented here).

BOX 1: Role of endogenous TRAIL/TRAIL-R signalling in cancer

The TRAIL/TRAIL-R system can elicit the induction of cell death and of gene activation. Mice deficient for TRAIL or TRAIL-R are viable, fertile and do not exhibit any overt phenotype [138, 139]. However, TRAIL signalling has been implicated in diverse roles upon pathological challenge, ranging from immune-surveillance in anti-viral and anti-tumor defence to tumor-supportive effects, including modulation of the tumor microenvironment [13].

Regarding immune-regulatory effects, natural killer (NK) cells in particular utilize surface TRAIL to promote their cytolytic antiviral and anti-tumor effector functions; more extensively reviewed elsewhere [140, 141]. For regulatory T cells (Tregs), TRAIL expression can also establish immune tolerance by eliciting potent immune-suppressive functions, thereby enhancing survival of mice in an allogeneic skin graft model [142]. Regarding functionality of endogenous TRAIL in tumor physiology, several lines of evidence exist for both anti- and pro-tumor functions of the

TRAIL/TRAIL-R system [13]. TRAIL knockout mice exhibited enhanced tumor burden upon transplantation with A20 B cell lymphoma in comparison to wildtype counterparts [3, 139]. In accordance, surface expression of TRAIL on liver NK cells enables TRAIL-mediated anti-tumor immune surveillance; particularly regarding metastasis suppression [140]. Indeed, TRAIL-deficient mice were more susceptible to experimental liver metastasis [139, 143]. This metastasis-suppressive effect results from detachment-induced sensitization to TRAIL-mediated apoptosis in metastasizing tumor cells [144].

Dependent on the oncogene mutation status, TRAIL can, however, also promote the formation of metastases in TRAIL apoptosis-resistant cancer cells [15, 16]. As such, cancer cell-endogenous mTRAIL-R expression promotes progression, invasion and metastasis in autochthonous KRAS-driven murine pancreatic and lung cancer models [16]. Next to cell-autonomous roles, endogenous TRAIL/TRAIL-R signalling mediates cytokine production, thereby modulating the composition of the tumor immune microenvironment, in a FADD/caspase-8-dependent manner. As such, endogenous TRAIL-signalling was recently shown to promote the accumulation of tumor-supportive M2-like myeloid cells via a CCL2/CCR2 axis [14, 16].

BOX 2: Regulators of the ubiquitin code

Ubiquitination is the attachment of the C-terminal glycine residue of ubiquitin to the epsilon-amino-group of a lysine residue of a substrate. This process is mediated by the coordinated action of three classes of ‘writer’ enzymes, the E1, E2 and E3. Ubiquitin possesses seven lysines (K) which are also targeted for ubiquitination, leading to the formation of poly-ubiquitin chains. The N-terminal methionine (M1) of the proximal ubiquitin can also serve as a target for the formation of chains which are

exclusively generated by the specific E3-Ligase, Linear ubiquitin chain assembly complex (LUBAC). LUBAC is composed of HOIL-1, SHARPIN and the catalytically active component HOIP. Poly-ubiquitin linkages are categorized depending on the modified residue of the target ubiquitin, which designates chain functionality. Thus K6, K11, K27, K29, K33, K48, K63 and M1 linkages can be distinguished and differentially combined on a given target or residue forming a 'ubiquitin code' [137] (**Figure 1**).

Chain recognition occurs by Ubiquitin Binding Domains (UBD) present in multiple 'reader' proteins, which elicit crucial complex modulating functions, deciphering the ubiquitin code. In the context of TNF signalling, K63 and M1 chains recruit the TAB/TAK and the IKK complex (readers), followed by K48 ubiquitination and consequent proteasomal degradation of I-κB, consequently ensuring optimal gene activation. Hence, ubiquitination modulates the function and fate of both, the ubiquitin targets and the respective reader proteins, thereby enabling a coordinated downstream signalling output.

The final actors involved in regulation of the ubiquitin code belong to a specific class of isopeptidases, named deubiquitinases (DUBs) which are able to hydrolyse ubiquitin linkages, therefore referred to as 'erasers' of the ubiquitin code. Certain proteins are believed to fulfil multiple of these functions with A20 perhaps being thought of as the most versatile player, as it has been suggested to act as writer, reader and eraser of the ubiquitin code. In TNF signalling, A20 has been proposed to negatively regulate NF-κB activation and death by removing K63-chains from RIPK1 via its OTU domain and by subsequently K48-ubiquitinating RIPK1 via its ZnF4 [123, 145]. Moreover, A20 can elicit DUB activity-independent functions. Importantly, wild type and OTU mutants of A20 equally restrict TNF-induced NF-κB activation and cell

death while a ZnF7 mutant of A20, which is not able to bind to linear ubiquitin chains, fails to do so, demonstrating that A20 can act as a scaffold/ubiquitin-protective protein [28, 124, 125, 146, 147]. Contrary to A20-deficent mice, A20 OTU mutant or A20 ZnF4 mutant mice do not develop any signs of inflammation [148-150]. The generation of ZnF7 mutant mice would thus likely unravel the physiological importance of the different functions of A20 in regulating the ubiquitin code.

Outstanding questions

Do the TRAIL-R1 and TRAIL-R2 signalling complexes differ in composition and, if so, is differential complex formation regulated by ubiquitination?

How and where is the TRAIL-induced necroptosis-mediating complex formed and how do ubiquitination events exactly regulate its formation and function?

Do $cFLIP_L$ and $cFLIP_S$ differentially modulate TRAIL-induced gene activation and, if so, what is the contribution of ubiquitination to this difference?

Is caspase-10 ubiquitinated upon TRAIL signalling and, if so, to what extent does this affect TRAIL-R signalling output?

What are the specific roles of the LUBAC components SHARPIN and HOIL-1 in TRAIL signalling?

What are the distinct molecular mechanisms underlying HOIP's activity-dependent and -independent roles in TRAIL signalling?

To which extent do cIAP1/2 affect TRAIL signalling independently of their role in HOIP recruitment?

What are the molecular requirements for recruitment of Cullin-3, TRAF2/cIAPs to TRAIL complex I and II?

What is the relative contribution of the different roles of A20 (writer, reader and eraser of the ubiquitin code) to its function in TRAIL signalling?

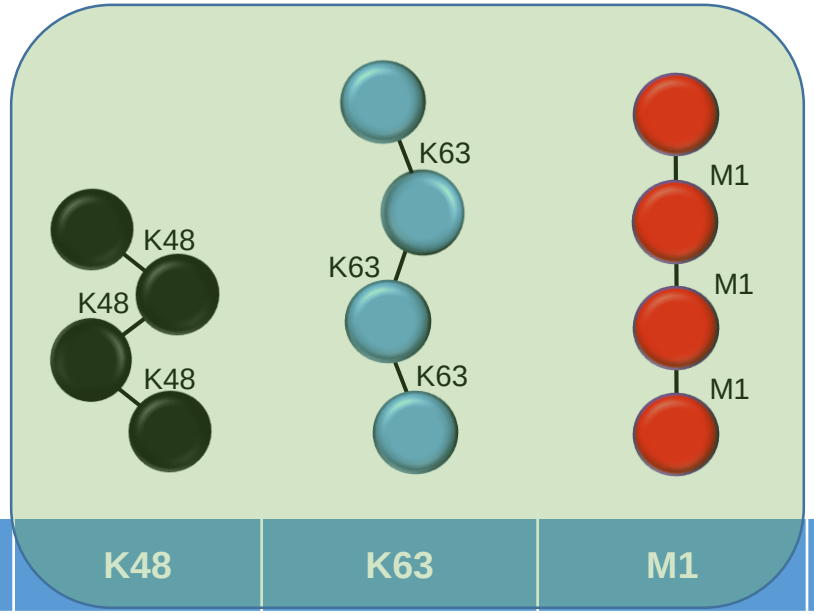
How does the caspase substrate CYLD regulate TRAIL signalling?

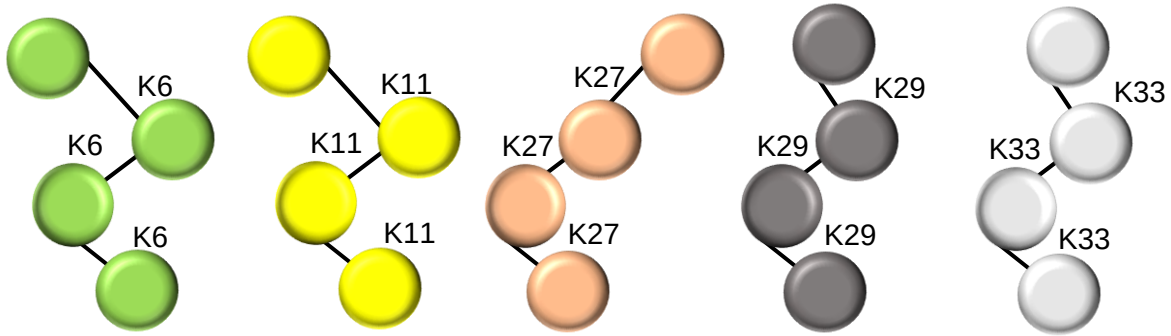
How do ubiquitin events impact on the cross-talk between cancer cells and their microenvironment *in vivo*?

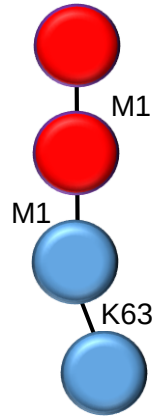
Could ubiquitin modifiers and binders be used as biomarkers or possibly targets for rendering TRAIL/TRAILR-based cancer therapies more effective?

Figure 1

Associated with TRAIL/TRAIL-R signalling

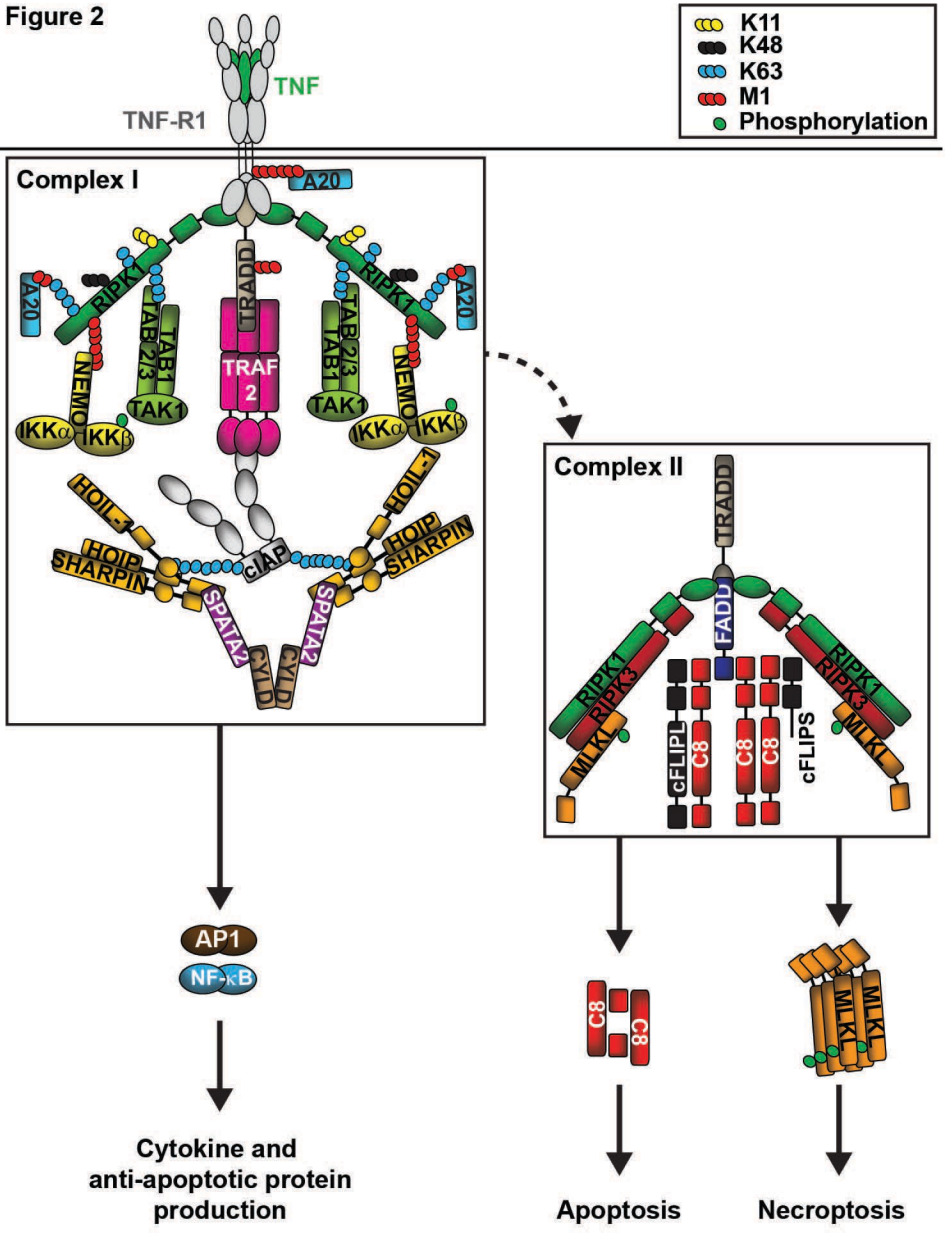






Linkage type	K48	K63	M1	K6	K11	K27	K29	K33	K63-M1 (Hybrid)
Function	-Protein turnover -Degradation	-Gene activation -Innate immunity -DNA damage response -Scaffold	-Gene activation -Innate immunity -Scaffold	-Mitophagy -DNA damage response	-Mitophagy -Protein Turnover -Trafficking -Degradation	-Autoimmunity -Mitochondrial damage response -Scaffold -Degradation	-Degradation	-Trafficking -Adaptive immunity	-Scaffold
Signalling platforms	-TNF -IL-1α/β -TLR3/4…	-TNF -IL-1α/β -TLR3/4…	-TNF -IL-1β -TLR3/4…	N/D	-TNF	-TNF	-Wnt/β catenin	-AMPK kinase -TCR signalling	-TNF -IL-1α/β -TLR1/2/3

Figure 2


K11
K48
K63
M1
Phosphorylation
TNF
TNF-R1
Complex I
A20
RIPK1
TRADD
TAB2/3
TAB1
TAK1
TRAF 2
NEMO
IKKα
IKKβ
HOIL-1
HOIP
SHARPIN
cIAP
SPATA2
CYLD
Complex II
FADD
cFLIPL
C8
cFLIPS
RIPK3
MLKL
AP1
NF-κB
Cytokine and anti-apoptotic protein production
Apoptosis
Necroptosis

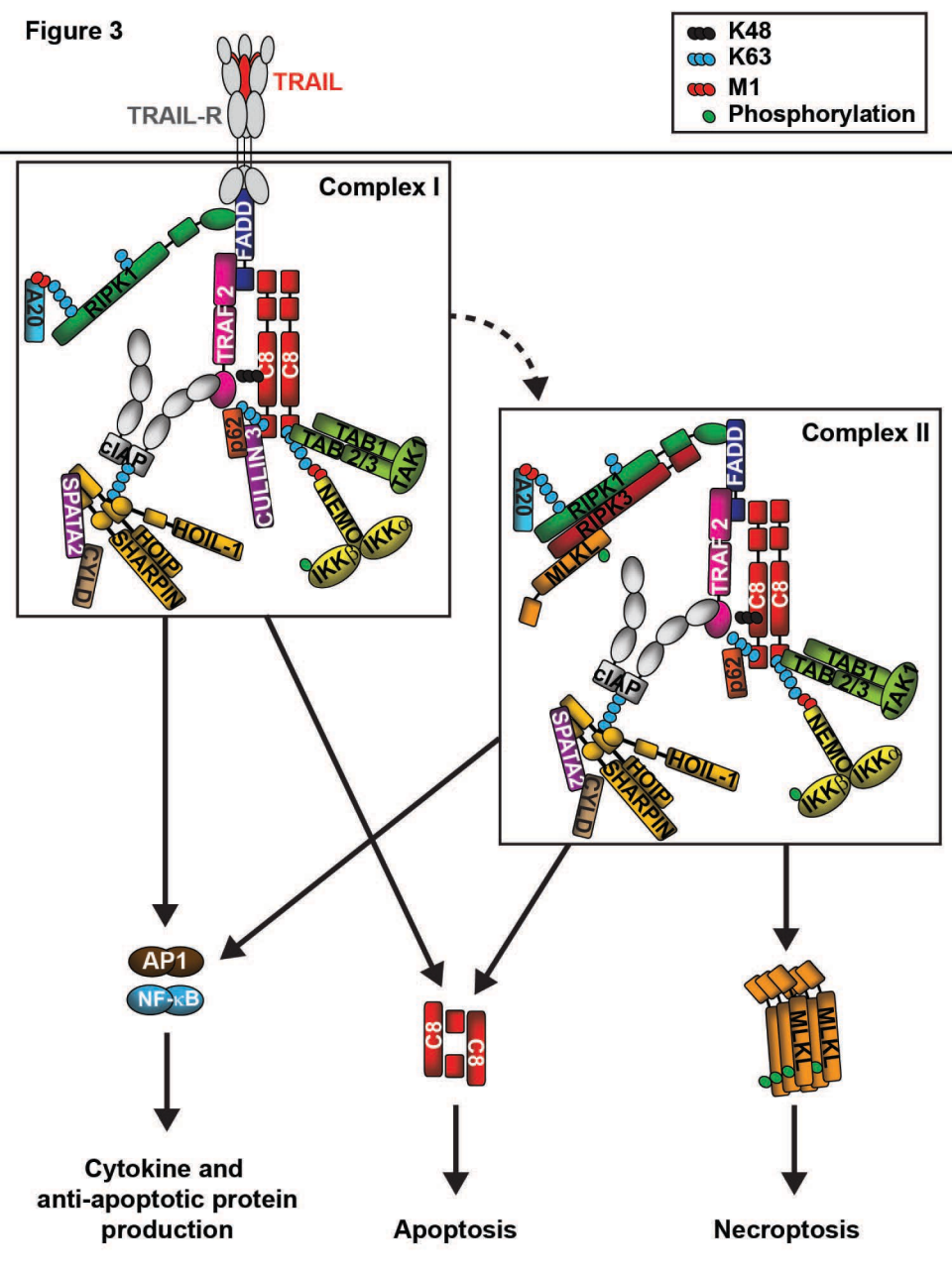


Figure 3
TRAIL
TRAIL-R
K48
K63
M1
Phosphorylation
Complex I
FADD
RIPK1
A20
TRAF2
C8
C8
cIAP
p62
CULLIN-3
TAB1
TAB2/3
TAK1
SPATA2
HOIL-1
HOIP
SHARPIN
CYLD
NEMO
IKKγ
IKKβ
Complex II
FADD
RIPK1
RIPK3
A20
MLKL
TRAF2
C8
C8
cIAP
p62
TAB1
TAB2/3
TAK1
SPATA2
HOIL-1
HOIP
SHARPIN
CYLD
NEMO
IKKγ
IKKβ
AP1
NF-κB
C8
C8
MLKL
MLKL
Cytokine and anti-apoptotic protein production
Apoptosis
Necroptosis

Key Table 1. Regulators of the ubiquitin code in TRAIL signalling

Regulator of the ubiquitin code (function)	Substrate Targeted residue Ubiquitination type	Impact on TRAIL-induced cell death	Impact on TRAIL-induced gene activation/cytokine production	References
MARCH 8 (E3)	TRAIL-R1 (K273) K48?	↓ Apoptosis Necroptosis N/D	N/D	[49]
MKRN1 (E3)	FADD K48?	↓ Apoptosis Necroptosis N/D	N/D	[56]
Cullin-3 (E3)	Caspase-8 (K461) K48/K63	↑ Apoptosis Necroptosis N/D	N/D	[68]
HECTD3 (E3)	Caspase 8 (K215) K63	↓ Apoptosis Necroptosis N/D	N/D	[69]
TRAF2 (E3)	Caspase-8 (K224/229/231) K48	↓ Apoptosis ↓ Necroptosis	↑ NF-κB and JNK signalling ↑ Cytokine production	[47, 70, 99, 101, 115]
cIAP1/2 (E3)	RIPK1?	↓ Apoptosis ↓ Necroptosis	↑ NF-κB signalling ↑ Cytokine production	[14, 43, 44]
LUBAC (E3)	RIPK1, Caspase-8 M1	↓ Apoptosis ↓ Necroptosis	↑ NF-κB and ERK signalling ↑ Cytokine production	[43]
Itch (E3)	$cFLIP_{L/S}$ K48?	↑ Apoptosis Necroptosis N/D	N/D	[84, 85, 90]
USP8 (DUB)	$cFLIP_L$, Itch K48?	↓ Apoptosis Necroptosis N/D	N/D	[89, 90]
A20 **(E3)** **(DUB)** **(UBD)**	 RIPK1 K63 Caspase-8 (K461) K48/K63 K63, M1 chains?	 ↓ Apoptosis Necroptosis N/D	 ↓ Cytokine production	[43, 68, 77, 91]