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PERSPECTIVES

Genotoxicity-Stimulated and CYLD-Driven Malignant Transformation

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Abstract: Oxidative stress, which can cause DNA damage, can both activate TNF-R1 directly in the absence of TNF stimulation and phosphorylate c-Abl, thus promoting its cytoplasmic translocation. Persistent cytoplasmic localization of c-Abl has been associated with cellular transformation. c-Abl phosphorylates OTULIN at tyrosine 56, thereby disrupting its relationship with LUBAC. OTULIN-released LUBAC interacts with SPATA2 and is recruited to the TNF-R1sc, facilitating SPATA2-CYLD interaction. All these interactions are required for the activation of IKKβ to stimulate NF-κB transcriptional activity following genotoxic stress. IKKβ also induces the critical phosphorylation of CYLD at serine 568 to increase its deubiquitinating (DUB) activity required for the termination of signaling cascades. Contrary to the widespread belief that CYLD is an absolute tumor suppressor, CYLD initiates and terminates NF-κB activity by alternately using its oncoprotein and tumor suppressor activities, respectively. If IKKβ fails to achieve the DUB activity-inducing phosphorylation at serine 568, CYLD would operate in a sustained mode of oncogenic activity. The resulting dysregulated NF-κB activation and other accompanying pathologies will disrupt cellular homeostasis in favor of transformation.

Keywords: CYLD, DNA damage, NF-κB, OTULIN, c-Abl, IKKβ, TNF-R1

Plain Summary

•Oxidative stress can cause both DNA damage and ligand-free TNFR stimulation.

•DNA damage disrupts the OTULIN-LUBAC interaction and initiates NF-kB transcription via the LUBAC-SPATA2-CYLD-mediated IKKβ activation pathway.

•IKKβ also terminates the NF-κB signaling by activating the deubiquitinase function of CYLD through Serine 568 phosphorylation.

•Following oxidative DNA damage, failure to phosphorylate CYLD at serine 568 could initiate a myriad of signaling that can trigger a series of feedback cycles that can force uncontrolled cell proliferation.

Introduction

There is extensive evidence indicating that reactive oxygen species (ROS)-induced DNA damage accumulation leads to genomic instability, which is important for malignant transformation.[1] DNA damage can also be caused by a variety of factors other than oxidative stress, including exogenous agents such as ultraviolet light and ionizing radiation, as well as endogenous triggers such as errors in DNA replication (replication stress). Unrepaired damage to DNA can eventually cause cell death if the damage is too extensive. In case of the damage is tolerable, it can be retained and potentially transmitted to future progeny if the cell maintains its ability to divide.[1,2] As evidence, the vast majority of human cancers contain pathological changes that exhibit persistent DNA damage and genomic instability.[1] Several characteristic alterations occur during the transformation of a cell, including the capacity to proliferate autonomously, escape apoptosis, invade surrounding tissues, and metastasize to distant sites. Since many of these properties accompany the dysregulated activation of nuclear factor-kappa B (NF-kB), a transcription factor is accepted as one of the leading mediators capable of inducing cell transformation. Consistently, NF-κB is aberrantly activated in tumor cells to promote the cellular advantage in survival and proliferation.[3]

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Nuclear Processes Due to Oxidative DNA Damage

The genome of eukaryotic cells is under continuous attack from a variety of endogenous and exogenous DNA-damaging agents, which lead to many types of DNA lesions. ROS are not known to directly cause DNA double-strand breaks (DSBs). Instead, DSBs could be generated if two single-strand breaks (SSBs) oppose each other on complementary strands during an intermediate step in a repair process.[4] The major mechanism that cells use to repair oxidative damage lesions is base excision repair (BER). In other words, many, but not all, of the DNA lesions repaired by BER, are products of ROS attack. DNA polymerase-β (Pol-β) is a key enzyme implicated in the BER pathway, where it mainly repairs SSBs.[5,6]

Despite its error-prone nature, non-homologous end-joining (NHEJ) is the predominant form of DSB repair in human somatic cells.[7] Ku70 and Ku80, important components of NHEJ, likely influence BER, independent of the Ku heterodimer. Ku80 actively repairs the base lesion, most probably through facilitating the accumulation of other BER components. Remarkably, if Ku80 has no impact, Ku70 interferes with the repair of these lesions.[4] On the other hand, Pol-β of the BER pathway physically interacts with Ku70 of the NHEJ pathway, and this association is enhanced by DNA damage. When SSB occurs, Ku70 binds to Pol-β and promotes its polymerase activity, thus accelerating BER. Reciprocally, when DSB occur, Pol-β binds to Ku70 and participates in NHEJ to promote repair of the DSB lesions. Thus, two-way crosstalk between Ku70 and Pol-β modulates DNA repair through BER and NHEJ.[7]

The ataxia-telangiectasia-mutated (ATM) is activated by oxidative stress. Interestingly, oxidation of ATM directly induces ATM activation in the absence of DNA break and the Mre11-Rad50-Nbs1 (MRN) complex.[8] ATM phosphorylates histone H2AX at serine 139 (Ser139) into γ-H2AX around the DSB site. The activated ATM also phosphorylates the scaffolding subunit of PP2A, PR65, at Ser401, leading to the disassociation of the ATM-PR65 complex.[9] Furthermore, Ser401 phosphorylation of PR65 could result in the dissociation of the holoenzyme and causes its translocation to the cytoplasm, leaving the catalytic domain of PP2A, PP2A(C), in the nucleus[8,9] (Figure 1). Catalytically inactive PP2A may associate with CYLD in the cytoplasm,[10] which may indirectly regulate the phosphorylation of Akt. Oxidative stress-induced activation of ATM[11] and DNA-PK[12] results in direct Akt phosphorylation. Altogether, DNA damage-induced signaling may promote AKT activity and prosurvival signaling by increasing phosphorylation and reducing dephosphorylation. On the other hand, PP2A(C), retained in the nucleus, directly interacts with γ-H2AX and dephosphorylates it and potentially other factors at the DNA break, causing an inefficient repair.[13] Thus, premature dephosphorylation of γH2AX could facilitate DNA replication and increase mutations and genomic instability.[14]

ATM also contributes to the oxidative BER pathway by activating DNA glycosylases that recognize base damage.[15] Importantly, ATM was shown to activate the Ku70/80 heterodimer in regions of oxidatively damaged DNA. Thus, ATM also plays a role in the regulation of NHEJ in response to oxidative stress.[16] Classical NHEJ is initiated by DSB recruitment of the Ku70/80 heterodimer, which constrains the DSB and engages with the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) to form the DNA-PK complex.[2] Ku70/Ku80 constitutively interacts with checkpoint kinase 2 (CHK2), suggesting that CHK2 may be recruited to DNA-PK through an interaction with the Ku heterodimer in response to DNA damage.[17] Another important component of the DNA-PK complex is the Monopolar spindle 1 (MPS1, also known as TTK), an important mitotic checkpoint kinase, which regulates the expression of DNA-PK.[18] Furthermore, CHK2 interacts with the MPS1 in response to DNA damage; subsequently, MPS1 can directly phosphorylate CHK2 at threonine 68 (Thr68).[19] Interestingly, CHK2 can also phosphorylate MPS1 at Thr288 and can regulate the stability of the kinase, thus forming a positive feedback loop.[20] The activated MPS1 as a part of BER machinery can colocalize with MDM2 and phosphorylate it, which in turn promotes histone H2B ubiquitination and chromatin relaxation in response to oxidative stress.[21] Altogether, several key checkpoint proteins, including DNA-PK, CHK2, and Mps1, which are important for mitotic activity, form a phosphorylation-specific large nuclear complex in response to oxidative DNA damage.[22] I would like to suggest calling this complex as DNAPKsome, which arises in response to oxidative DNA damage.

Cytoplasmic c-Abl-Induced Significant Alterations Prior to NF-κB Activation

Remarkably, upon exposure to oxidative DNA damage, activated MPS1, as a component of DNAPKsome, phosphorylates c-Abl, a non-receptor tyrosine kinase, at Thr735 and promotes its cytoplasmic translocation[23] (Figure 1). Cytoplasmic c-Abl is considered an inducer of cellular transformation by promoting cell proliferation and inhibiting cell death.[24,25] c-Abl interacts

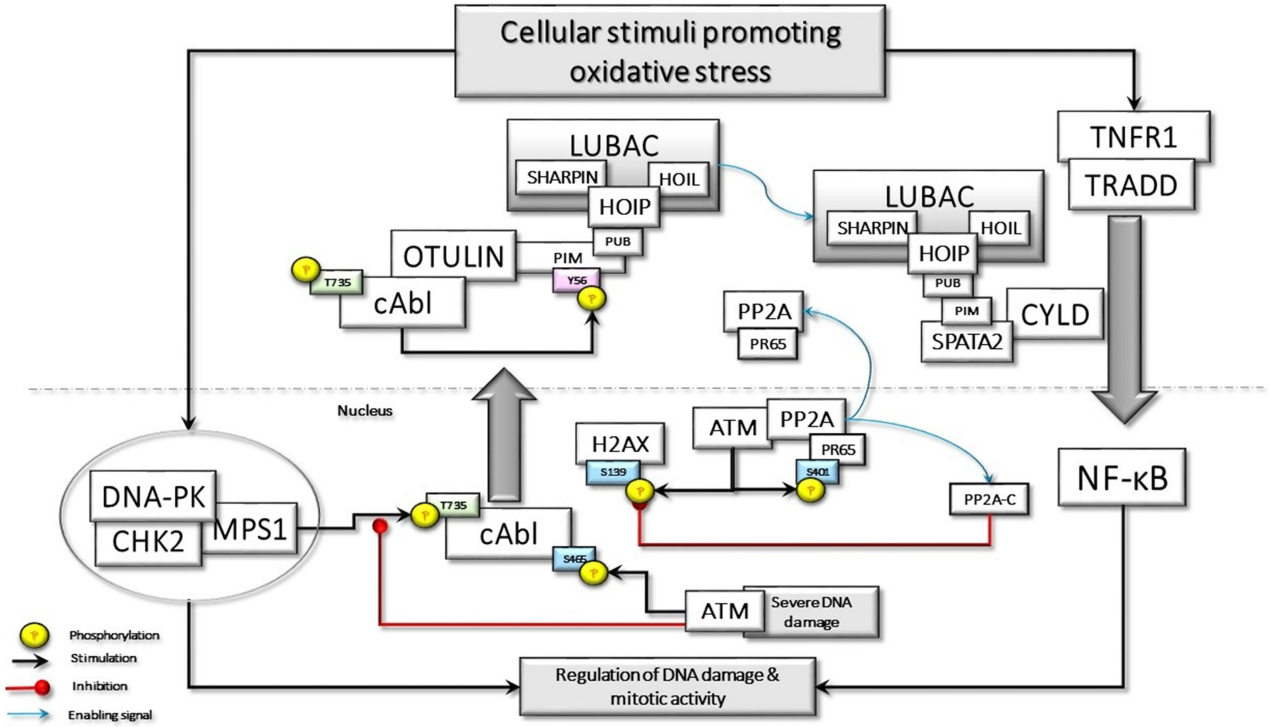

Figure 1 Oxidative stress-induced DNA damage may stimulate the assembly of DNAPKsome formation and activation of Mps1, which phosphorylates c-Abl at Threonine 735 (T735), promoting its cytoplasmic translocation. c-Abl phosphorylates the Tyrosine 56 (Y56) in the PIM domain of OTULIN, disrupting its association with LUBAC. The released LUBAC interacts with SPATA2 and is involved in the TNF-R1-mediated signaling pathway, which could also be activated by oxidative stress. Oxidative stress may also directly activate ATM, which phosphorylate the scaffolding subunit of PP2A, PR65, at Serine 401 (S401). This phosphorylation disassembles the holoenzyme, causing the translocation of phosphorylated PR65 to the cytoplasm and retention of the catalytic subunit, PP2A(C), in the nucleus. P2A(C) interacts with and dephosphorylates γ-H2AX. In case of severe DNA damage, nuclear ATM activation may lead to the phosphorylation of c-Abl at S465, which may interfere with DNAPKsome formation and facilitate apoptotic processes.

with Mucin 1 (MUC1) and phosphorylates it at Tyrosine 60 (Tyr60), which blocks c-Abl signaling to the nucleus and the apoptotic response. MUC1 is an oncoprotein, which is overexpressed by most human carcinomas and blocks the induction of apoptosis by genotoxic agents.[26] Transcription of the ARF (p19) locus is repressed by binding of the Cdc6 replication-licensing factor. c-Abl activates the ubiquitin ligase, CUL-4, which promotes the nuclear export of Cdc6 and relieves repression of ARF transcription. The ARF tumor suppressor stabilizes and activates p53 by directly inhibiting MDM2. However, cytoplasmic c-Abl-MUC1 interaction abrogates the c-Abl-driven CUL-4 activation, thus suppressing the ARF-mediated activation of the p53 signaling pathway.[27]

c-Abl when expressed in the nucleus inhibits NF-κB transcriptional activity, whereas cytosolic c-Abl is unable to share the same function.[28] ATM kinase interacts and activates c-Abl in response to DNA damage in the nucleus.[29] c-Abl is phosphorylated by ATM at serine-465 in response to severe DNA damage, resulting in c-Abl activation.[30] ATM-activated c-Abl, in turn, phosphorylates DNA-PKcs, which inhibits the ability of DNA-PK to form a complex with DNA.[31] In addition, nuclear c-Abl stabilizes the histone deacetylase, HDAC1,[32] which is a negative regulator of inducible NF-κB activity.[33] Taken together, nuclear c-Abl harms DNAPKsome formation and NF-κB transcription.

Cytoplasmic translocation of c-Abl may interfere with the ATM-mediated DNA damage response (DDR). Following DNA damage, H3K9me3 histone tagged nucleosomes are recognized by the TIP60/KAT5 acetyltransferase.[34] TIP60 is activated by c-Abl-mediated phosphorylation. In turn, activated Tip60 acetylates and facilitates the binding of ATM to H3K9me3 on chromatin, which may promote ATM-mediated phosphorylation of downstream effectors.[35] However, c-Abl is not an obligatory upstream activator for ATM functions; instead, modifications between c-Abl, Tip60, and ATM may be important for the activation of their pro-apoptotic function.[36] Taken together, DNAPKsome-mediated cytoplasmic transfer of c-Abl would be a barrier against the apoptotic effect of ATM (Figure 1).

Another consequence of the DNAPKsome-mediated cytoplasmic accumulation of c-Abl as a result of genotoxic stress is OTULIN phosphorylation at Tyr56, which may have critical outcomes[37] (Figure 1). OTULIN is a deubiquitinating (DUB) enzyme with high activity and unique specificity for methionine 1 (Met1)-linked polyubiquitin chain, which is assembled by the linear ubiquitin chain assembly complex (LUBAC). LUBAC is a multi-subunit E3 ligase consisting of HOIP, HOIL-1, and SHARPIN. LUBAC function is required for the full activation of the inhibitor κB (IκB) kinase (IKK) complex and hence the productive inflammatory signaling pathway.[38] The N-terminus of OTULIN contains the PUB-interacting motif (PIM) that mediates the interaction with the PUB domain of the catalytic HOIP subunit and includes the Tyr56 phosphorylation site.[39]

OTULIN is implicated in angiogenesis and Wnt signaling. LUBAC-induced Met1-ubiquitination suppresses canonical Wnt signaling.[40] c-Abl-driven Tyr56 phosphorylation within the PIM domain of OTULIN terminates its interaction with the catalytic HOIP component of LUBAC upon genotoxic stress. Subsequently, OTULIN interacts with β-catenin, inhibiting its linear ubiquitination, thereby robust activation of Wnt/β-catenin signaling.[37] Linear ubiquitination has also been demonstrated to play an important role in the maintenance of proteasome function. OTULIN deficiency leads to excessive linear ubiquitination on proteasome subunits, which disrupts proteasome assembly and function.[41] Therefore, Ty56 phosphorylation of OTULIN may increase its DUB activity on the proteasome, potentiating the ubiquitin-proteasome system (UPS) activity that may have profound effects on oncogenesis.[42]

Cylindromatosis protein (CYLD), another DUB, is recruited to LUBAC via the adaptor SPATA2, which binds to both CYLD and HOIP via PUB-PIM interactions. Since SPATA2 and OTULIN bind to the same PUB domain of HOIP, their binding is mutually exclusive.[43–45] Therefore, the OTULIN-HOIP relationship, which is disrupted as a result of genotoxicity, may increase the CYLD-SPATA2-HOIP association. In other words, DNAPKsome-induced cytoplasmic c-Abl activity may promote the interaction between CYLD and LUBAC. CYLD-LUBAC association is crucial for NF-kB activation and cell-fate determination by interacting with regulatory proteins[46] (Figure 1).

The CYLD gene encodes a 956 amino acid protein with several functional domains: three N-terminal cytoskeleton-associated protein glycine-rich (CAP-Gly) domains and a C-terminal ubiquitin-specific catalytic domain [or ubiquitin-specific protease (USP) domain].[47] The first and second CAP-Gly domains interact with microtubules.[48] Third CAP-Gly (CAP-Gly3) interacts with several important effectors, including NF-κB essential modulator (NEMO),[49] mind bomb homolog 2 (MIB2), an E3 ligase,[50] and Aurora-B, a mitotic kinase.[10] Remarkably, in a recent study, Elliott et al reported that CAP-Gly2 and CAP-Gly3, in addition to microtubule-binding activities, are novel ubiquitin-binding domains (UBDs) of CYLD. Thus, they may increase CYLD activity through the linkage preference to lysine 63 (K63)-linked ubiquitin chains.[45]

CYLD is regulated by post-translational mechanisms. IKKβ and IKKε, one of the non-canonical IKKs, have been shown to phosphorylate CYLD at a cluster of serine residues located in the region between the second and third CAP-Gly domains, around Ser418.[45,51,52] Furthermore, a novel and atypical phosphorylation site, Ser568 in the linker between the CAP-Gly3 and the USP domain of CYLD, is demonstrated. Intriguingly, IKKε-mediated Ser418 phosphorylation alone can potentiate LUBAC activity by reducing its autoubiquitination through the stimulation of CYLD. In contrast, UBD activity of CAP-Gly3 and phosphorylation of both Ser418 and Ser568 are indispensable for full CYLD DUB activity on K63-linked ubiquitin chains.[45] Altogether, while UBD and Ser418 phosphorylation-driven CYLD activity facilitate the recruitment of LUBAC and the deposition of Met1-ubiquitin, Ser568 phosphorylation of CYLD stimulates its DUB activity and preferentially hydrolyzes K63-ubiquitin chains at signaling complexes.

Until recently, OTULIN has been considered a negative regulator of NF-κB signaling in response to tumor necrosis factor receptor 1 (TNF-PP6R1) activation.[38] However, the notion that OTULIN functions primarily by preventing LUBAC-mediated activation of proinflammatory NF-κB or MAPK signaling has recently been challenged.[53] OTULIN supports rather than counteracts LUBAC function by preventing its autoubiquitination. Thus, OTULIN stabilizes TNF-R1-associated complex I, which is required for receptor-interacting protein kinase 1 (RIPK1) scaffold function and NF-kB activation.[53,54]

Since two different DUBs, OTULIN and CYLD, can activate the LUBAC function after TNFR stimulation and only one of them can bind to LUBAC, the question is, what determines the choice of one of them. TNF is a physiologically important pro-inflammatory cytokine for the function of the innate immune system. Through the stimulation of pattern recognition receptors, it increases the expression of genes required to control tissue inflammation and injury. However, it is also involved in the

pathophysiology of many inflammatory disorders. Therefore, changes in TNF concentration, tissue and cell type, TNF receptor distribution, and duration of TNF stimulation will determine whether the reaction will be physiological or pathological as a result of a complex interaction.[55]

The increased TNF/TNF-R1 signaling was shown to be sufficient to induce genotoxicity. Elevation of intracellular reactive oxygen and nitrogen species and redox imbalance may be responsible for TNF-α/TNF-R1 signalling-induced DNA breaks.[56] Interestingly, TNFR signaling can be activated independently of TNF-α in the presence of oxidative stress by self-dimerization of the receptor. Moreover, TNF-α can induce stronger downstream signaling to NF-κB in the presence of ROS.[57] Taken together, it seems reasonable to assume that OTULIN functions in the TNFR signaling complex in response to physiological TNFα stimulation that does not accompany by oxidative base damage. CYLD has been shown to be activated following DNA damage to suppress tumorigenesis.[58] Considering that, following genotoxicity, CYLD is activated and cytoplasmic transfer of c-Abl enables CYLD-LUBAC interaction, CYLD will take over the OTULIN function in the case of pathological genotoxicity (Figure 1).

Cytoplasmic NF-κB Activation Signaling Pathway Following DNA Damage

Multiple early reports have been published suggesting that DNA damage can activate NF-κB. Genotoxic stress-induced NF-κB activation shares some features with the mode of NF-κB stimulation by TNF-R1.[59] Although TNF-R1-mediated NF-κB stimulation is relatively well defined, the mechanisms to describe the NF-κB signaling pathway in response to DNA damage are complicated and remain to be elucidated. Previous studies suggested that NF-κB activation by genotoxic stress is also mediated by cell surface receptors, such as TNF-R1 and IL-1 receptors.[60] Nevertheless, the activation of NF-κB, after intertwined molecular interactions for the repair and survival of cells exposed to genotoxic stimuli, induces a large number of inflammatory genes, including those encoding TNF-α.[60,61] Increased TNF expression by the ATM-centered NF-κB activation in response to DNA damage triggers a second wave of NF-κB activation mediated by autocrine TNF-TNFR1 signaling. This second wave may determine cell fate according to the extent of DNA damage.[61]

Soluble TNF-activated TNF-R1 recruits the TNFR1-associated death domain (TRADD) protein, which in turn recruits TNF Receptor Associated Factor 2 (TRAF2) and RIPK1 to form the membrane-bound pro-survival complex I.[62] TRAF2 is an E3 Ub ligase required for itself and K63-linked ubiquitination of RIPK1. In unstimulated cells, cytosolic TRAF2-interacts with TRAF-interacting protein (TRIP/TRAIP), which is a negative regulator of TRAF2-mediated NF-κB activation.[63] Following TNF stimulation, TRADD-associated TRAF2 binds to sphingosine kinase 1 (SpK1) that generates the pro-survival lipid mediator sphingosine-1-phosphate (S1P). S1P specifically binds to the N-terminal RING domain and stimulates TRAF2 E3 ligase activity by removing TRIP[64] (Figure 2).

TNF-α-induced activation of c-Jun N-terminal kinase (JNK), but not that of IKK, depends on the integrity of the TRAF2 E3 ligase activity. K63-linked ubiquitin chains on autoubiquitinated TRAF2 have been reported to interact with components of the JNK signaling pathway, including the MAPK kinase kinases, such as MEKK1.[65] TRAF2-MEKK1-MEKK7 signaling and activation of JNK[66] is critical for TNF regulation of the AP-1 group of transcription factors.[67] K63-linked ubiquitin chains at TRAF2 also create a platform for TRAF family member-associated NF-κB activator (TANK).[68] Thus, TRAF2 forms a complex with TANK, and two noncanonical IKKs, TBK1 and IKKε[68] (Figure 2).

The BIR1 domain of cellular inhibitor of apoptosis proteins (cIAPs) is required for TNF signaling and readily associated with the cIAP-interacting motif (CIM) of TRAF2 immediately after TNF-R1 signaling.[69] However, the UBA domain of c-IAPs binds only ubiquitinated TRAF2. cIAP1/TRAF2 E3 ligase complex is responsible for IKKε ubiquitination. K63-linkage-specific ubiquitination of IKKε at K30 and K401, which is required for its kinase activity, is essential for IKKε-mediated cell transformation.[70] Activated IKKε phosphorylates TRAF2 on Ser11, leading to the stabilization of TRAF2, which is required for the recruitment of the UBA domain of cIAP. UBA-mediated TRAF2 binding facilitates cIAP-mediated K63-linked ubiquitination of RIPK1 at K377.[71] Thus, TRAF2-cIAP interaction represses RIPK1 kinase activity, which is believed to promote malignant transformation by the RIPK1-mediated NF-κB activation[72] (Figure 2).

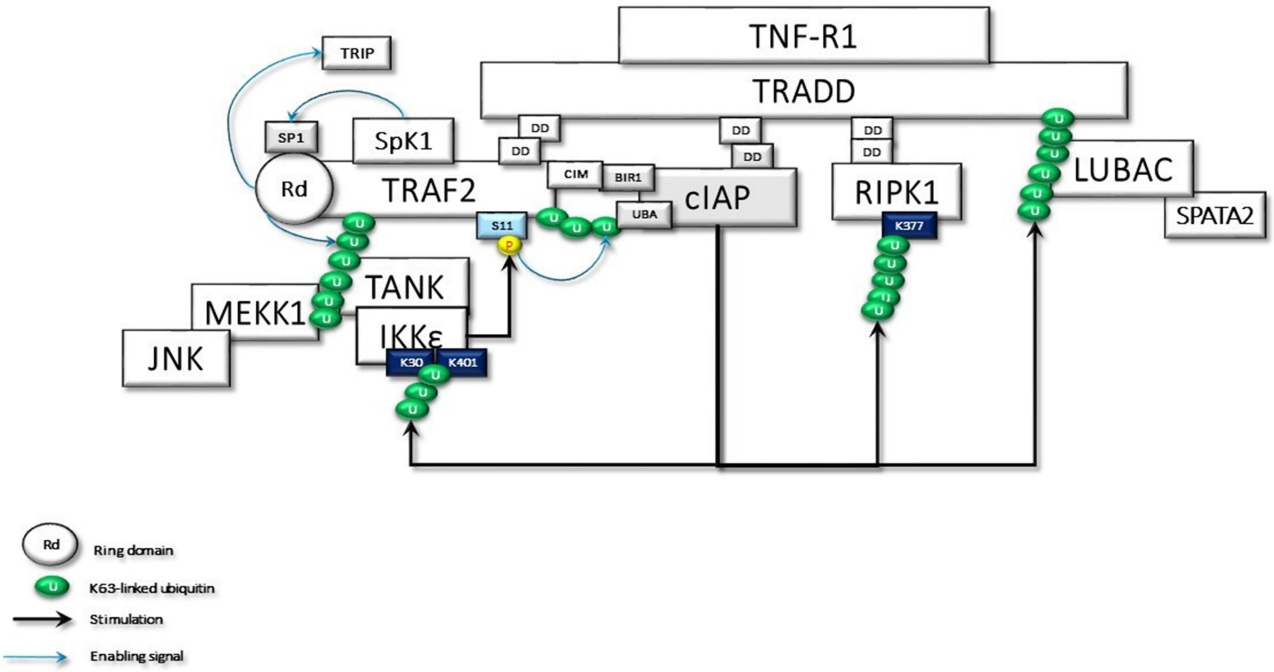

Figure 2 TRADD in the TNFR1sc recruits the TRAF2, c-IAPs, and RIPK1 through dead domain (DD) interactions. Membrane-associated TRAF2 comes close to sphingosine kinase (SpK1) and binds with it. SpK1 may activate the E3 ligase function of TRAF2 through its product sphingosine-1-phosphate (SP1). Subsequently, K63-linked autoubiquitination of TRAF2 promotes the recruitment of downstream effector kinases, such as MEKK1 and TANK. TRAF2-TANK interaction stimulates the activation of IKKϵ and TBK1. IKKϵ phosphorylates TRAF2 at Ser11, which may promote the interaction of ubiquitinated TRAF2 with the UBA domain of c-IAPs. Then, the stabilized TRAF2-c-IAP interaction drives the K63-linked ubiquitination of IKKϵ, RIPK1, and TRADD for the recruitment of LUBAC.

Under resting conditions, CYLD interacts with TRAF2.[51] Following stimulation, LUBAC is recruited to the TNF-R-signaling complex (TNF-Rsc) through TRADD, TRAF2, and c-IAP interaction and c-IAP-generated ubiquitin chains[73] (Figure 2). cIAP-driven ubiquitination and activation of IKKϵ promote its homo-dimerization.[70,74] The dimerized ubiquitinated IKKϵ most likely interacts with the second CAP-Gly domain (amino acid 232–303) of CYLD monomers, one of the UBDs, upstream of the TRAF2 binding site (amino acid 453–457).[75] Subsequently, IKKε can phosphorylate a cluster of serines between residues 418 and 444 of CYLD[52] (Figure 3).

SPATA2 is a LUBAC-associated component of TNF-Rsc and has an important function in regulating the outcome of TNFR signaling through recruitment of CYLD to TNF-Rsc. The B-box-mediated dimerization of CYLD is essential for SPATA2 interaction. Thus, CYLD and SPATA2 form a highly stable heterotetramer.[43] Phosphorylation-mediated allosteric changes of a protein can reason to dimerization, which facilitates the binding of many response regulators to their partners.[76] Therefore, homo-dimerized IKKϵ-mediated Ser418 phosphorylation could promote dimerization of CYLD and thus facilitate the interaction of CYLD with SPATA2. This association could be considered an important step that can trigger the linear ubiquitination required for NF-κB activation. Since the autoubiquitination of LUBAC inhibits its activity in TNF-Rsc, a signal is required for LUBAC to be active. CYLD-SPATA2 association, possibly by inhibiting LUBAC autoubiquitination, operates as a triggering mechanism for LUBAC activity, thus promoting linear ubiquitination.[45] CYLD dimerization not only increases LUBAC activity in TNF-Rsc but also creates a bonding opportunity for the increasing amount of proteins (Figure 3).

MIB2, RING-type Ub-E3 ligase, interacts with RIPK1 through its MZM region only when RIPK1 is recruited to TNF-R1. Therefore, MIB2 may sense the activity status of RIPK1. The MIB2-RIPK1 interaction will have crucial implications for NF-κB activation and cell fate determination. First, MIB2 ubiquitinates RIPK1 at K377 and K634, contributing to the suppression of the RIPK1 kinase activity-dependent cytotoxic function.[77] Second, MIB2 binds and polyubiquitinates the caspase-like domain of the long-form of cellular FLICE-inhibitory protein ($cFLIP_L$), a catalytically inactive homolog of caspase 8. Although caspase 8 and ubiquitinated $cFLIP_L$ are recruited to the RIPK1/FADD complex, ubiquitinated cFLIPL suppresses TNF-induced apoptosis by inhibiting the formation of a proper oligomer with caspase-8.[78] Third, MIB2 is an E3 ligase, known to ubiquitylate itself. CYLD may interact with the ubiquitinated MIB2, which

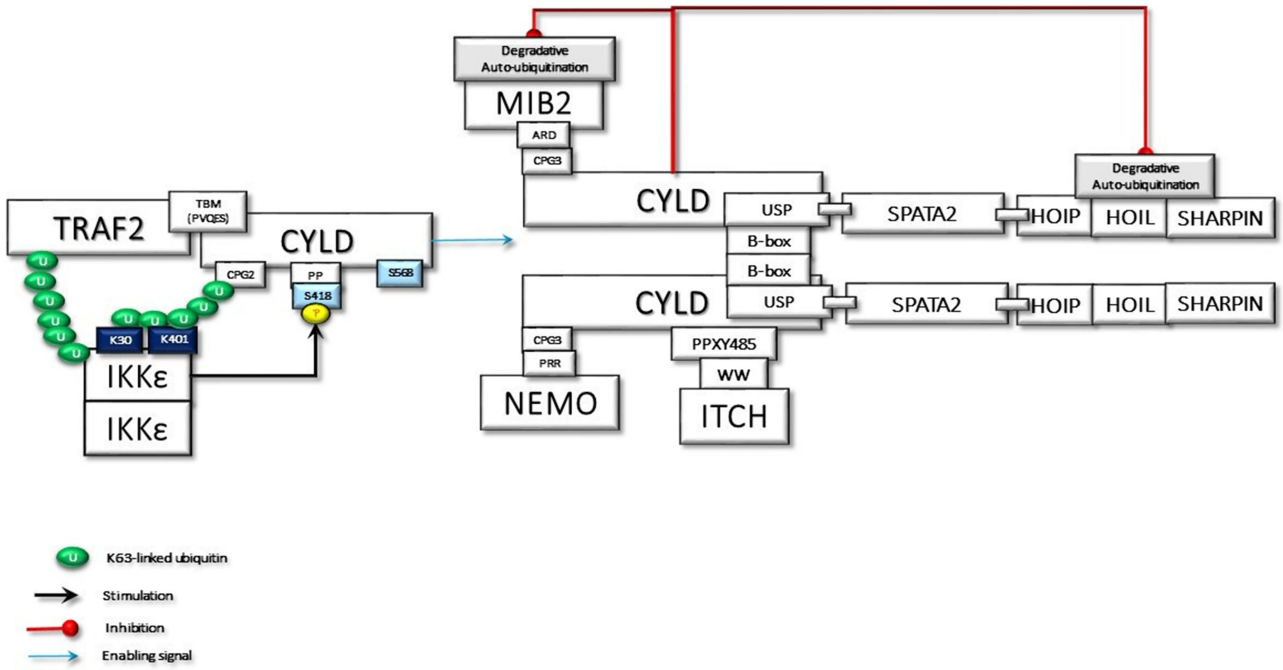

Figure 3 The activated and ubiquitinated IKKϵ may interact with the second CAP-Gly UBD domain of TRAF2-associated CYLD. This interaction enables IKKϵ to phosphorylate Ser418, which may induce the dimerization of CYLD. Then, the dimerized CYLD is associated with the SPATA2-LUBAC complex. Thus, IKKϵ phosphorylation promotes the LUBAC-associated transportation of CYLD to TNF-Rsc. Furthermore, the dimerization of CYLD also may induce the interaction with MIB2, NEMO, and ITCH. In addition to augmenting protein interactions through scaffolding activity, CYLD protects the MIB2 and LUBAC degradation caused by autoubiquitination.

may facilitate the binding of the third CAP-Gly domain of CYLD with the ankyrin repeat domain (ARD) of MIB2. Deubiquitinases and E3 ligases frequently interact. MIB2 is not a deubiquitination target of CYLD but rather is stabilized by CYLD in a non-catalytic-dependent manner (Figure 3). Furthermore, while the expression of CYLD alone inhibits LUBAC-stimulated NF-κB activation, CYLD association with MIB2 rescues the NF-κB activity[50,79] (Figure 4). Fourth, K63-linked ubiquitin chains on MIB2 recruit ubiquitin-binding TAB proteins along with transforming growth factor-β-activated kinase 1 (TAK1) and NEMO (IKKγ) to support NF-κB activating signal transduction. MIB2-TAK1 interaction results in increased kinase activity of TAK1 following its phosphorylation.[80] Several lysine residues of TAK1 are potential sites for polyubiquitination. MIB2 may directly stimulate K63-linked polyubiquitination of TAK1 at K562, which has been reported to be required for the autophosphorylation of TAK1 at Thr187[81] (Figure 4).

The dimerized CYLD directly interacts with NEMO with its third CAP-Gly domain,[49,82] which may facilitate the association of MIB2 with NEMO. In addition, the PPXY motif of CYLD interacts with the WW domain of another important E3 ligase, ITCH,[83] which is also associated with TAK1[84] and c-FLIP$_L$[85] (Figures 3 and 4).

MIB2-dependent recruitment of CYLD to RIPK1 may also facilitate SPATA2-mediated transport of the LUBAC as well.[43,86] Subsequently, LUBAC conjugates M1-linked ubiquitin chains on cFLIP$_L$, further stabilizing it to protect cells from TNFα-induced apoptosis.[87] Moreover, CYLD-MIB2-RIPK1 interaction may facilitate HOIL-mediated direct interaction of RIPK1 with LUBAC.[88] Consequently, LUBAC-induced linear ubiquitination of RIPK1 on K612 and caspase-8 may restrict necroptosis by limiting the formation of RIPK1/RIPK3/MLKL containing complex[89,90] (Figure 4).

The ubiquitination of RIPK1 at K377 is indispensable for the activation of NF-κB. K63-linked poly-ubiquitinated RIPK1 interacts with the polyubiquitin binding adaptors TAB2/TAB3, which promotes the recruitment and activation of TAK1 kinase.[91] TAK1-driven p38/MK2 kinase cascade directly phosphorylates RIPK1 at Ser321, thus blocking the RIPK1 kinase-dependent apoptosis and necroptosis.[92] In addition, the K63-linked ubiquitin chains facilitate the recruitment of LUBAC, enabling the deposition of Met1-Ub on the existing K63-ubiquitin chains.[93] In turn, the K63-Ub and Met1-Ub facilitate the recruitment of NEMO-canonical IKK kinase subunits of the IKKα and IKKβ complex. TAK1-catalyzes the phosphorylation of IKKβ at Ser177, which is a priming event that enables IKKβ to activate itself by

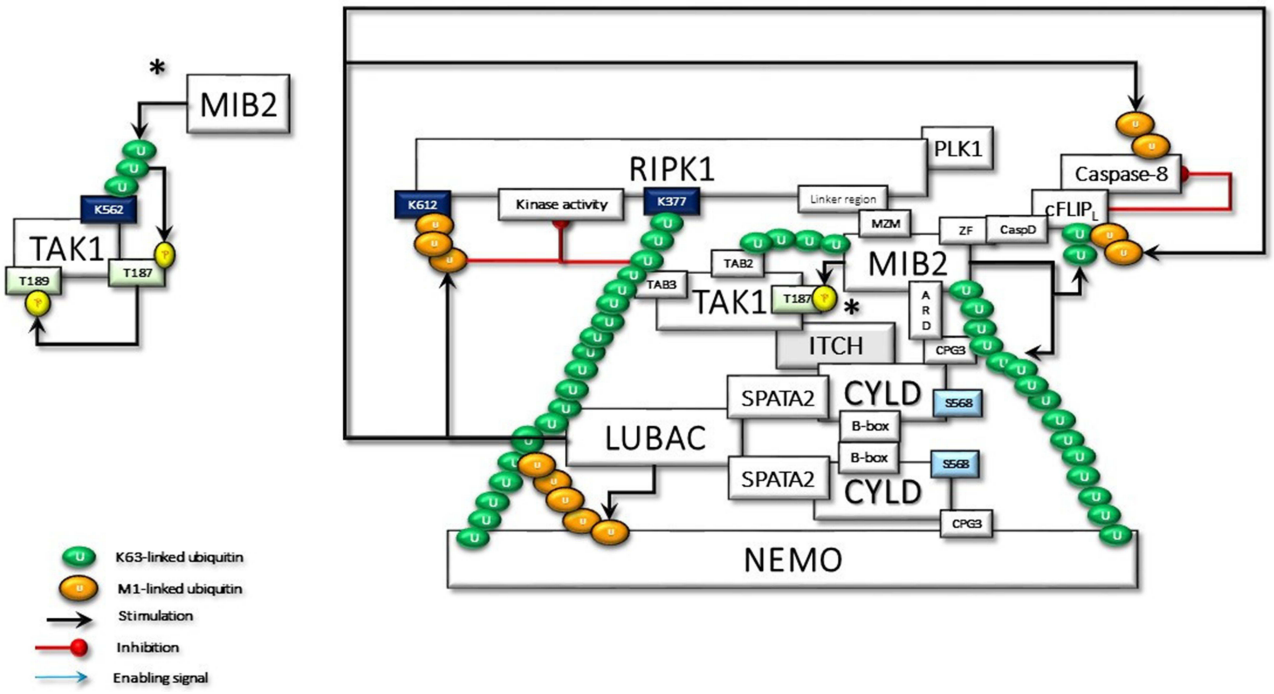

Figure 4 The CYLD-SPATA2-LUBAC association enhances the linear ubiquitination function of LUBAC. Activated LUBAC, associated with K63 linked ubiquitin chains on RIPK1, adds Met1-linked ubiquitin chains on K63-linked ubiquitin chains. K63/Met1-hybrid ubiquitin chains on RIPK1 recruit NEMO for the activation of canonical IKKs. RIPK1 in the TNF-R1sc interacts with the MZM motif of MIB2 through its linker region. Thus, activated MIB2 autoubiquitinates itself with K63-linked chains, which facilitates its interaction with the third CAP-Gly domain of CYLD by its ankyrin repeat domain (ARD). MIB2 also interacts by its zinc finger (ZF) motif to the caspase domain (CasD) of c-FLIP$_L$ and then decorates it with K63-linked ubiquitin chains. Furthermore, CYLD-associated LUBAC conjugates M1-linked ubiquitination chains on c-FLIP$_L$. Thus, the hybrid K63/Met1 ubiquitin chains stabilize c-FLIP$_L$ to inhibit the caspase-8 activity. In addition to the scaffolding activities, K63-linked and Met1-linked poly-ubiquitin chains on RIPK1 block its kinase activity. K63-linked ubiquitin chains on MIB2 interact with TAB proteins for the recruitment and the activating phosphorylation of TAK1, which is also associated with CYLD-bound ITCH. Following TAB-driven interaction with TAK1, MIB2 may ubiquitinate the K562 residue, which may promote autophosphorylation of TAK1 at T187 and subsequent phosphorylation at T189 (significance was denoted by an asterisk (*) and it has been moved to a free area and detailed in order not to confuse the main shape further in the figure). Besides K63-linked poly-ubiquitin chains on RIPK1, K63-linked chains on MIB2 may also provide support for the activation of CYLD-associated NEMO by providing an additional scaffold. PLK1 interaction with CYLD and RIPK1 may alter its mitotic activity as detailed in the text.

phosphorylating Ser181.[94] Activated IKKβ phosphorylates the NF-κB inhibitor IkBα, which results in proteasomal degradation of IκBα and subsequent nuclear translocation of NF-κB and activation of NF-κB target genes.[77] NFκB regulates more than 500 genes involved in inflammation, cellular transformation, survival, proliferation, angiogenesis, invasion, and metastasis (Figure 5). In cases where NF-κB activity cannot be controlled, increased genetic expressions of some proteins, including, TNFα, TRAF2, c-IAPs, Bcl2, CYLD, and c-FLIP$_L$, could stimulate NF-κB activity, thus promoting a positive feedback loop.[95–97]

Termination of signaling is a crucially important factor to prevent the inappropriate activity of NF-κB. In addition to its essential role in stimulating NF-κB activity, even termination of this activity has been shown to require the function of IKKβ. IKKβ–mediated phosphorylation of CYLD at Ser568,[45] which resides in the third CAP-Gly domain of CYLD, increases its DUB activity, promoting deconjugation of K63-linked ubiquitin chains on NEMO, TRAF2, and MIB2 (Figure 6). Moreover, in response to oxidative stress, IKKβ was found to translocate into the nucleus and directly phosphorylate ATM, promoting DNA repair. This mechanism would be beneficial for the maintenance of homeostasis by repairing DNA damage that occurs in normal cells. However, the same mechanism may also enable cancer cells to resist chemotherapy.[98]

IKKβ-stimulated activation of CYLD may enable the CYLD-ITCH complex to promote proteasomal degradation. CYLD-ITCH complex may sequentially cleave K63-linked ubiquitin chains and catalyze the degradative K48-linked polyubiquitin chain on TAK1[83] and c-FLIP[85] to terminate TAK1 and c-FLIP-mediated pro-survival signals. Similarly, following CYLD-mediated cleavage of K63 ubiquitin chains, MIB2 catalyzes K48 ubiquitin chains on CYLD for degradation, thus completely terminating the CYLD activity.[50] Altogether, while CYLD initially functions as a bridging and adaptor protein for signal progression independent of its DUB activity, it eventually terminates signaling by supporting the degradation of key molecules, including itself through its DUB activity (Figure 6).

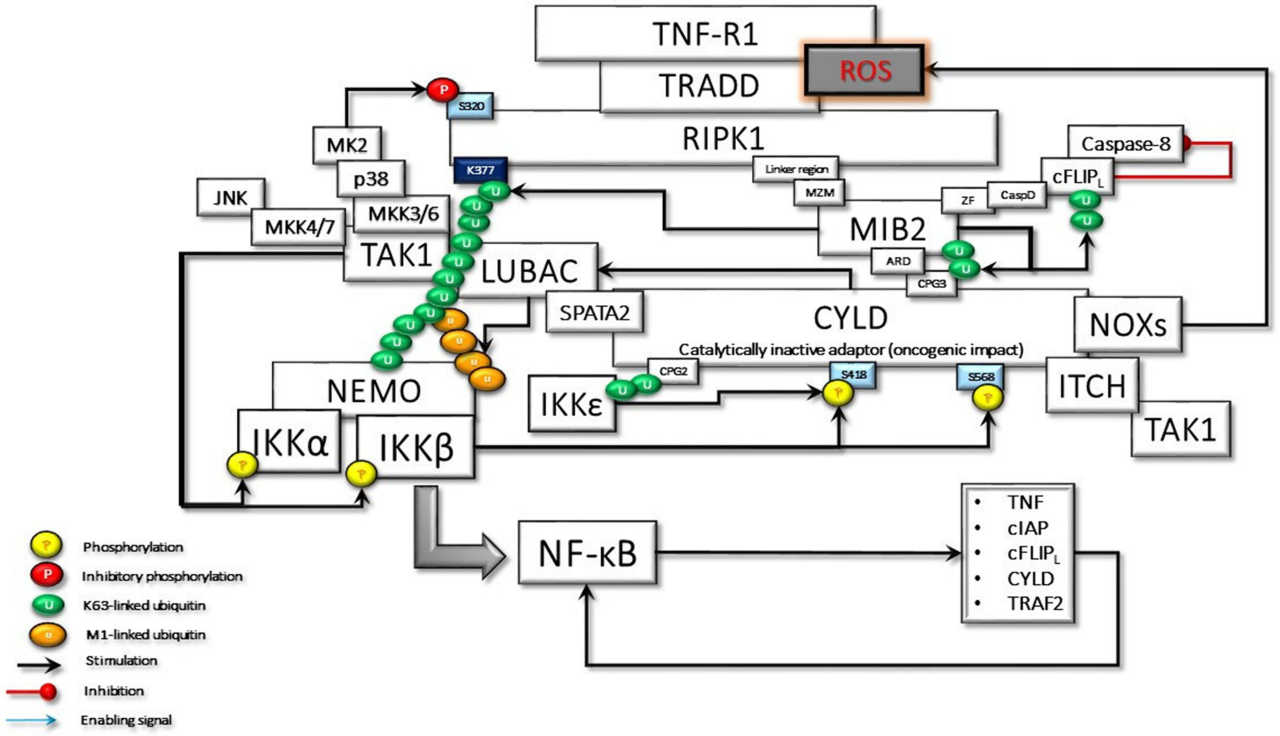

Figure 5 The activated TAK1 phosphorylates IKKα and IKKβ complexed with NEMO, which is required for the NF-κB transcriptional activity. In the absence of regulation to terminate the signaling pathway, NFK stimulates the expression of many genes, some of which promote persistent activity. IKKβ also phosphorylates Ser568 of CYLD to stimulate its DUB activity. The activated CYLD deconjugates K63-linked ubiquitin chains that trigger a cascade of reactions to terminate signal transduction.

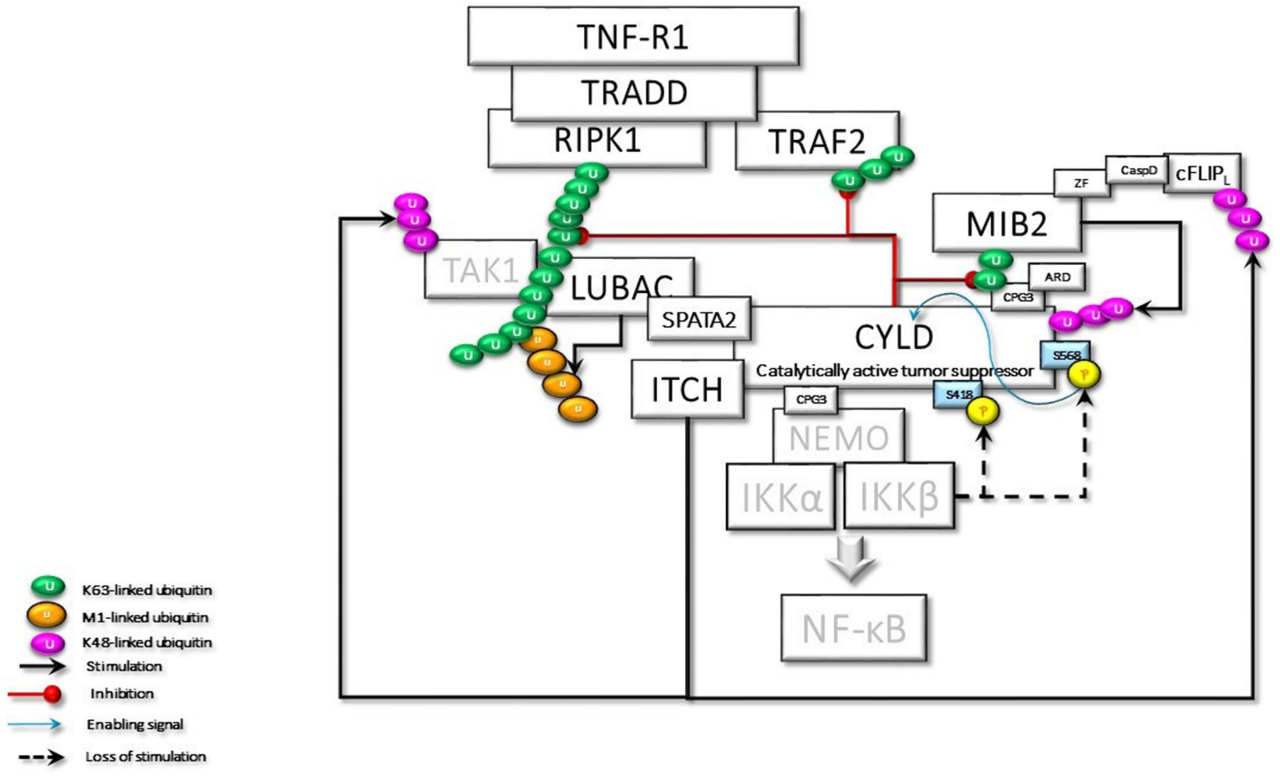

Figure 6 The activated CYLD behaves as a tumor suppressor and deubiquitinates the K63-linked ubiquitin chains on TRAF2, RIPK1, and MIB2. Loss of K63-linked chains reason for the stimulation of the K48-linked degradative ubiquitination of TAK1 and c-FLIP$_L$ by ITCH, and CYLD by MIB2, thus terminating CYLD-mediated signaling events.

Novel CYLD-Centered Model of Malignant Transformation

In addition to its role in innate immunity, IKKε, an upstream regulator of the transcription factor NF-κB, is also accepted as an oncogene that is amplified and overexpressed in up to 30% of breast cancers.[99] According to a highly regarded study from Cantley Lab,[52] TRAF2-interacted and c-IAP-ubiquitinated IKKϵ[70,71] phosphorylates CYLD at Ser418, contributing to cell transformation. In fact, besides IKKϵ, canonical IKKs can also cause CYLD Ser418 phosphorylation.[51] Consistent with these early reports, all subsequent studies, particularly, Elliot et al reaffirmed that Ser418 phosphorylation inhibits CYLD DUB activity.[44] They also demonstrated that phosphorylation of a hitherto unknown atypical serine residue, Ser568, increases CYLD's DUB activity toward K63-linked ubiquitin chains. It is therefore questionable why the Ser568 residue might have been overlooked in previous studies. The explanation is most likely that while results from previous studies were obtained with transformed cell lines, the recent study used CYLD KO U2OS/NOD2 cells for reconstitution of wild-type CYLD expression. Taken together, there might be a possibility that malignant cells were unable to express phosphorylatable Ser568.

Serine is a high probability target of mutations; therefore, it mutates very often and evolves very quickly. Encoded by two separate codon sets, AGY and TCN, serine is unique among the 20 amino acids; therefore, it will be more easily reached from another amino acid after mutation.[100] Phosphoserines tend to occur in intrinsically disordered regions (IDRs), which play essential roles in a wide range of biological processes and can function as linear motifs, linkers, or entropic chains.[101] IDRs are integral parts of many cancer-associated proteins and can be direct targets of cancer-inducing mutations.[102] Since Ser568 is the unique phosphorylatable site within the linker region (IDR) between third CAP-Gly and the USP domain of CYLD,[45] it could be considered a residue with high mutability. Another mechanism that may affect the phosphorylation of CYLD Ser568 is the methylation of cytosines within serine codons. The differentially methylated cytosines may lead to alternative splicing mechanisms resulting in the expression of alternative or less sensitive proteins.[103] So, in the light of the data summarized above, let us try to evaluate the alterations that might occur when a cell that could not phosphorylate CYLD Ser568 is exposed to oxidative stress and genotoxicity (Figure 7).

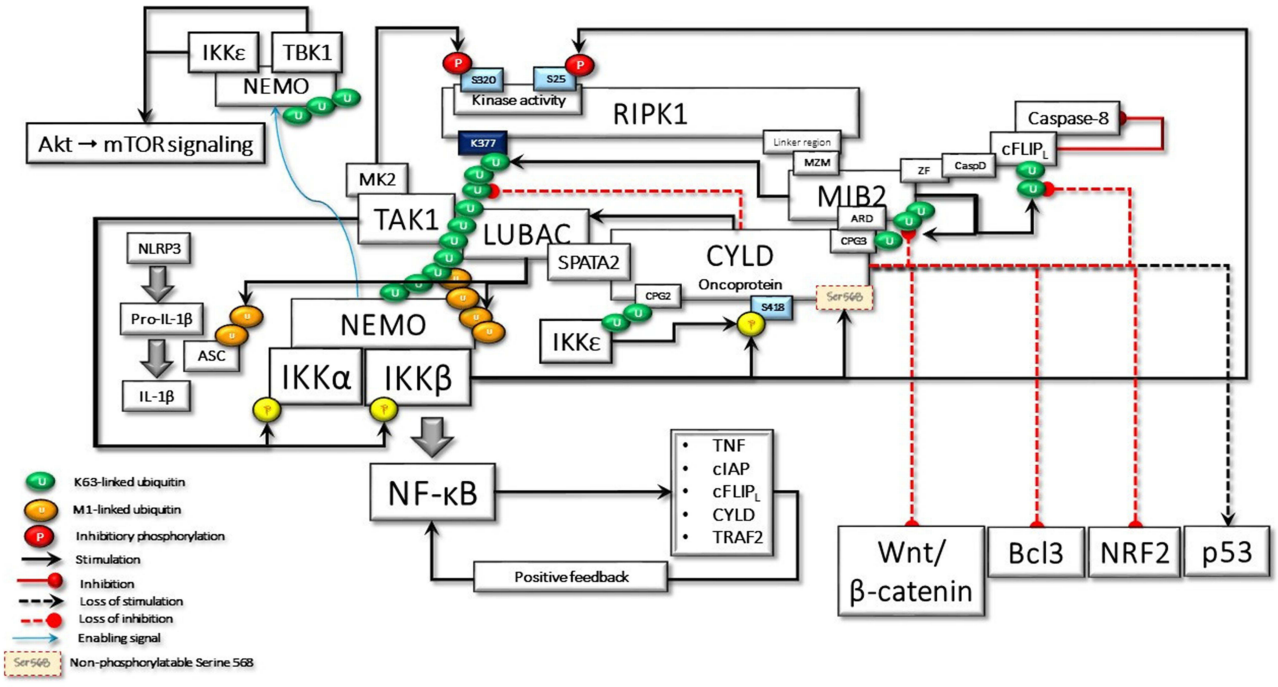

Figure 7 In the case of hypothetical loss of IKKβ-mediated Ser568 phosphorylation, CYLD behaves as an oncoprotein, keeping its scaffolding activity. Here, some of the important cyclical activities that can lead to cell transformation due to the loss of CYLD DUB activity are summarized: i) uncontrolled NF-κB activation due to positive feedback regulation; ii) RIPK1 kinase activity-dependent and -independent resistance to apoptosis; iii) IKKε- and TBK1-stimulated increased Akt and mTOR signaling; iv) LUBAC-induced increased secretion of IL-1β; v) Bcl3-driven increased transcriptional activity; vi) NRF2-driven increased transcriptional activity; vii) increased Wnt-β-catenin signaling; viii) loss of p53-mediated transcriptional activity; ix) MIB2-c-FLIP$_L$-mediated caspase 8 inhibition; x) Increased Akt-mTOR signaling; xi) increased IL-1β secretion.

1. Cytosolic c-Abl phosphorylates Tyr56 of OTULIN, facilitating SPATA2-LUBAC interaction. LUBAC-free OTULIN may increase Wnt-β-catenin signaling and UPS activity.
2. Cytosolic c-Abl interacts with MUC1, which blocks nuclear targeting of c-Abl and therefore the apoptotic response to genotoxic stimuli. Furthermore, c-Abl may alter cell polarity, and facilitate epithelial-to-mesenchymal transition (EMT) program, invasion, or growth.[104]
3. Cytosolic c-Abl blocks the ARF-mediated p53 activation, bypassing p53-mediated tumor suppression.
4. While CYLD could not exert DUB activity, its scaffolding function leads to uncontrolled NF-κB transcription. Thus, overexpression of CYLD and anti-apoptotic proteins may increase cell survival as a result of positive feedback signaling.
5. CYLD directly interacts with and deubiquitinates p53, promoting DNA damage-induced p53 stabilization and activation in response to oxidative stress-induced genotoxicity.[58] The stabilized p53 transcription factor is a key tumor suppressor and maintains genomic stability after cellular stresses.[105] The lack of CYLD catalytic DUB activity may impair the tumor suppressor function of p53.
6. Cancer cells, even in oxygen-rich conditions, prefer the less efficient use of glycolysis to metabolize glucose, a prominent metabolic change called the Warburg effect or aerobic glycolysis. Therefore, active metabolic reprogramming by altered oncoproteins and tumor suppressors is considered a key hallmark of cancer "even Warburg did not anticipate".[106] p53 regulates cellular metabolism; it represses glycolysis and the synthesis of lipids and nucleotides that all contribute to its tumor-suppressive function.[107] p53 can limit the activity of phosphofructokinase-1 (PFK-1), the rate-limiting enzyme in glycolysis.[108] p53 suppresses transcription of 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3) as well, which is also a rate-limiting enzyme that promotes glycolysis.[109] p53 stabilization by catalytically active CYLD was reported to suppress glycolysis through the inhibition of PFK1 and PFKFB3 transcriptions.[110] Furthermore, CYLD interacts with Fizzy-related protein 1 (FZR1, also named Cdh1), an important component of the anaphase-promoting complex/cyclosome (APC/C), a ubiquitin E3 ligase, and enhances FZR1 activity, which promotes the degradation of PFKFB3, cyclin B1, and cyclin D1.[110] Taken together, it would not be difficult to speculate that CYLD, unable to exploit its catalytic activity, may support the operation of metabolic reprogramming.
7. Loss of CYLD DUB activity increases the expression of proto-oncogene Bcl-3-mediated pro-survival, pro-inflammatory, and genes that control cell cycle activity.[111,112]
8. Loss of CYLD DUB activity increases the nuclear translocation of the transcription factor nuclear factor erythroid 2-related factor 2 (NRF2).[113] NRF2 is the major mediator of oxidative stress responses and DDR pathways stimulate NRF2 signaling. The increased NRF2 signaling plays important role in cancer initiation, progression, metabolic reprogramming, metastasis, and resistance to therapy. Intriguingly, similar to CYLD, NRF2 has been increasingly believed to operate as both a tumor suppressor and an oncoprotein.[114]
9. NF-κB signaling has been reported to increase the expression of nicotinamide adenine dinucleotide phosphate oxidases (NOXs).[115] Interestingly, the DUB activity of CYLD negatively regulates NOXO1 protein expression,[116] Therefore, the loss of CYLD DUB activity may potentiate NOX-mediated ROS generation, thus contributing to the oncogenesis.[117]
10. CYLD interacts with PLK1 and regulates mitotic entry.[118] Furthermore, active PLK1 is recruited into the RIPK1-Caspase 8 complex, which cleaves PLK1 during mitosis. Since caspase 8 activity is blocked, active PLK1 accumulates, resulting in defects in chromosome congression, segregation, and higher aneuploidy.[119] Thus, unregulated PLK1 activity may favor tumor evolution, drug resistance, and risk for tumor relapse.
11. In addition to the resistance to cell death, CYLD-MIB2 interaction may also increase Notch signaling, which may impart a stem-like phenotype.[79]
12. CYLD binds tubulin primarily through its first CAP-Gly domain and promotes tubulin polymerization into microtubules, which is important for cell migration.[48] CYLD is also associated with end binding protein (EB1). They act in concert to regulate microtubule nucleation at the centrosome, and microtubule growth at the cell periphery, thus further contributing to the directional cell migration.[120] Importantly, this function of CYLD is independent of its DUB activity.

13. The overexpression of Aurora B is a trigger for tumorigenesis and has been associated with a poor prognosis for cancer. The ubiquitin E3-ligase protein Skp2, which is also a cell-cycle regulatory protein, is required for the activation of Aurora B through K63-linked ubiquitination.[121] CYLD interacts with the catalytic domain of Aurora B, inhibiting its activity. The third CAP-Gly domain and the deubiquitinase domain are required for CYLD to inhibit Aurora B activity. CYLD also interacts with the scaffolding subunit of PP2A and promotes inactive PP2A/Aurora B interaction.[10] EB1 is an Aurora-B-interacting protein as well, and it stimulates Aurora-B activity through antagonizing its dephosphorylation/inactivation by PP2A.[122] Taken together, due to EB1 interaction and lack of DUB activity of CYLD, the decreased K63-linked deubiquitination, and dephosphorylation of Aurora B would be critical for cell proliferation toward malignancy.
14. CYLD suppresses Wnt signaling through deubiquitination of K63-linked ubiquitin chains on Dvl, an adapter protein that transduces proximal Wnt signals.[123] Consistently, loss of CYLD catalytic activity has been reported to fuel tumorigenesis and aggressiveness through hyperactivation of the Wnt pathway.[124]
15. The localization of cell polarity protein disheveled (Dvl) at the cell cortex is important for spindle orientation. Cortical polarity proteins can recruit the nuclear mitotic apparatus (NuMA) protein, which can generate pulling forces on astral microtubules to rotate the spindle. CYLD, through removing K63-linked polyubiquitin chains, stabilizes astral microtubules and stimulates the formation of the Dvl-NuMA-dynein/dynactin complex at the cell cortex, thereby promoting proper spindle orientation.[125] Thus, the loss of CYLD DUB activity may lead to misoriented cell division in epithelial cells, which is also important for carcinogenesis.
16. CYLD also binds HDAC6 through its first CAP-Gly domain and inhibits HDAC6 activity through its DUB function, leading to acetylated α-tubulin around the nucleus.[126] HDAC6 is a microtubule-associated deacetylase and HDAC6-mediated deacetylation regulates microtubule-dependent cell motility.[127] In the case of CYLD DUB activity loss, elevated HDAC6 activity, and increased deacetylation of microtubules, may promote tumor formation and oncogenic transformation by facilitating anchorage-independent proliferation, which allows cells to survive by escaping anoikis.[128]
17. LUBAC-mediated linear ubiquitination may also participate in the NLRP3 inflammasome activation pathway-mediated IL-1β secretion by promoting caspase-1 activation.[129] IL-1β is claimed as a cancer marker by some, due to its pleiotropic effects on immune cells, angiogenesis, cancer cell proliferation, migration, and metastasis.[130]
18. TBK1 and IKKϵ synergize with TANK to promote interaction with the canonical IKKs. The TANK binding domain within NEMO is required for the proper functioning of these IKK subunits.[131] K63-linked polyubiquitin chains on NEMO and TANK link TAK1 and the canonical IKK complex to TBK1/IKKε, enabling Ser172 phosphorylation and activation.[132] Sustained activation of noncanonical IKKs, IKKϵ and TBK1, also promotes the oncogenic phenotype.[133] They can activate Akt, which is involved in several critical cellular pathways including survival, proliferation, invasion, apoptosis, and angiogenesis,[134] by direct phosphorylation on both Thr308 and Ser473.[135] In addition to the Akt-mediated mTORC1 signaling axis, IKKϵ and TBK1 can also positively regulate mTORC1 activity.[136,137] Furthermore, they directly phosphorylate and activate mTORC2 which controls cellular metabolism, proliferation, and survival.[138] Importantly, uninterrupted mTOR activation has been recalling Blagosklonny's "hyperfunction theory".[139,140] The tight relationships between oxidative stress, DNA damage, ROS, and mTOR are critical to the concept of hyperfunction. Thus, a defective CYLD-mediated "hyperfunctional" state following oxidative DNA damage could be considered to force senescence in postmitotic cells, while providing support for malignant transformation in cells with mitotic activity. Altogether, non-canonical IKK-driven deregulated mTOR signaling contributes significantly to carcinogenesis with its myriad functions.[141]
19. In addition to NF-κB activating function, IKKβ promotes tumoral transformation by phosphorylating several other proteins that regulate many cellular processes from the cell cycle to metabolism and differentiation.[142]
20. NF-κB is a key positive regulator of programmed cell death ligand (PD-L1) expression in cancer.[143] NF-κB directly induces PD-L1 gene transcription by binding to its promoter, and it can also regulate PD-L1 post-transcriptionally through indirect pathways. Thus, cancer cells exploit the PD-L1-driven inhibitory pathway to their benefit as a key mechanism of immune evasion.[144]

21. Continuous linear ubiquitination of NEMO increases TRAF3 association and disrupts the MAVS-TRAF3 complex formation that inhibits type I Interferons (IFNs) activation.[145] IFNs are key regulators of natural host defense against viral infection and cancer.[146]
22. Oxidative stress-induced DNA breaks and subsequent abnormal use of the non-conservative NHEJ DNA repair through the activation of the NF-κB pathway may increase genome instability and favor transformation.[147,148]
23. Oxidative stress-induced direct activation of ATM may reason in the dissociation of the PP2A holoenzyme, which may promote the translocation of the scaffolding unit to the cytoplasm and the retention of the catalytic unit in the nucleus. Direct interaction of PP2A(C) with γ-H2AX causes premature dephosphorylation and consequent defects in DNA repair, which may increase the mutation rate.
24. Suppression of CYLD DUB activity and TAK1-facilitated activation of JNK stimulates the promoter activation of AP-1, leading to the transcription of genes important for cell proliferation, such as cyclin D1 and c-Myc.[149] c-Myc-driven epigenetic reprogramming promotes the formation, and maintenance of tumor-initiating cells and their attainment of metastatic capacity.[150]
25. Finally, evidence directly suggesting a relationship between loss of CYLD DUB activity and carcinogenesis came from a remarkable recent study. The researchers reported that transgenic mice lacking the deubiquitinase function of CYLD spontaneously develop tumors of various origins.[151]

Treatment Option for This Novel Postulate

Tyrosine kinase inhibitors (TKIs), with multiple targets, including c-Abl, are the major medicines for targeted therapy of cancer. Imatinib is a first-generation BCR-ABL TKI, which selectively targets the ATP binding site of the BCR-ABL protein. It was approved in 2001 by the FDA for treating chronic myelogenous leukemia. Since abnormally activated Abl kinases are implicated in a variety of pathologies, including various solid tumors, inflammatory disorders, neurodegenerative diseases, and even COVID-19, targeting Abl kinases with small molecule inhibitors is considered an option to treat different pathologies with hyperactive c-Abl.[152,153] However, while treatment with these kinase inhibitors has a predominant role on the cytoplasmic c-Abl, they cannot provide its nuclear localization.[154] If the hypothetical mechanisms operate as described, cytoplasmic translocation of c-Abl would be indispensable for triggering CYLD-mediated pathological outcomes. Therefore, the design of small molecules and future perspectives for the application of drugs targeting Thr735 of c-Abl could interfere with its cytosolic functions. This approach can also re-establish the apoptotic response in cancer cells by increasing ATM-c-Abl interaction.[155]

Conclusion

Contrary to the general belief that CYLD is an absolute tumor suppressor, it could operate both as a tumor suppressor and an oncoprotein in a context-dependent manner. Physiological alternately regulation of these two activities is crucially important for the maintenance of cellular homeostasis. If one of these features dominates independently of the other, the pathological outcome would be inevitable. Most cylindroma lesions in which CYLD cannot be expressed are benign. Contrary to common belief, lack of CYLD, which would also cause concomitant loss of CYLD-mediated oncogenic activity, may limit tumor growth and prevent progression to malignancy. In the case of non-phosphorylatable CYLD Ser568, oxidative stress that can lead to DNA damage could enable the malignant transformation of the cell as a result of the self-sustaining signaling network of the resistance to apoptosis, accelerated mitotic activity, and other concomitant unregulated processes.

Disclosure

The author reports no conflicts of interest in this work.

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