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Ubiquitylation in apoptosis: a post-translational modification at the edge of life and death

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Abstract | The proper regulation of apoptosis is essential for the survival of multicellular organisms. Furthermore, excessive apoptosis can contribute to neurodegenerative diseases, anaemia and graft rejection, and diminished apoptosis can lead to autoimmune diseases and cancer. It has become clear that the post-translational modification of apoptotic proteins by ubiquitylation regulates key components in cell death signalling cascades. For example, ubiquitin E3 ligases, such as MDM2 (which ubiquitylates p53) and inhibitor of apoptosis (IAP) proteins, and deubiquitinases, such as A20 and ubiquitin-specific protease 9X (USP9X) (which regulate the ubiquitylation and degradation of receptor-interacting protein 1 (RIP1) and myeloid leukaemia cell differentiation 1 (MCL1), respectively), have important roles in apoptosis. Therapeutic agents that target apoptotic regulatory proteins, including those that are part of the ubiquitin–proteasome system, might afford clinical benefits.

Thioester linkage
An ATP-dependent linkage formed between the carboxy-terminal group of ubiquitin and the Cys thiol group of E1 enzymes.

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Apoptosis is mediated by the assembly of signalling complexes that culminates in the activation of a cell death programme. It is evident that these complexes are subject to substantial post-translational regulation through modification by the 76-amino-acid protein ubiquitin. The ubiquitylation of constituent components in the apoptotic pathway often destabilizes them by targeting them for proteasomal degradation. However, just as importantly, the ubiquitin chain-mediated assembly of apoptotic signalling complexes illuminates how ubiquitylation can have non-degradative functions. Recent progress provides insight into how these seemingly disparate outcomes of ubiquitylation in apoptosis are mediated.

This Review addresses the intersection of these two exciting fields: apoptosis and the ubiquitin–proteasome system (UPS). We first describe the biochemistry of ubiquitylation and the various forms of ubiquitin modification, ranging from monoubiquitylation to linkage-specific polyubiquitin chain formation. This is followed by an introduction to the apoptotic pathways. We then discuss how apoptotic pathways are regulated by various components of the UPS, including ubiquitin E3 ligases and deubiquitinases (DUBs), before describing how the deregulation of ubiquitylation, and subsequently of apoptosis, can result in human diseases, such as cancer.

The UPS machinery

Ubiquitylation, which describes the covalent modification of target proteins with ubiquitin, has a profound bearing on the fate and function of its substrates and requires the enzymic activity of an E1, an E2 and an E3 protein (FIG. 1). Ubiquitin, in an initial energy-dependent step, associates with these enzymatic components through a labile thioester linkage. This facilitates the covalent ligation of ubiquitin to the target through a more stable isopeptide linkage to the ε-amino group of acceptor Lys residues or, less commonly, the amino terminus. The enzymatic cascade of ubiquitylation has remarkable combinatorial complexity and specificity, as dictated by the diversity of its constituent enzymes: two known E1s, tens of E2s and hundreds of E3s[1].

Ubiquitin ligases may exist as multisubunit complexes or as single proteins, and they may contain one of a number of domains that promote ubiquitylation, including the RING domain or HECT domain[2]. The largest subclass of E3 enzymes is that of the cullin RING ligases (CRLs). The CRLs are multicomponent E3 ligases, which, at their simplest, are composed of a RING domain-containing protein (RBX1 or RBX2), a regulatory cullin, and a substrate-binding adaptor. A variable number of linker proteins may increase the complexity. The prototypical example of a multisubunit E3 ligase is the Skp–cullin–F-box (SCF) complex. Because there

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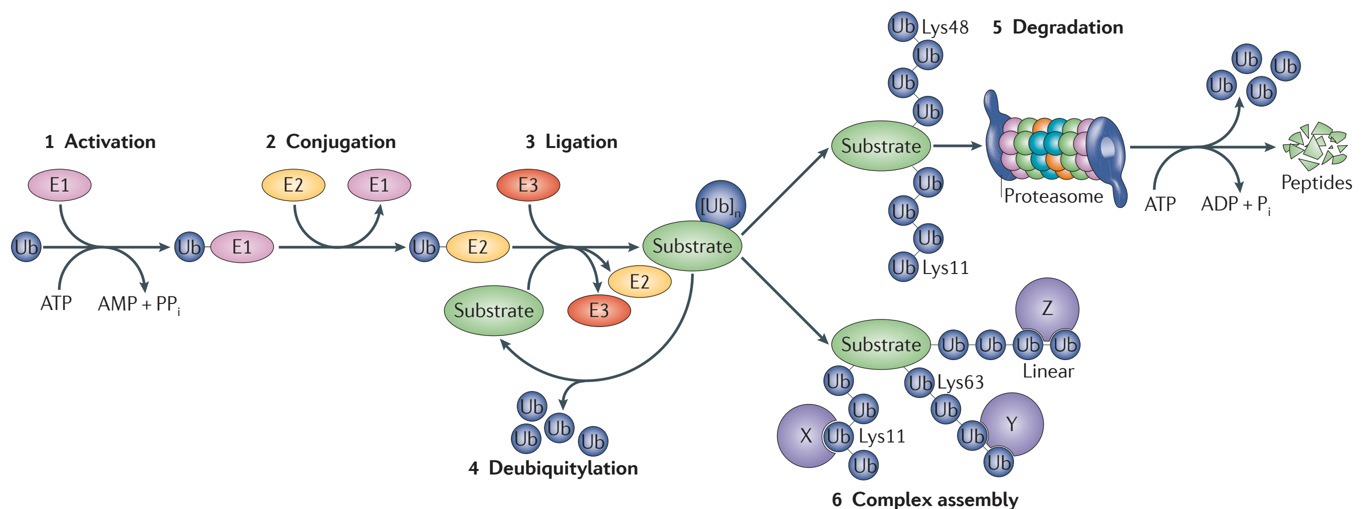

Figure 1 | **The enzymes and reactions of the UPS.** The ubiquitylation and degradation of substrate proteins is achieved by a series of reactions mediated by the enzymes of the ubiquitin–proteasome system (UPS). In the activation reaction, ubiquitin is transferred to an E1 enzyme in an ATP-dependent manner (step **1**). The activated ubiquitin is subsequently transferred to an E2 enzyme in the conjugating reaction (step **2**). The E2 enzyme then carries the ubiquitin to the E3 enzyme, which is also known as a ubiquitin ligase (step **3**). The E3 is important not only because it covalently ligates ubiquitin to Lys residues on the substrate protein, but also because it mediates substrate specificity. This process of ubiquitin ligation may be repeated with a Lys of the ubiquitin protein itself serving as the substrate, which leads to the formation of a polyubiquitin chain on the target protein. Deubiquitylating enzymes may reverse substrate protein ubiquitylation (step **4**). Ligation of polyubiquitin has diverse biological consequences for the recipient protein. For example, Lys11- and Lys48-linked polyubiquitin chains serve as tags to target substrate proteins for proteasomal degradation (step **5**). Conversely, linear, Lys63- and Lys11-linked chains promote the assembly of signalling complexes (step **6**). X, Y and Z indicate ubiquitin-binding proteins. P$_i$, inorganic phosphate; PP$_i$, inorganic diphosphate; Ub, ubiquitin. Image is modified, with permission, from REF. 126 © (2010) Macmillan Publishers Ltd. All rights reserved.

are 69 mammalian F-box proteins that act as substrate-binding adaptors, the number of proteins under SCF regulation is considerable[3].

Substrates are monoubiquitylated when the carboxy-terminal group of ubiquitin covalently links with the ε-amino group of a substrate Lys. However, as ubiquitin itself possesses seven Lys residues and a free N terminus, there are eight amino groups available for the synthesis of polyubiquitin chains through isopeptide linkages. All of these linkage types are observed in organisms from yeast to man, underscoring the importance of the biological information conveyed by the various topologies. Lys48-linked and Lys11-linked ubiquitin chains generally target substrates for proteasomal degradation. By contrast, Lys63-linked chains, linear polyubiquitin chains that are formed through the free N terminus of ubiquitin, and, in some cases, Lys11-linked chains provide scaffolding for the recruitment and assembly of signalling complexes[4] (FIG. 1). There is growing evidence that ubiquitin ligases, E2 enzymes and ubiquitin collaborate to dictate ubiquitin chain linkage specificity[5–7].

How is the information embedded in the linkages deciphered? A vast family of ubiquitin-binding domains, which are discrete entities from different structural classes, provides the decoding apparatus: some, such as the ubiquitin-interacting motif (UIM), are largely helical, whereas others, such as the zinc-finger ubiquitin-binding protein in ubiquitin-specific protease 5 (USP5), are zinc-finger proteins[8]. A subset of these ubiquitin-binding proteins displays linkage specificity, enabling them to preferentially bind particular chains. Specific examples pertinent to the apoptotic pathways are discussed below.

Ubiquitylation, similarly to phosphorylation, is a reversible modification and, in mammals, approximately 100 DUBs function to depolymerize and remove ubiquitin adducts (FIG. 1). The DUBs fall into five major classes: four Cys protease classes and one metalloprotease class[9]. The realization that the dynamic interplay between ubiquitylation and deubiquitylation sets the threshold for apoptotic signalling has unleashed a torrent of interest and has significant therapeutic ramifications.

Apoptotic pathways

Apoptotic cell death is mediated through an intrinsic (mitochondrial) pathway and/or an extrinsic (death receptor-mediated) pathway and results in the activation of caspases — Cys-dependent aspartyl-specific proteases that are the central executors of apoptosis[10]. Irradiation, growth factor withdrawal or chemotherapeutic agents initiate the mitochondrial pathway by activating BCL-2 homology 3 (BH3)-only motif proteins such as BH3-interacting domain death agonist (BID), BCL-2 antagonist of cell death (BAD), p53 upregulated modulator of apoptosis (PUMA; also known as BBC3), NOXA (also known as PMAIP1) or BCL-2-interacting mediator of cell death (BIM; also known as BCL-2L11)[11] (FIG. 2a). These BH3-only proteins neutralize the anti-apoptotic B-cell lymphoma 2 (BCL-2) family members BCL-2,

Isopeptide linkage
An amide bond that forms between a side-chain carboxyl group and amino group and is not present on the main chain of a protein. In the case of ubiquitylation, isopeptide linkages form between the ε-nitrogen of Lys side chains and the C-terminus of the incoming ubiquitin, and constitute the basis of polyubiquitin chains.

RING domain
A ubiquitin ligase domain that is defined by the presence of a catalytic zinc-finger-like module that chelates two zinc ions in a unique 'cross-brace' structure.

HECT domain
Homologous to the E6AP (also known as UBE3A) carboxyl terminus, the HECT domain is a ubiquitin ligase domain that contains a catalytic Cys residue, allowing it to accept the charged ubiquitin from the E2 enzymes and transfer it directly to a substrate.

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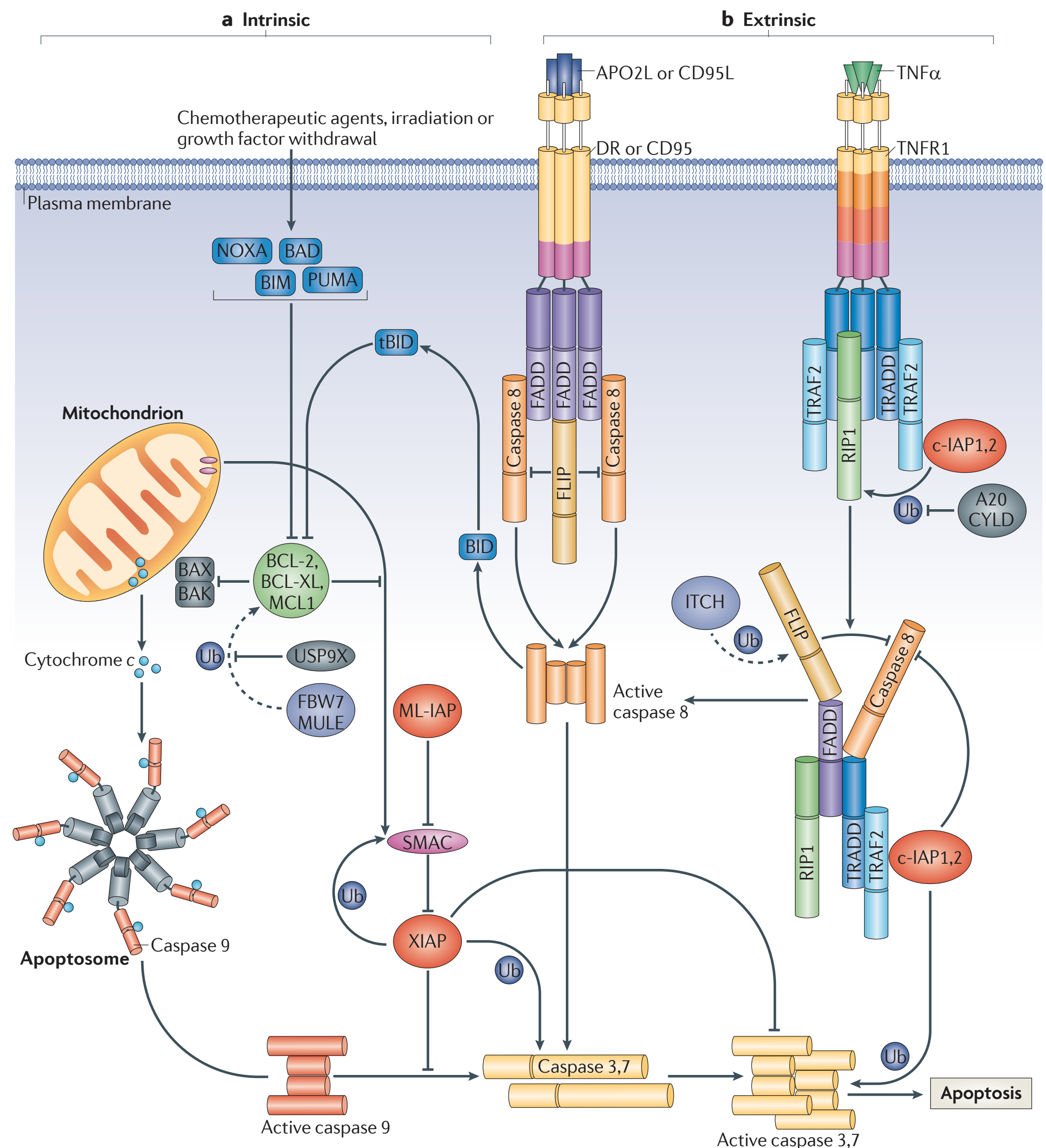

Figure 2 | **The intrinsic and extrinsic apoptotic pathways.** Apoptosis is executed via mitochondrial (or intrinsic) and death receptor (or extrinsic) pathways, and it culminates with the activation of caspases, which are proteases that execute cell death. **a** | The intrinsic pathway is activated by chemotherapeutic agents, irradiation or growth factor withdrawal, leading to the activation of BCL-2 homology 3 (BH3)-only B-cell lymphoma 2 (BCL-2) family proteins (BH3-interacting domain death agonist (BID), BCL-2 antagonist of cell death (BAD), p53 upregulated modulator of apoptosis (PUMA), NOXA or BCL-2-interacting mediator of cell death (BIM)) and neutralization of anti-apoptotic BCL-2 proteins (BCL-2, BCL-extra large (BCL-XL) and myeloid leukaemia cell differentiation 1 (MCL1)). Consequent disruption of mitochondrial integrity causes the release of cytochrome c and second mitochondrial activator of caspases (SMAC) and apoptosome-dependent activation of caspase 9. This leads to the activation of caspases 3 and 7 and, ultimately, to apoptosis. **b** | Death ligands, such as CD95 ligand (CD95L; also known as FASL), APO2 ligand (APO2L; also known as TRAIL) or tumour necrosis factor-α (TNFα) engage their cognate receptors to instigate the formation of death-inducing signalling complex (DISC) and the execution of the extrinsic death pathway. DISC recruitment leads to the activation of caspase 8 and the subsequent activation of effector caspases 3 and 7. Several ubiquitin ligases are critically involved in the inhibition of cell death, including inhibitor of apoptosis proteins (IAP) proteins, whereas others, such as FBW7 (F-box- and WD repeat-containing 7) and MULE (MCL1 ubiquitin ligase E3), promote apoptosis through their E3 ligase activity. Dashed arrows indicate apoptosis-promoting action of E3 ligases. Deubiquitinases can either enhance cell death, as is the case for CYLD, or inhibit it, as for ubiquitin-specific protease 9X (USP9X). BAK, BH antagonist or killer; c-IAP, cellular IAP; DR, death receptor; FADD, FAS-associated DEATH domain protein; FLIP, FLICE-inhibitory protein; ML-IAP, melanoma IAP; RIP1, receptor-interacting protein 1; tBID, truncated BID; TNFR1, TNF receptor 1; TRADD, TNFR1-associated DEATH domain protein; TRAF2, TNFR-associated factor 2; Ub, ubiquitin; XIAP, X chromosome-linked IAP.

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Table 1 | **Ubiquitin–proteasome system proteins affecting apoptosis**

Enzyme	Substrate(s)	Biological process(es)
Ubiquitin ligases		
XIAP	Caspase 3, caspase 7, SMAC	Apoptosis
c-IAP1, c-IAP2	RIP1, TRAF2, NIK, caspase 3, caspase 7, XIAP, SMAC, c-IAP1, c-IAP2	Canonical and noncanonical NF-κB signalling, apoptosis
MDM2	p53	Cell cycle, apoptosis, senescence
βTRCP	IκBα	Canonical NFκB signalling
FBW7	MCL1, MYC, JUN	Apoptosis, proliferation
ITCH	FLIP, p63	Apoptosis
SIAH1, SIAH2	HIPK2, PHD1, PHD3	DNA damage, hypoxia
VHL	HIF1α	Hypoxia
MULE	MCL1	Apoptosis
Deubiquitinases		
A20	RIP1, RIP2, TRAF2, TRAF6, UBCH5, UBC13, caspase 8, NEMO, MALT1, others	Downregulates NF-κB activity and promotes apoptosis
CYLD	BCL-3, RIP1	Downregulates NF-κB activity
USP14	Various proteins destined for proteasomal degradation	Attenuates proteasomal function to induce cell death
USP9X	β-catenin, TGFβ, SMADs, MCL1, others	Anti-apoptotic
USP7	MDM2, p53, MDMX, FOXO4, PTEN, claspin, others	Anti-apoptotic

βTRCP, β-transducin repeat-containing protein; BCL-3, B-cell lymphoma 3; c-IAP, cellular IAP; FBW7, F-box- and WD repeat-containing 7; FLIP, FLICE-inhibitory protein; FOXO4, forkhead box O4; HIF1α, hypoxia-inducible factor 1α; HIPK2, homeodomain-interacting protein kinase 2; IAP, inhibitor of apoptosis; IκBα, inhibitor of NF-κBα; MALT1, mucosa-associated lymphoid tissue lymphoma translocation 1; MCL, myeloid leukaemia cell differentiation; MULE, MCL1 ubiquitin ligase E3; NEMO, NF-κB essential modulator; NF-κB, nuclear factor-κB; NIK, NF-κB-inducing kinase; PHD, prolyl hydroxylase; PTEN, phosphatase and tensin homologue; RIP, receptor-interacting protein; SMAC, second mitochondrial activator of caspases; TGFβ, transforming growth factor-β; TRAF, TNFR-associated factor; UBC, ubiquitin-conjugating enzyme; USP, ubiquitin-specific protease; VHL, von Hippel–Lindau disease tumour suppressor; XIAP, X chromosome-linked IAP.

BCL-extra large (BCL-XL; also known as BCL-2L1) or myeloid leukaemia cell differentiation 1 (MCL1) to liberate the pro-apoptotic, multi-BH domain proteins BAX and BH antagonist or killer (BAK)[12]. The subsequent disruption of mitochondrial membrane potential, which is likely to be initiated by the insertion of BAX and BAK into the mitochondrial membrane, results in the release of cytochrome *c* and second mitochondrial activator of caspases (SMAC) from the mitochondrial intermembrane space into the cytoplasm[13–15]. Newly released cytochrome *c*, along with dATP, binds the adaptor protein apoptotic protease-activating factor 1 (APAF1), which leads to the formation of the so-called apoptosome complex that recruits and activates initiator caspase 9. Activated caspase 9 in turn activates effector caspases 3 and 7, leading to the precipitous cleavage of protein substrates and cell death[16].

Extrinsic apoptosis is initiated by the binding of death ligands of the tumour necrosis factor (TNF) family to their cognate receptors, such as CD95 (also known as FAS), death receptor 5 (DR5; also known as TNFRSF10B) or TNF receptor 1 (TNFR1)[17] (FIG. 2b). Ligand binding triggers receptor aggregation and the assembly of the receptor-associated death-inducing signalling complex (DISC)[18]. Recruitment of the adaptor FADD (FAS-associated DEATH domain protein), or in the case of TNFR1 the adaptor TRADD (TNFR1-associated DEATH domain protein), and zymogen caspase 8, leads to DISC formation. Through an induced proximity mechanism, caspase 8 is activated and the death signal is amplified by the subsequent proteolytic activation of the downstream effectors caspase 3 and caspase 7; this culminates in apoptotic death. Apoptotic pathways are often connected, which provides an opportunity for crosstalk and signal amplification. For example, activated caspase 8 can cleave BID to generate its active form, truncated BID (tBID), which translocates to mitochondria to stimulate cytochrome *c* and SMAC release[19].

Anti-apoptotic proteins counter the execution of apoptosis. In the mitochondrial pathway, the anti-apoptotic proteins BCL-2, BCL-XL and MCL1 oppose BH3-only and multidomain pro-apoptotic BCL-2 family members to preserve mitochondrial integrity[12]. In the extrinsic pathway, FLICE-inhibitory protein (FLIP; also known as CFLAR) is recruited to the DISC where it attenuates the activation of caspase 8 (REF. 20). Inhibitor of apoptosis (IAP) proteins block cell death at converging points in both pathways, namely, at the level of caspase activation. X chromosome-linked IAP (XIAP) can directly bind to and inhibit caspases 3, 7 and 9, whereas cellular IAP1 (c-IAP; also known as BIRC3) and c-IAP2 (also known as BIRC2) negatively regulate caspase 8 activation in the context of TNFR1 signalling[21]. SMAC, however, can bind XIAP and prevent it from inhibiting caspases[13,15]. Other IAP proteins, like melanoma IAP (ML-IAP; also known as BIRC7), can sequester SMAC away from XIAP and thereby limit its pro-apoptotic activity[22].

In addition to the core components of the apoptotic machinery outlined above, many other proteins have a profound impact on cellular fate. Some of these, such as nuclear factor-κB (NF-κB) and p53 (REF. 23), regulate the expression of apoptotic modulators, whereas others, such as kinases and acetyl transferases, promote their post-translational modification[24,25]. As ubiquitylation affects the protein-binding preferences, subcellular localization and stability of so many crucial apoptotic proteins, it is little wonder that it also plays a critical part in the regulation of apoptotic pathways[26]. Indeed, a diverse group of proteins belonging to the UPS, including ubiquitin ligases and DUBs, regulates the stability and activity of numerous pro-apoptotic and anti-apoptotic proteins[26,27] (TABLE 1), and this is the focus of the remainder of our Review article.

Ubiquitin ligases in apoptotic pathways

Several ubiquitin ligases, including RING domain-containing MDM2, which ubiquitylates p53, and IAP proteins that affect caspase and SMAC stability, have important roles in apoptotic pathways (TABLE 1). In addition, E3 ligases also regulate signalling processes that greatly influence cellular proliferation and survival.

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Regulation of apoptotic proteins by IAP proteins. The IAP family of E3 ligases, some members of which contain a RING domain, has a conserved role in regulating apoptosis. Invertebrate model systems revealed that the *Drosophila melanogaster* IAP antagonists Reaper (RPR), Head involution defective (HID; also known as Wrinkled) and Grim can form a multimeric complex, which directly binds to insect and baculovirus IAP proteins, causing their autoubiquitylation and subsequent proteasomal degradation[28,29]. *D. melanogaster* IAP1 is crucial for fly survival and the control of caspases in *D. melanogaster*, and temporal regulation of IAP1 can determine if caspases will exert their nonapoptotic role in cellular morphogenesis or cause cell death[30]. It has also been proposed that IAP1 can polyubiquitylate and neddylate *D. melanogaster* caspases in both a degradative and nondegradative manner to cause the catalytic inactivation of these proteins[28,31,32]. Another important fly protein that regulates cell death, and like *D. melanogaster* IAP1 contains a baculovirus IAP repeat domain (BIR domain) , is Bruce. Interestingly, Bruce associates with an SCF complex, the activity of which is required for caspase activation during cellular remodelling in *D. melanogaster*[33].

A principal regulator of caspase activity in mammalian cells is XIAP. Through its BIR2 domain and preceding linker region, and its BIR3 domain, XIAP binds and inhibits caspases 3, 7 and 9 (REF. 34). In addition to blocking the catalytic activity of these caspases, XIAP also promotes their ubiquitylation to limit their levels and exert an anti-apoptotic effect[35]. Murine cells expressing an E3 ligase-deficient version of XIAP contained elevated levels of caspase 3 activity and showed increased sensitivity to apoptotic stimuli, consistent with the importance of XIAP ubiquitin ligase activity in negatively regulating apoptosis[36].

Whereas c-IAP proteins are not physiological inhibitors of caspases, c-IAP1 can promote the ubiquitylation of caspase 3 and caspase 7 (REF. 37). In general, c-IAP1 and c-IAP2 regulate apoptotic pathways through their ubiquitin ligase activity. In a similar fashion to *D. melanogaster* IAP1, which can stimulate the ubiquitylation of RPR-like proteins, c-IAP1 mediates the ubiquitylation and degradation of SMAC, thus enhancing cell survival[28,38,39]. Additionally, c-IAP1 can mediate the ubiquitylation of other IAP proteins, namely XIAP and c-IAP2 (REFS 40,41), which probably fine-tunes their levels to ensure an optimal balance. IAP proteins also promote the ubiquitylation of many other proteins[42,43]. Some substrates, such as receptor-interacting protein 1 (RIP1; also known as RIPK1) and TNFR-associated factor 2 (TRAF2)[44], are discussed below in the context of NF-κB signalling, but many others await additional studies to clearly establish their physiological relevance.

Regulation of signalling pathways by IAP proteins. Signalling pathways that regulate the expression of apoptotic regulators, such as the NF-κB and mitogen-activated protein kinase (MAPK) pathways, are important regulators of cellular fate. The NF-κB transcription factor family comprises five members that function as homodimers and heterodimers and regulate the expression of TNFα, c-IAP2, BCL-2 and many other core components of the apoptotic machinery. In the canonical NF-κB pathway, which is activated by TNFα, ligand binding leads to the recruitment of signalling adaptor proteins, including TRADD and TRAF2, to TNFR1; these recruit c-IAP proteins and RIP1 to assemble a proximal TNFR1-associated signalling complex[45,46] (FIG. 3a). Within this complex, c-IAP1 and c-IAP2 ubiquitylate RIP1, TRAF2 and themselves through a variety of non-degradative linkages that include, but are not limited to, Lys63 and Lys11 polyubiquitin chains[47–51,52]. The critical E2 partners of c-IAP1 and c-IAP2 in this signalling pathway are the UBCH5 (also known as UBE2D1) family of ubiquitin conjugating enzymes[48,49,53]. The c-IAP-polymerized chains form a platform for the recruitment of the inhibitor of NF-κB (IκB) kinase (IKK) complex and IKKβ-activating TGFβ-activated kinase 1 (TAK1) with its associated TAK1-binding proteins (TABs), as well as auxiliary signalling components including HOIL1-interacting protein (HOIP; also known as RNF31), a member of the linear ubiquitin chain assembly complex (LUBAC)[54,55]. Activated IKKβ phosphorylates the inhibitory NF-κB subunit IκB, leading to its ubiquitylation and proteasomal degradation. This liberates NF-κB dimers, which consist of p50 and RELA (also known as p65), to enter the nucleus and promote the expression of pro-inflammatory, anti-apoptotic genes. HOIP, along with its LUBAC partners haeme-oxidized IRP2 ubiquitin ligase 1 (HOIL1L; also known as RBCK1) and SHANK-associated RH domain-interacting protein (SHARPIN), promotes linear ubiquitylation and ensures persistent activation of NF-κB signalling and inhibition of TNF-induced cell death[52,56,57].

The noncanonical NF-κB pathway is negatively regulated by the continual ubiquitylation, and consequent proteasomal degradation, of NF-κB-inducing kinase (NIK) by c-IAP1 and c-IAP2 as part of the c-IAP–TRAF2–TRAF3 complex[58–61] (FIG. 3b). Binding of TNF-related weak inducer of apoptosis (TWEAK; also known as TNFSF12), CD40 or several other TNF ligands to their cognate receptors disrupts this protein complex by promoting the degradation of its components, thus enabling the accumulation of NIK[58,59,62]. NIK then phosphorylates and activates IKKα, which in turn phosphorylates the latent NF-κB transcription factor p100 (also known as NF-κB2); this triggers its partial proteasomal processing to generate transcriptionally active p52, which translocates to the nucleus as a homodimer, or as a heterodimer with RELB[63,64]. Although the complete mechanistic details of JUN N-terminal kinase (JNK) and p38 activation have yet to be unravelled, the E3 ligase activities of c-IAP1 and c-IAP2 are also necessary for TNF family-induced MAPK activation[65,66]. Therefore, ubiquitin ligases and their substrates, as well as proteins that depend on ubiquitin modification for recruitment to the receptor complexes, are crucial signalling components for these pro-inflammatory and anti-apoptotic (that is, survival) pathways.

The importance of E3 ligases in apoptosis is best illustrated by TNFR1 signalling. Although in the presence of c-IAP proteins TNFR1 predominantly activates canonical NF-κB and MAPK pro-inflammatory and

BIR domain
(Baculovirus inhibitor of apoptosis (IAP) repeat domain). Coordinates zinc binding and is required for the anti-apoptotic activity of IAP proteins.

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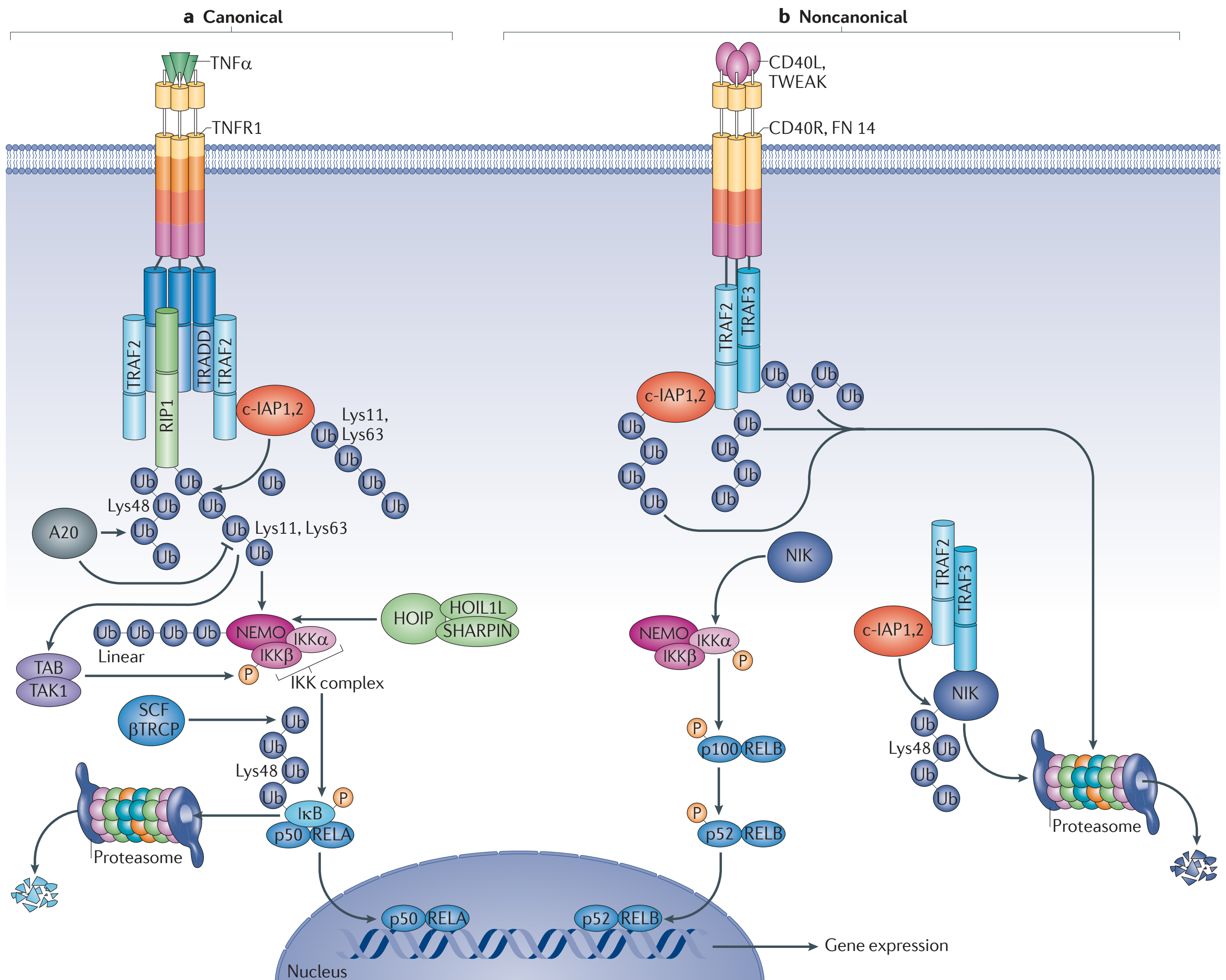

Figure 3 | **Canonical and noncanonical NF-κB signalling pathways.** Nuclear factor-κB (NF-κB) signalling is activated via canonical and noncanonical pathways. **a** | During the tumour necrosis factor-α (TNFα)-stimulated canonical pathway, the E3 ligases cellular inhibitor of apoptosis 1 (c-IAP1) and c-IAP2 promote Lys11 or Lys63 nondegradative polyubiquitylation of receptor-interacting protein 1 (RIP1), and also of themselves. These c-IAP-polymerized ubiquitin chains form a platform for the recruitment and activation of the inhibitor of NF-κB (IκB) kinase (IKK) complex, which phosphorylates the inhibitory NF-κB subunit IκB and the linear ubiquitin chain assembly complex (LUBAC), allowing HOIL1-interacting protein (HOIP)-mediated linear ubiquitylation of NF-κB essential modulator (NEMO), which ensures persistent activation of NF-κB signalling. Phosphorylation of IκB triggers its Lys48-linked ubiquitylation and subsequent degradation, thus liberating the NF-κB p50–RELA dimers, and allowing them to translocate to the nucleus and activate gene expression. Activated genes include the anti-apoptotic genes *c-IAP2* and B-cell lymphoma 2 (*BCL-2*). A20-mediated deubiquitylation of Lys63 chains on RIP1 and A20-mediated Lys48 ubiquitylation of RIP1 can attenuate this signalling pathway. **b** | During the TNF-related weak inducer of apoptosis (TWEAK)- or CD40-induced noncanonical pathway, negative regulation by c-IAP1- and c-IAP2-mediated NF-κB-inducing kinase (NIK) polyubiquitylation is abrogated by the recruitment and subsequent degradation of c-IAPs, TNFR-associated factor 2 (TRAF2) and TRAF3 at the receptor complexes, which liberates NIK and allows the activation of signalling. c-IAP1, c-IAP2, TRAF2 and TRAF3 in this receptor signalling complex are ubiquitylated with a variety of linkages that have yet to be confirmed. CD40L, CD40 ligand; CD40R, CD40 receptor; FN14, FGF-inducible 14; HOIL1L, haeme-oxidized IRP2 ubiquitin ligase 1; SCF$^{\beta TRCP}$, Skp–cullin–F-box–βTRCP; SHARPIN, SHANK-associated RH domain-interacting protein, TAB, TAK1-binding protein; TAK1, TGFβ-activated kinase 1; TNFR1, TNF receptor 1; TRADD, TNFR1-associated DEATH domain protein; Ub, ubiquitin.

survival signalling, the absence of c-IAP proteins transforms TNFα into a death ligand[47,49,50]. This 'Jekyll and Hyde' transformation is achieved through several interdependent molecular steps. First, in the absence of c-IAP proteins, RIP1 is not ubiquitylated and LUBAC and IKK complexes cannot be recruited to the TNFR1 complex, thus preventing the activation of the canonical NF-κB and MAPK pathways and the expression of pro-survival

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genes[47–49,54]. Second, nonubiquitylated RIP1 readily associates with FADD and caspase 8 to from a secondary death-promoting signalling complex that dissociates from the receptor[47,67]. Third, in limited circumstances when caspase activity is blocked, nonubiquitylated RIP1 will bind RIP3 to promote necroptosis, an alternative, caspase-independent cell death pathway (reviewed in REF. 68). However, as c-IAP proteins are negative regulators of noncanonical NF-κB pathways, their absence can promote the survival and proliferation of B cells.[58–61,66,69]. Therefore, through the regulation of NF-κB- and TNFα-stimulated signalling pathways, the E3 ligase activity of c-IAP proteins can determine cell fate in a variety of tissues and cellular settings.

Apoptosis regulation by other single-protein ligases. MDM2 is another critical ligase that regulates cell viability, and it is the primary ubiquitin ligase for the potent tumour suppressor p53. The tumour-suppressive function of p53 is attributable to its ability, as a transcription factor, to upregulate a range of target genes that are responsible for cell cycle arrest or apoptosis in response to various cellular stresses[70]. The tight regulation of p53 by MDM2 is most definitively revealed by murine knockout studies. MDM2-null mice are embryonic lethal, but crossing these mice with p53-null mice restores their viability. This suggests that p53 is the primary MDM2 target and is constitutively elevated in the absence of MDM2. Moreover, p53-null mice and mice that are deficient in both MDM2 and p53 succumb to the same types of tumours and have essentially identical survival curves, again underscoring the critical MDM2–p53 relationship and its role in regulating cell survival and tumorigenesis[71,72].

Despite these convincing genetic data, a substantial number of studies indicate that MDM2 targets a number of other proteins that regulate cell survival, including MDMX (also known as MDM4), another p53 inhibitory protein[73]. In general, MDM2 functions as an oncogene to either directly or indirectly inactivate tumour suppressors. For example, MDM2-dependent ubiquitylation contributes to the degradation of forkhead box O (FOXO) family transcription factors to promote cell survival and proliferation[74]. Additionally, MDM2 targets insulin-like growth factor 1 receptor (IGF1R) for degradation independently of p53, and loss of MDM2 promotes significant IGF1R stabilization in wild-type and p53-null cells. As such, the absence of MDM2 is required for IGF1R to exert its protective effects against DNA damage-induced apoptosis[75]. Other MDM2 targets include dihydrofolate reductase (DHFR), which desensitizes cells to methotrexate-induced cell death[76], and the ribosomal protein S7, which enhances the apoptotic response to stress signals[77]. Substantial evidence suggests that MDM2 may also autoubiquitylate and target itself for destruction; however, the generation of a RING-mutant MDM2 knock-in mouse suggested that other MDM2 ligases exist[78]. One such ligase is SCF–βTRCP ($SCF^{βTRCP}$), which promotes MDM2 degradation in response to DNA damage to permit p53 upregulation and either DNA repair or apoptosis (see below)[79].

Although MDM2 regulates the stability of p53, it does not affect the stability of the p53-related proteins p63 and p73 (REFS 80,81). This role is actually attributable to a HECT domain E3 ligase called ITCH[82]. In addition to ubiquitylating p63 and p73, ITCH regulates the stability of FLIP. TNFα-stimulated JNK activation promotes JNK-mediated phosphorylation and activation of ITCH[83]. ITCH, in turn, ubiquitylates FLIP and induces its proteasomal degradation, preventing it from inhibiting caspase 8. Accordingly, the absence of ITCH protects mice from TNFα-induced acute liver failure, as this prevents FLIP ubiquitylation and degradation, allowing it to inhibit apoptosis[83].

Another group of ubiquitin ligases with multiple substrates that affect cellular survival pathways are the RING-domain containing SIAH proteins. SIAH1 mediates the polyubiquitylation and consequent degradation and inactivation of homeodomain-interacting protein kinase 2 (HIPK2), a protein that promotes cell death induced by DNA damage[84]. Through the kinase activity of ataxia-telangiectasia mutated (ATM) and ataxia-telangiectasia- and RAD3-related (ATR), DNA damage causes disruption of HIPK2–SIAH1, leading to HIPK2 stabilization and activation and apoptosis[84]. It has also been suggested that SIAH1 also targets XIAP for proteasomal degradation, independently of the E3 ligase activity of XIAP[85,86]. SIAH1 relies on ARTS (apoptosis-related protein in the TGFβ signalling pathway; also known as SEPT4), a XIAP-binding protein, to physically link it to XIAP[85,86]. In addition to promoting the ubiquitylation of TRAF2, SIAH2 modulates the cellular hypoxia response by regulating the stability of prolyl hydroxylase 1 (PHD1) and PHD3 (REF. 87). This consequently affects hypoxia-inducible factor 1α (HIF1α), a critical mediator of the cellular hypoxic response that is also a substrate of the von Hippel–Lindau disease tumour suppressor (VHL) E3 ligase complex. HIF1α is kept at low levels in the presence of oxygen as the hydroxylation of select Pro residues within the α-subunit of HIF1α allows VHL binding and the subsequent polyubiquitylation and proteasomal degradation of HIF1α[88].

Regulation of apoptosis by ubiquitin ligase complexes. Thus far, we have highlighted the roles of single-protein ubiquitin ligases in the regulation of cell death, but ubiquitin ligase complexes also have critical roles in regulating cellular demise. Two substrate-binding, F-box subunits of the SCF class of multisubunit ubiquitin ligases, FBW7 (F-box- and WD repeat-containing 7; also known as FBXW7 and SEL10) and β-transducin repeat-containing protein (βTRCP), are important regulators of apoptosis by virtue of the substrates that they target for proteasomal degradation. Humans express two βTRCP paralogues that have indistinguishable properties, βTRCP1 (also known as FBXW1, FBW1A and FWD1) and βTRCP2 (also known as FBXW11 and FBXW1B); in this Review, we refer to both paralogues as βTRCP. Other F-box proteins, including S-phase kinase-associated protein 2 (SKP2; also known as FBXL1), have less direct or less extensive roles in regulating apoptosis and are reviewed elsewhere[89,90]. Readers are also referred to a comprehensive review

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that summarizes the role of cullin-based ubiquitin ligase complexes in the regulation of non-apoptotic functions of caspases in invertebrate systems[91].

The F-box is a ~40-amino-acid protein–protein interaction motif that is characteristic of F-box proteins and mediates their binding to SKP1; F-box proteins also bind to ubiquitylation substrates, thereby recruiting them to the SCF ligase core complex[92]. FBW7 and βTRCP also contain WD40 domains, which direct substrate binding, although the substrates that they regulate are mostly distinct. Genetic ablation of both βTRCP1 and βTRCP2 has not been reported and βTRCP1-null mice do not show gross tissue abnormalities or reduced viability, which is probably due to the redundant regulation of substrates by βTRCP2 (REFS 93,94). However, inhibition of both βTRCP isoforms by RNA interference (RNAi), or expression of dominant-negative mutants, promotes apoptosis, probably owing to an accumulation of pro-apoptotic substrates[95,96]. $SCF^{\beta TRCP}$ substrates have been comprehensively reviewed[89,90], so here we highlight the substrates that are thought to be major contributors to $SCF^{\beta TRCP}$-mediated cell death. The most overtly pro-apoptotic $SCF^{\beta TRCP}$ substrates are BIMEL (an extra-long isoform of BIM) and procaspase 3. BIMEL is a pro-apoptotic BCL-2 family member with remarkably high affinity for pro-survival BCL-2 family members, and it effectively neutralizes their effects on binding[12,97]. Procaspase 3 is the zymogen form of active caspase 3; thus, elevated levels of the precursor form of this cell death protease augment cell death[98]. The tumour suppressor protein p53 and related protein p63, which function as transcription factors, may also promote apoptosis, and both are $SCF^{\beta TRCP}$ substrates[99,100]. Signal transducer and activator of transcription 1 (STAT1) is another pro-apoptotic transcription factor and tumour suppressor that is targeted by $SCF^{\beta TRCP}$ for degradation[101]. In addition, $SCF^{\beta TRCP}$ regulates apoptosis at the translational level by promoting the degradation of translation inhibitor programmed cell death 4 (REF. 102). Although βTRCP ablation promotes cell death, which suggests that the net effect of stabilizing its targets is the promotion of apoptosis, the pro-survival protein MCL1 has also been identified as an $SCF^{\beta TRCP}$ substrate, and this may serve to fine-tune cell death in certain contexts[103]. Furthermore, the $SCF^{\beta TRCP}$ ligase has additional substrates that have primarily pro-survival effects. As discussed above, the NF-κB transcription factors promote cell survival, and $SCF^{\beta TRCP}$ targets various IκBs, as well as the inhibitory precursors p100 and p105, for degradation[90]. Additionally, the ubiquitin ligase MDM2, which directs p53 degradation, is also an $SCF^{\beta TRCP}$ substrate[79]. $SCF^{\beta TRCP}$ also regulates proper cell cycle progression by degrading the CDC25 phosphatase, thereby keeping hyperproliferation 'in check' (REFS 95,104).

FBW7 isoforms are encoded by three transcripts that are produced by alternative splicing, and ablation of all three isoforms is embryonic lethal[105]. A number of additional conditional or isoform-specific FBW7-knockout models exist, all of which confirm that FBW7 promotes the degradation of pro-apoptotic substrates in neurons, T-cells and haematopoietic stem cells[106–108]. The numerous FBW7 substrates have been extensively reviewed elsewhere[89,106,108]; here, we focus on the most direct regulators of cell death. Elevated levels of Notch proteins are thought to be key mediators of the cellular demise that is induced by FBW7 ablation[105], as Notch-mediated signalling influences cell fate decisions, cell proliferation, differentiation and apoptosis in a context-dependent manner[109]. Cyclin E was the first identified, and is the best-characterized, FBW7 substrate, and elevated cyclin E levels may promote cell death by disrupting normal cell cycle progression and DNA replication[110]. The oncogenic transcription factors MYC and JUN are also FBW7 substrates and may promote cell death in the absence of FBW7 (REFS 111–114). Given that FBW7 is often inactivated in human tumours, it was unclear how tumour cells could survive and proliferate despite harbouring elevated levels of FBW7 substrates, such as JUN, MYC and NOTCH1, which can activate apoptosis[115]. Identification of the pro-survival protein MCL1 as an FBW7 substrate provided a solution to this conundrum — MCL1 is likely to counteract accumulated pro-apoptotic FBW7 substrates and sustain inappropriate tumour cell survival when FBW7 is inactivated[116,117]. MCL1 stability is also regulated by HECT domain-containing MCL1 ubiquitin ligase E3 (MULE; also known as HUWE1)[118] and the APC/C (anaphase-promoting complex, also known as the cyclosome) bound to its co-activator CDC20 (REF. 119). MULE is unique among E3 ligases as it possesses a BH3 domain that allows it to bind to MCL1. Given the seminal role of MCL1 for cellular survival, proliferation and resistance to various apoptotic insults, it is not unexpected that its turnover is regulated by multiple ubiquitin ligases.

DUBs in apoptotic pathways

DUBs are the enzymes that remove ubiquitin modifications from substrate proteins. Notable DUBs, such as A20 (also known as TNFAIP3 and encoded by the *TNFAIP3* gene) and USP9X, which regulate the ubiquitylation and degradation of RIP1 and MCL1, respectively[1,120,121] (TABLE 1), are important regulators of apoptosis.

A dual role for A20 in regulating apoptotic pathways. Because A20 has both DUB and ubiquitin ligase activities, it serves as a fitting transition from ubiquitin ligases to DUBs that regulate apoptosis[122–124]. *TNFAIP3* was discovered as a primary response gene of inflammatory cytokines[125]. Because A20 downregulated its own expression, it was proposed to participate in a negative feedback loop to attenuate inflammatory responses[126]. This early hypothesis has been substantiated by several models[127], most convincingly the generation of A20-null mice that succumb perinatally to systemic inflammation and multi-organ failure as a result of unchecked NF-κB activity[128]. RNAi studies also suggest a role for A20 in attenuating NF-κB-independent immune responses[129]. A20 primarily attenuates inflammatory signalling by modifying the polyubiquitylation status of substrates via ubiquitin editing. As comprehensive reviews have detailed the numerous A20 substrates, we focus on the overlying concepts of A20-mediated regulation of cell death[127,129].

WD40 domains
Protein domains that comprise multiple WD40 repeats that form a scaffold for protein–protein interactions. WD40 repeats are structural motifs of ~40 amino acids that terminate in Trp (W) and Asp (D) residues.

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Ubiquitin editing can be broadly conceptualized as the removal of modifications that promote signalling complex assembly and activation, such as Lys63-linked polyubiquitylation, linear ubiquitin chains or even ligation with small ubiquitin-like modifier (SUMO), followed by the addition of modifications that promote substrate degradation, such as Lys11 or Lys48 polyubiquitylation[120]. The net result of ubiquitin editing is attenuation of signalling. For example, A20 attenuates TNFα-induced NF-κB signalling by removing Lys63 chains from RIP1 via its OTU DUB domain and promotes the addition of degradative polyubiquitin chains on RIP1 via its zinc-finger 4 motif[122,123,130] (FIG. 3a). Given that A20 attenuates NF-κB signalling, and NF-κB generally activates pro-survival responses, it would logically follow that enhanced A20 activity should promote cell death. Although this effect is likely to contribute to the tumour-suppressive function of A20 in lymphomas, it appears to be cell type- and context-specific. That is, it is unclear why certain A20-null cells, such as splenocytes and enterocytes, show enhanced sensitivity to TNF-induced apoptosis[128,131], yet the reintroduction of wild-type A20 in A20-inactivated lymphoma cells promotes cell death[127,132]. Given the crosstalk between the signalling complexes assembled downstream of the receptors mediating extrinsic apoptosis (FIG. 2), it is possible that the interaction of A20 with caspase 8 could play a part[133]. It will be important to clarify the basis for this dichotomous role of A20 in regulating cell survival, which may also include apoptosis-independent mechanisms, in order to fully understand how A20 regulates pathogenesis.

CYLD regulates apoptotic pathways. The DUB CYLD was originally identified through a gene mutation found in a genetic condition with predisposition for the development of tumours of skin appendages, a familial cylindromatosis[134]. CYLD is thought to act as a tumour suppressor as it is often mutated in multiple myeloma and several other cancers, and the ablation or reduced expression of the *CYLD* gene contributes to tumorigenesis and causes increased sensitivity to chemically induced skin tumours in mice[134,135]. The deubiquitylating activity of CYLD resides in its C-terminal USP catalytic domain and is critical for its tumour-suppressive function.

Most CYLD substrates participate in NF-κB signalling, although a few have roles in the cell cycle and calcium signalling[136,137]. One CYLD substrate, BCL-3 (an IκBα homologue), is a transcriptional co-activator for the NF-κB DNA-binding subunits p50 and p52 (REF. 138). By eliminating the Lys63-linked polyubiquitylation and nuclear translocation of BCL-3, CYLD limits BCL-3's proliferative activity[136]. RIP1 is another NF-κB and apoptosis regulator that has been proposed to be a CYLD substrate; however, the role of CYLD in TNFα-induced RIP1 deubiquitylation is less clear. For example, there were no differences in RIP1 ubiquitylation between wild-type and CYLD-null peritoneal macrophages after TNFα treatment[139]. In CYLD-null testicular germ cells, ubiquitylation associated with RIP1 was persistent but TNFα-independent[140].

Because Lys63-polyubiquitylated RIP1 mediates canonical NF-κB signalling and also prevents the formation of the pro-apoptotic signalling complex that includes caspase 8 and FADD, DUBs that deubiquitylate RIP1 will not only block NF-κB activation but will also stimulate caspase 8-dependent apoptosis[141]. In addition, recent studies suggest that nonubiquitylated RIP1 associates with RIP3 to promote necroptosis when caspase activation is blocked[68,142]. Thus, RIP1 deubiquitylation can promote caspase-dependent and caspase-independent cell death pathways. As such, much effort is currently focused on identifying the particular DUBs that deubiquitylate RIP1 in specific contexts and cell types.

Other USPs regulate apoptosis. In addition to CYLD, several other USP-type DUBs, USP14, USP9X and USP7, also regulate apoptosis. USP14 is a proteasome-associated DUB that antagonizes substrate degradation by disassembling polyubiquitin chains from their distal end, a process known as chain trimming[143]. USP14 is crucial for general homeostasis, as its deletion is embryonic lethal[144]. Therefore, ataxia mutant mice, which harbour an insertion in the USP14 gene that results in the almost complete loss of USP14 expression, are a useful model of USP14 function. The most striking phenotypes of ataxia mutant mice are severe tremors, hindlimb paralysis and death by two months of age[145]. Rescue of ataxia mutants with neuronal-targeted expression of USP14 restored viability and motor system function[146] and revealed that USP14 is required for the proper development and function of the male reproductive system[144]. The recent characterization of a small-molecule USP14 inhibitor, IU1, confirmed the role of USP14 in ubiquitin chain trimming[147]. Functionally, USP14 inhibition increased proteasomal clearance of damaged proteins and enhanced the viability of HEK293 cells challenged with oxidizing agents[147]. Thus, under conditions of cell stress, enhancement of proteasome activity via USP14 inhibition may have beneficial effects on cell viability.

USP9X is the mammalian orthologue of *D. melanogaster* FAM (also known as FAFX), which has a critical role in eye development[148]. Given the marked evolutionary conservation of this DUB, it is unsurprising that USP9X controls fundamental processes, such as preimplantation development and the signalling pathways that regulate cell viability in postnatal development[149]. In general, USP9X enhances cellular survival, probably as it deubiquitylates and stabilizes numerous pro-survival substrates[150]. More specifically, USP9X stabilizes β-catenin, thereby enhancing pro-survival Notch and WNT signalling, as well as the self-renewal of embryonic stem cell-derived neural progenitors[151,152]. USP9X also enhances transforming growth factor-β (TGFβ) signalling via deubiquitylation of TGFβ receptors and of their SMAD intracellular mediators, and it stabilizes the pro-survival BCL-2 family member MCL1 (REFS 150,152,154). However, the pro-survival effects of USP9X may be context-specific, as it can also stabilize oxidative stress-activated apoptosis signal-regulating kinase 1 (ASK1; also known as MAP3K5) to promote cell death[155].

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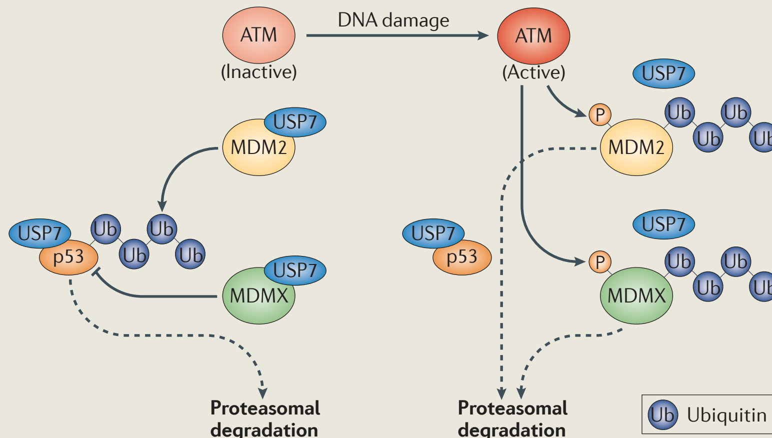
Box 1 | **The complex interrelationships among p53, MDM2 and USP7**



The pivotal role of ubiquitin-specific protease 7 (USP7) in controlling cell death is best illustrated by its context-specific regulation of p53 and MDM2, a critical p53 ubiquitin ligase[191]. USP7 can associate with, deubiquitylate and stabilize both p53 and MDM2. This dichotomy could theoretically set up a futile cycle of p53 ubiquitylation and deubiquitylation, particularly given that *MDM2* is a p53 target gene. Nevertheless, MDM2 seems to be the preferred USP7 substrate in normal cycling cells, as definitively demonstrated in USP7-null cells and embryos, in which p53 accumulates as a result of MDM2 destabilization in the absence of USP7 (REFS 192–194). So, what determines whether p53 or MDM2 is the primary USP7 substrate? The answer appears to be context-dependent, and a complex, yet finely-tuned, mechanism of response to DNA damage has been elucidated. In this scenario, DNA damage activates the ataxia-telangiectasia mutated (ATM) kinase, which phosphorylates MDM2 and the related p53 inhibitory protein MDMX (see the figure). This phosphorylation lowers their affinities for USP7. The net result is MDM2 and MDMX destabilization, p53 stabilization and subsequent p53-mediated DNA repair or cell death[195]. Thus, in the event of DNA damage, p53 inhibitors are no longer protected from degradation by USP7, thereby permitting p53 to exert its tumour-suppressive functions. Dashed arrows indicate proteins being targeted for proteasomal degradation.

USP7 was first identified as a herpesvirus-associated USP, hence its alternative name HAUSP. USP7 associates with and stabilizes the herpesvirus protein VMW110 (also known as ICP0), which is required for efficient initiation of the viral lytic cycle. As a result, USP7 facilitates viral lytic growth[156]. Since this initial discovery, USP7 has also been shown to destabilize or otherwise compromise the effectiveness of a number of critical tumour suppressors, including p53, FOXO4 and phosphatase and tensin homologue (PTEN)[150], and to enhance the oncogenic properties of claspin (a protein that is involved in the DNA replication checkpoint)[153]. Thus, USP7 is, by inference, an oncogenic pro-survival protein. The unique interrelationship of p53, USP7 and MDM2, a critical p53 ubiquitin ligase, is discussed further in BOX 1.

Apoptosis and ubiquitylation in disease

We have described ubiquitin ligases and DUBs that are key determinants of cell death. Given the critical role that cell death plays in regulating tumorigenesis[157], inflammatory disorders[127], neurodegeneration[158] and other diseases, it follows that the aberrant regulation or inactivation of these ubiquitin-modifying enzymes may result in pathophysiology and disease. Additionally, alterations to their function may enhance or attenuate the responsiveness of the diseased cells to therapeutic agents. Below, we highlight some of the recent findings in this area (see also BOX 2).

IAP proteins and antagonists in cancer. An increase in the level of IAP proteins in tumours, and their ability to engage survival signalling pathways, strongly implicates them in human malignancies. For example, ML-IAP displays a strong cancer-expression bias, and the expression of XIAP and c-IAP1 and c-IAP2 is associated with poor prognosis in several tumour types[159,160]. The chromosomal region that harbours the c-*IAP1* and c-*IAP2* genes is amplified in several human and murine tumour types, implicating c-IAP proteins as potential oncoproteins[160,161]. In support of the oncogenic role of c-IAP proteins, a significant proportion of extranodal non-Hodgkin mucosa-associated lymphoid tissue lymphoma translocation (MALT) lymphomas harbour a t(11;18)(q21;q21) chromosomal translocation that fuses the N-terminal region of *c-IAP2* with the central and C-terminal portion of *MALT1* (also known as paracaspase)[162,163]. The c-IAP2–MALT1 fusion protein promotes constitutive activation of the NF-κB pathway, which enhances pro-survival and pro-inflammatory signalling, promotes cancer progression and enhances resistance to anticancer therapies[164]. These features define IAP proteins as attractive targets for therapeutic intervention. Among several targeting strategies that have been explored thus far, the one using small-molecule IAP antagonists has garnered the most attention[165] (BOX 2).

Small molecule IAP antagonists mimic the N-terminal end of active SMAC protein (or of *D. melanogaster* HID protein) and bind select BIR domains with high affinities to promote cell death and inhibit tumour growth in *in vivo* models[165]. Interestingly, IAP antagonists seem to trigger conformational changes in c-IAP proteins that increase their ubiquitin ligase activity[59,165,166]. This promotes the ubiquitylation of several proteins that are found in the same receptor-associated or cytoplasmic protein complexes; of note, c-IAP-mediated ubiquitylation of RIP1 activates canonical NF-κB signalling to promote cell survival[47,49]. However, this burst of ubiquitin ligase activity also causes the rapid autoubiquitylation and proteasomal degradation of c-IAP proteins[47,59,60]. The absence of c-IAP proteins, in turn, allows NIK accumulation and the consequent activation of the noncanonical NF-κB pathway[59,60]. TNFα that is produced as a result of these signalling events induces TNFR1-mediated signalling, which leads to the death of tumour cells in the absence of c-IAP proteins[47,59,60,67]. Thus, IAP antagonists that were initially designed to block IAP protein interactions with caspases and other pro-apoptotic proteins were, unexpectedly, found to activate IAP E3 ligase activity, a feature that is critical for the induction of apoptosis in tumour cells.

A20 and the attenuation of constitutive inflammation. A20 polymorphisms are associated with several autoimmune disorders, including Crohn's disease, psoriasis, rheumatoid arthritis and systemic lupus erythematosis[167]. Furthermore, A20 is a critical tumour suppressor in a range of lymphomas, including diffuse large B cell

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Box 2 | **Therapeutic regulation of apoptotic pathways**

Small-molecule inhibitor of apoptosis (IAP) antagonists mimic the amino-terminal end of active second mitochondrial activator of caspases (SMAC) and bind select baculovirus IAP repeat domains (BIR domains) with high affinities to promote the death of cancer cells. Restoring A20 activity is not currently possible; thus, several approaches are being trialled to functionally mimic A20 reactivation (see the table). These include: antagonizing pro-inflammatory receptors, including tumour necrosis factor receptor 1 (TNFR1) and TNFR2 using infliximab (Remicade; Centocor Otho Biotech), adalimumab (Humira; Abbott Laboratories) or etanercept (Enbrel; Pfizer); attenuating nuclear factor-κB (NFκB) activation with IAP antagonists or proteasome inhibitors; and inhibiting anti-apoptotic proteins using B-cell lymphoma 2 (BCL-2) family antagonists or IAP antagonists. Restoration of p53 function can be achieved by a number of strategies, including blocking p53–MDM2 interaction with nutlins, JNJ-26854165, MI-219 (also known as AT-219) or RITA (RBPJ-interacting and tubulin-associated) to protect p53 from MDM2-directed ubiquitylation and stabilize functional p53 protein. The development of small molecules that inhibit ubiquitin-specific protease 7 (USP7) deubiquitinase activity in order to enhance MDM2 degradation and stabilize p53 is also in progress. Bortezomib (Velcade; Millennium Pharmaceuticals) and other protease inhibitors (that is, carfilzomib or NPI-0052) probably cause cell death through various cellular pathways, including stabilization of pro-apoptotic BCL-2 family members, inhibition of canonical and noncanonical NF-κB pathways, and accumulation of misfolded proteins. In contrast to malignant states, in which proteasome inhibition is desirable, enhanced proteasome activity is favourable in neurodegenerative diseases. Thus, enhancing proteasomal activity using IU1, an inhibitor of the proteasome-associated deubiquitinase USP14, may be a promising therapeutic strategy for a variety of pathologies induced by proteotoxicity.

Targeting agent	Targeted protein or pathway	Outcome
IAP antagonists	IAP proteins, apoptosis, necroptosis, NF-κB signalling	Tumour cell death
Infliximab, adalimumab, etanercept	Pro-inflammatory receptors, such as TNFR1 and TNFR2	Attenuation of the chronic inflammation that may promote tumorigenesis
Nutlins, JNJ-26854165, MI-219 (AT-219), RITA	Block p53–MDM2 interaction	Stabilization of p53, tumour suppression
USP7 inhibitors	Enhance MDM2 degradation	Stabilization of p53, tumour suppression
Bortezomib, carfilzomib, NPI-0052	Proteasomal proteases	Tumour cell death
IU1	USP14 inhibition	Enhancement of proteasomal activity

lymphoma (DLBCL), MALT lymphoma, and classic Hodgkin's lymphoma[127]. The A20 gene is inactivated by various mechanisms, including deletion, promoter methylation, frame shift mutations and/or nonsense mutations that result in truncations or point mutations in the A20 protein. As is often the case with tumour suppressors, both A20 alleles are commonly disabled. The protease MALT1 is constitutively active in certain lymphomas and may also cleave and inactivate A20 to enhance NF-κB signalling[168,169]. These various modes of A20 inactivation contribute to autoimmune disorders and lymphomagenesis by promoting unchecked NF-κB activity, resulting in constitutive inflammation and enhanced cell survival[127,170].

It would be therapeutically beneficial to selectively restore A20 activity in chronically inflamed and malignant cells. However, as this is not feasible with our current technology, we must rely on alternative strategies. These could include three specific approaches: antagonizing pro-inflammatory receptors, including CD20 (using rituximab (Rituxan; Biogen Idec)) or TNFR1 and TNFR2 (using infliximab (Remicade; Centocor Otho Biotech), adalimumab (Humira; Abbott Laboratories) or etanercept (Enbrel; Pfizer)); attenuating NF-κB activation, for example with IAP antagonists[171] or proteasome inhibitors[172,173], or by blocking growth factors that signal through the NF-κB pathway[174,175]; or inhibiting anti-apoptotic proteins using BCL-2 family antagonists or IAP antagonists[171,176]. Indeed, the more general approaches of using nonsteroidal anti-inflammatory drugs (NSAIDs) to reduce inflammation or antibiotics to eliminate infection have already provided striking therapeutic benefit in the treatment of MALT lymphoma and colorectal cancer[177].

p53 inactivation and p53-modulating compounds. Given that p53 is inactivated in many cancer types by a variety of mechanisms, much energy has been invested in researching how to reinstate p53 activity in tumours. This is reflected in the number of ongoing clinical trials and different approaches that researchers are using to try and achieve this[178–181]. The strategies for restoring p53 function can be grouped into three broad categories. The first is focused on enhancing or restoring the transcriptional activity of wild-type p53, whereas the second category comprises strategies to selectively regulate the viability of cells depending on their p53 status. Given that neither of these strategies modulates p53 function via the UPS, the reader is referred to several excellent reviews on these topics[178,179,181]. The third category of reagents aims to increase the levels of wild-type p53 protein. For example, a number of small molecules, such as the nutlins, JNJ-26854165 and MI-219 (also known as AT-219), bind MDM2 to block p53 binding, whereas RITA (RBPJ-interacting and tubulin-associated) binds p53 to block MDM2 binding; with both approaches, p53 is protected from MDM2 ubiquitylation and degradation[178,179,182,183]. The development of small molecules that inhibit USP7 DUB activity in order to enhance MDM2 degradation and stabilize p53 is in progress[184,185], and antisense MDM2 oligonucleotides are also in preclinical development[178–181]. Clearly, the efficacy of these p53-directed therapeutics will rely on the identification of the appropriate biomarkers and facile assays to identify the patients who are most likely to respond.

Unfolded proteins and protease inhibitors. The rationale for targeting the UPS for the treatment of cancer is validated by the US Food and Drug Administration (FDA)'s approval, and the clinical efficacy, of bortezomib (Velcade; Millennium Pharmaceuticals) for the treatment of multiple myeloma and mantle cell lymphoma[186]. Bortezomib and other protease inhibitors (that is, carfilzomib or NPI-0052) probably cause cell death through a multitude of cellular pathways, including stabilization of pro-apoptotic BCL-2 family members, inhibition of canonical and noncanonical NF-κB pathways, disruption of tumour–stroma interactions and the accumulation of misfolded proteins[187]. More specifically, proteasome inhibition may selectively promote the death of multiple myeloma cells because they have an exceptionally high rate of production of immunoglobulin-γ (IgG) proteins. Thus, perturbation of proteasome activity might overwhelm

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the quality-control machinery of these malignant cells to cause cell death[188]. In contrast to malignant states, in which proteasome inhibition is desirable, enhanced proteasome activity is favourable in neurodegenerative diseases. Cells within the neuromuscular system are particularly sensitive to the aberrant accumulation of oxidized and otherwise misfolded proteins[189]. Because the UPS is one of the most important conduits of degradation of aggregated and misfolded proteins, enhanced proteasomal activity via inhibition of the proteasome-associated DUB USP14, for example, with the small-molecule inhibitor IU1, may be a promising therapeutic strategy for a variety of pathologies induced by proteotoxicity[147].

Conclusions and future perspectives

The controlled degradation of apoptotic regulators, activation of signalling pathways and modulation of many other cellular processes by ubiquitylation affect apoptotic outcomes in normal and diseased cells. Our understanding of these processes is far from complete and a number of important questions remain unanswered, including the identity of ubiquitin ligases and DUBs that regulate the stability of apoptotic proteins, the spatial and temporal nature of ubiquitylation during apoptotic signalling and the relationship and interdependence with other post-translational modifications. An improved understanding of ubiquitin networks and molecular and physiological mechanisms that govern them should usher in novel ways for targeting crucial components of ubiquitin pathways.

In addition to targeting ubiquitin ligases or DUBs for therapeutic intervention, future studies should examine the targeting of crucial oncogenes that have been chemically intractable so far (for example, RAS) by selectively affecting their protein stability. This could be achieved by inhibiting a DUB that is critical for keeping an oncogene in the deubiquitylated state or by activating an E3 ligase that could target oncogenic proteins for proteasomal degradation. Such approaches have shown promising initial results and they could open new therapeutic opportunities to address large, unmet medical needs in the treatment of human cancers and immune diseases[190].

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Competing interests statement
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