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# Ubiquitin-chains dynamics and its role regulating crucial cellular processes

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## Abstract

The proteome adapts to multiple situations occurring along the life of the cell. To face these continuous changes, the cell uses posttranslational modifications (PTMs) to control the localization, association with multiple partners, stability, and activity of protein targets. One of the most dynamic protein involved in PTMs is Ubiquitin (Ub). Together with other members of the same family, known as Ubiquitin-like (UbL) proteins, Ub rebuilds the architecture of a protein in a few minutes to change its properties in a very efficient way. This capacity of Ub and UbL is in part due to their potential to form complex architectures when attached to target proteins or when forming Ub chains. The highly dynamic formation and remodelling of Ub chains is regulated by the action of conjugating and deconjugating enzymes that determine, in due time, the correct chain architecture for a particular cellular function. Chain remodelling occurs in response to physiologic stimuli but also in pathologic situations. Here, we illustrate well-documented cases of chain remodelling during DNA repair, activation of the NF- $\kappa$ B pathway and autophagy, as examples of this dynamic regulation. The crucial role of enzymes and cofactors regulating chain remodelling is discussed.

**Keywords:** Ubiquitin chains, Ubiquitin enzymes, Proteasome, Autophagy, DNA repair, NF- $\kappa$ B signaling

## 1. Introduction

Proteins form a dynamic equilibrium in cells. Their quality, abundance and function must be highly regulated. This homeostatic protein equilibrium known as proteostasis contributes to maintaining cell viability, granting the ability to cope with external/internal stimuli or stress conditions. Various processes are involved in this equilibrium including protein synthesis, folding, posttranslational modifications (PTMs), assembly/disassembly of complexes, intracellular transport, and degradation. Disruptions of this equilibrium are associated with various pathologies such as inflammatory, neurodegenerative, or infectious diseases and different cancer types. A proteostasis disequilibrium is a common hallmark in the most aggressive types of cancers like those localised in the pancreas, lung, and prostate. Many enzymes and proteostasis regulators have been described to be dysregulated in tumorigenesis, cancer progression, metastasis and resistance to chemotherapy [1]. In addition, neurodegenerative diseases, in particular those associated with dementia, usually display a defective proteolytic activity generating a “proteostatic collapse”, a phenotype in which misfolded proteins aggregate together with ubiquitylated inclusion bodies [2].

PTMs like phosphorylation, acetylation, and modification by Ubiquitin (Ub) family members also known as (AKA) Ubiquitin-like proteins (UbLs), regulate the half-life of proteins and their localization, but also their activity and interactions with other cellular factors. The reversibility of PTMs has the great virtue of enabling reversible switches between different functional states, therefore a rapid and versatile manner of fine-tuning countless signaling cascades and cellular events. Historically, Ub was first involved in the regulation of proteasomal degradation, also called “Ub Proteasome System” (UPS). In the last decades, different functions and layers of complexity have however been attributed to this unique modification underlying a complex language that has been named as “*the Ub Code*”, involving different types of ubiquitylation and regulatory mechanisms [3]. The plasticity of the Ub Code allows the control of a large diversity of processes including cell cycle control, protein quality control, cell proliferation, apoptosis, development, DNA repair, autophagy, signal transduction pathways, endocytosis, intracellular trafficking, transcription and the regulation of multiple infections, among others [4–7].

Ub is a 76-residue amino-acid  $\beta$ -grasp structure that is highly conserved across eukaryotic organisms. Ub is generated through the processing of high molecular weight precursors that after cleavage expose the glycine residue 76 (G76) then used to attach it covalently to lysine or other residues on target proteins [3]. The complexity of Ub signals relies in part on its capacity to be attached as a monomer, one or several times on the target proteins, but also on one or more of the 7 reactive lysine residues present on Ub itself.

Ub attachment to protein substrates, or Ub conjugation, is a highly dynamic reversible process regulated by conjugating and deconjugating enzymes. Conjugation events involve a thiol ester cascade of reactions that are mediated by a group of enzymes that activate (E1), conjugate (E2) or ligate (E3) the Ub moiety to its target. This process is associated with the writing of the Ub Code and involves two E1, at least 37 E2s and around 600 putative ligases in human cells. While E1s and E2s form thiol-ester intermediates, not all E3s do it and Ub can be transferred from E2 directly to the lysine residue on the substrate. In this category of E3, we find the family of really interesting new gene (RING) finger type. Other enzymes like the homologous to E6-associated protein C-terminus (HECT) domain type form a thiol ester intermediate with an internal cysteine residue in the E3 before transferring Ub to the target protein. Literature describes two other major families of E3 ligases: the UFD2 homology (U-box), and the RING-in-between-RING (RBR) [3]. E3s confers specificity to the conjugation process as they control the decision for the substrate to be modified and the lysine residue to be conjugated.

Deconjugating enzymes are editors of this language and are specific for each member of the Ub family. In the case of Ub, these have been called DUBs for deubiquitylating enzymes. This group of enzymes proofread the architecture of Ub chains acting as “erasers” to control the quantity or number of Ub moieties modifying targets and chain architecture required to connect with a specific function. DUBs are proteases that specifically recognize monoubiquitin (monoUb) or polyubiquitin (polyUb) chains on modified targets and remove Ub by hydrolysis of isopeptide bonds from the carboxyl group of Ub G76 attached to the target. Some DUBs process high molecular weight precursors of Ub and others have a role on Ub chain remodelling by its endo- or exo-protease activity. DUBs have been classified in seven families, based on their sequence and structure of their catalytic domain; six are cysteine proteases, the Ub-specific proteases (USP), Ub C-terminal hydrolases (UCH), Machado-Joseph disease proteases (MJD), ovarian tumour domain proteases (OTUs), motif interacting with Ub containing novel DUB family (MINDYs) and the recently discovered zinc finger with UFM1-specific peptidase domain protein proteases (ZUFSPs). The seventh family encompasses the zinc dependent metalloproteases JAB1/MPN/MOV34 (JAMMs) [8,9]. The Ub chain architecture on a particular target protein can be simultaneously proofread by the action of one or more DUBs that will determine the final architecture.

The information encrypted in Ub chains is read and interpreted by different adaptor proteins or “readers” [10]. The modulation of the formation and processing of Ub chains is achieved through

Ubiquitin Binding Domains (UBD), some of them with a preference for specific Ub chains and topologies [11]. In this way, Ub chains, recognized by multiple UBDs present in effector proteins, can connect with multiple cellular functions. In some cases, this connection might rely on cofactors of different nature that will provide specificity or increase affinity of these temporal interactions. Since the Ub code can be regulated at the level of writing, editing, reading and interpretation, the complexity of this language is almost limitless. Improving our knowledge of the messages encrypted by distinct chain types is necessary to understand this cellular communication.

## 2. Ubiquitin chains diversity

Protein substrates can be modified by one Ub moiety over one or multiple amino acids of its target protein, referred to as mono- or multi-monoubiquitylation respectively. Ub attachment generally occurs on lysine (K) residues, but serine (S) and threonine (T) have been found to be ubiquitylated under particular circumstances too [12]. Since Ub has a globular shape, eight exposed residues can be conjugated to the C-terminus of another Ub via isopeptide bonds to form chains of various lengths, linkages, and structures. Seven lysine residues (K6, K11, K27, K29, K33, K48, and K63) and N-terminal methionine residue (M1) force Ub to adopt unique topologies forming distinct binding surfaces. Formation of polyUb chains can hold the same Ub linkage (homotypic chains) or a combination of different K linkages (heterotypic chains). Heterotypic chains provide another layer of complexity as they can be mixed with different Ub linkages and branched when Ub is modified at two or more K residues [11] (**Fig. 1**). Further complexity is generated by the order in which branched chains are formed resulting in different conformations.

Despite recent efforts, the mechanism underlying the formation of complex Ub chains is not fully understood [13]. To simplify our understanding, generic functions have been attributed to some chain linkages. While the functions of homotypic chains are well-defined, the structures and functions of heterotypic or branched chains have emerged more recently. Even if heterotypic K6/K11, K6/K48, K27/K29 and K29/K33 linkages have been detected *in vitro* or in cells, only a few examples have been found and their functions need to be better studied to define their role. Evidence also exists for branched M1/K63 and K11/K63 chains, though their structures are still to be identified [14]. Recent studies have shown that branched chains represent a significant fraction of all polyUb chains in cells, with current estimates ranging from 5 to 20% [15]. Finally, Ub moieties can also be modified by other PTMs, including phosphorylation, acetylation, SUMOylation or NEDDylation. These PTMs alter the Ub chain architecture and allow new interactions that connect with a distinct set of functions [16,17]. Examples of generic functions established for some Ub chains are summary in the table 1.

## 3. Ubiquitin chains implicated in proteasomal degradation

One of the most predominant Ub linkages found inside the cells is K48, which is the major signal for proteasome-mediated degradation. Quantitative mass spectrometry (MS) analyses of cells treated with the proteasome inhibitor MG132 provided evidence to support this idea, since K48-polyUb linkages quickly accumulate in cells after treatment. Initial studies suggested that at least four K48-linked Ub molecules were required for an efficient binding to the 26S proteasome. However, latest data indicate that the number of K48-polyUb chains may be more important than chain length since two di-Ub K48-chains provide a more efficient binding to the proteasome than a single tetraubiquitin K48-chain [18]. The requirement of four or more Ub moieties integrated into a polyUb chain to target proteasomal degradation was challenged by several studies. Ciechanover et al. demonstrated that multiple monoubiquitylation can also mimic the patch required for an optimal recognition by dedicated proteasome Ub receptors [19]. Dimova et al. also showed that multiple monoubiquitylation of cyclin B1 is an efficient signal for proteasomal degradation [20]. Consequently, these and other findings proved that multiple Ub moieties can be recognized by the proteasome and imply the existence of monoubiquitylation vs. polyubiquitylation decision mechanisms. Based on these observations, it was suggested that the chain

length necessary for proteasomal degradation is mainly determined by the size of the substrate: substrates smaller than 150 residues are degraded via multiple monoubiquitylation, whereas longer substrates need longer chains. Dimova and co-workers proved that by limiting the number of ubiquitylatable K residues, the degradation signal can change from multiple monoubiquitylations to polyubiquitylation [20]. These results suggest that masking K residues by protein–protein interactions or PTMs can affect the ubiquitylation pattern.

Since K48-linked chains became a canonical signal for proteasomal proteolysis, an interesting hypothesis proposed that intrinsic proteasome subunits with UBDs, such as Rpn10 and Rpn13, selectively bind with K48-polyubiquitin chains. Proteasome associated subunits with UBDs like hHR23A and hHR23B would also bind with K48-polyubiquitin conjugates to stimulate their binding to the 26S [21]. Interestingly, when isolated, these UBDs bind with K48- and K63-linked chains with similar affinity suggesting that other domains or cofactors could contribute to provide K48 Ub chain selectivity. Tsuchiya et al. described how the cell division cycle protein 48 (Cdc48) and its cofactor Npl4 direct K48 ubiquitylated proteins to the proteasome upstream Rad23/Dsk2 and proposed the Cdc48-Rad23/Dsk2 axis as a major route to drive proteasome-mediated proteolysis [22].

DUBs play a crucial role in the regulation of K48 Ub chain-mediated functions. Several members of the OTU family showed remarkable specificity for K48-linked chains including OTUB1, that limits its action towards this chain type even in the context of mixed chain linkages. K48 chains can also be regulated by DUBs associated with the 19S regulatory particle of the proteasome. This is the case for Ubp6/USP14 and Rpn11/PSMD14 (in yeast and human, respectively).

### **3.1 Heterotypic chains in proteasomal degradation**

Different evidences suggest that other Ub linkages are involved in proteasomal degradation as various Ub chain types are accumulated upon proteasome inhibition [3,23]. Among them, K11-linked Ub chains, which are the third most abundant linkages in cells, play a significative role during mitosis. However, in 2015, Grice et al. suggested that homotypic K11 Ub signals are not sufficient to bind the proteasome and drive proteasomal degradation [24]. Heterotypic K11/K48 chains are involved in the degradation of multiple cellular factors, but in the absence of tools and technology to monitor them, their characterization and the assessment of their role in the regulation of different functions remains problematic. By engineering a bi-specific antibody to detect K11/K48 Ub chains, Yau et al. discovered that the multi-subunit RING E3 ligase anaphase-promoting complex (AKA APC/C or cyclosome) assembles K11/K48 heterotypic chains on cell-cycle substrates [25]. They showed that K11/K48 chains were enriched upon proteotoxic stress generated by proteasome inhibition or HSP70/90 blockage. These findings underlined that K11/K48 Ub chains are involved in the control of cell-cycle and protein quality, as they provided a rapid and specific degradation of mitosis regulators like cyclin B and misfolded proteins [13]. The formation of K11/K48 branched chains, during mitosis, is mediated by the APC/C with the cooperation of two E2 conjugating enzymes, UBE2C and UBE2S. First, UBE2C attaches short chains containing mainly mixed K11/K48 linkages to substrates of the APC/C. Then, the K11-specific E2, UBE2S, adds multiple K11 linkages to these short chains, resulting in branched K11/K48. The proteotoxic stress response also involves E3 ligases, UBR4 and UBR5 to generate K11/K48 chains on misfolded and aggregation-prone proteins such as the Huntington's disease-associated HTT variant [25]. These modified proteins bind the p97/VCP complex and the proteasomal adaptor hHR23A. K11/K48 polyUb chains adopt conformations that can bind with the proteasome 19S subunit, likely via interaction with the I44 Ub-binding surface, or alternatively by the presence of K48 linkages permitting its association with 26S Ub receptors. It has been shown recently that the Rpn1 proteasomal subunit contains a high affinity towards K11/K48 chains [26]. In 2018, Samant et al. discovered that K48/K11 Ub chains are needed for cytoplasmic degradation in contrast to the K11-nuclear independent degradation of substrates, which only involves K48 Ub chains, also making a distinction in the regulatory elements associated in each proteolytic pathway [27].

The proteasome's capacity to differently degrade proteins modified with homotypic K11 and heterotypic K11/K48 chains has been associated with the capacity of proteasome-associated DUBs to disassemble these chains. Both homotypic K11 and heterotypic K11/K48-polyUb conjugates can be disassembled, but only K11/K48-polyUb conjugates can efficiently drive protein degradation [24]. Thus, the binding of ubiquitylated substrates to 19S receptors is not required to activate the proteasome-associated DUBs for the disassembly of homotypic K11 Ub chains. Deubiquitylation of APC/C substrates is mediated by the K11-linkage-specific DUB Cezanne (OTUD7B), a member of OTU family [28]. Cezanne depletion accelerates APC/C target degradation and provokes errors in mitotic progression. Cezanne also regulates ubiquitylation and stability of the hypoxia inducible factor HIF-1 $\alpha$  and HIF-2 $\alpha$  [29].

Whether the other Ub chain types can act as signal for proteasomal degradation is still unclear. Whereas K6, K27, K29, and K33 Ub linkages are all detected at low levels in cells, they all increase after treatment with proteasome inhibitors, though in different proportions. This means that their accumulation could be an indirect consequence of proteasome inhibition or that they could be involved in proteasomal degradation.

#### **4. Ubiquitin chains implicated in proteasome-independent functions**

Early structural studies demonstrated a drastic conformational distinction between Ub chains driving proteasomal degradation from those that do not. Ub chains recognized by the proteasome mainly adopt a packed "closed" conformation wherein the two neighboring Ub subunits form extensive interactions with each other. In contrast, non-proteolytic Ub chains form an extended configuration with no direct contact between neighboring Ub moieties. The many exceptions to this dogmatic theory argue in favor of other features and regulatory mechanisms determining the role of distinct Ub chains. Non-proteolytic chains are very diverse, and their functions are far from being fully understood. However, their role in the regulation of intracellular signaling cascades is without any doubt one of the most important. Cell signaling allows cells to perceive and respond to the extracellular environment, essential to their development, growth, and immune response. Ubiquitylation has emerged as a key mechanism that regulates signal transduction pathways in most biological processes. For instance, ubiquitylation regulates multiple receptor-mediated signaling cascades implicated in differentiation, cell cycle, innate and adaptive immune responses, among others. The first Ub chain implicated in cell signaling was the K63, reported to regulate the activation of protein kinases and protein trafficking for cell survival and proliferation. Defective formation of K63 chains is associated with the development of various disorders including inflammation, cancer, neurodegeneration and cardiac hypertrophy [30].

One of the best-known examples of signaling pathways regulated by K63 chains is the NF- $\kappa$ B pathway, an important signaling cascade involved in inflammation and cancer development. NF- $\kappa$ B is also regulated by M1 chains that will be discussed below. The following sections show some examples of regulation of crucial functions and cellular pathways by diverse Ub chains implicating multiple Ub E3s and DUBs in a dynamic way.

##### **4.1 Ubiquitin chains in the NF- $\kappa$ B pathway**

The nuclear factor  $\kappa$ B (NF- $\kappa$ B) was initially described in B cells as a transcription factor that binds the enhancer of the kappa light chain gene. NF- $\kappa$ B is a family of inducible heterodimeric transcription factors found at the center of cell signaling pathways, having a role in immune response, cell growth and apoptosis [31]. NF- $\kappa$ B plays an important role in inflammation since it is activated by cytokines such as interleukin-1 (IL-1) or the tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ). Under unstimulated conditions NF- $\kappa$ B is retained in the cytosol by natural inhibitors (see text below) and its nuclear translocation is

conditioned to the degradation of these molecules (see text below). The NF- $\kappa$ B pathway involves a signaling cascade starting with the receptor stimulation until the activation of transcription of inflammatory genes [32]. Two pathways lead to NF- $\kappa$ B activation, known as the canonical and non-canonical pathways (Fig. 2) [33,34]. The canonical one converges into phosphorylation of the inhibitor I $\kappa$ B by a multi-subunit I $\kappa$ B kinase (IKK) complex. The IKK complex composed by three subunits: IKK1 (AKA, IKK $\alpha$ ), IKK2 (AKA, IKK $\beta$ ) and a regulatory subunit NF- $\kappa$ B essential modulator (NEMO) (AKA, IKK $\gamma$ ) fundamental for NF- $\kappa$ B canonical activation. Phosphorylation on the N-terminal of I $\kappa$ B $\alpha$  is followed by ubiquitylation leading to its proteasomal degradation. A tight control of both pathways is necessary to maintain NF- $\kappa$ B homeostasis. Ubiquitylation and other PTMs participate in this fine regulation at different levels. We dissected the canonical pathway into 3 levels to illustrate the role of distinct Ub chains, E3s and DUBs: 1) Recruitment of adaptor proteins after receptor-ligand binding, 2) Recruitment of the main kinase complexes and 3) Release of NF- $\kappa$ B dimer.

**Recruitment of adaptor proteins after receptor-ligand binding.** In the prototype-signaling pathway induced by the tumor necrosis factor alpha (TNF- $\alpha$ ), the TNF receptor 1 (TNF-R1) trimerizes and triggers recruitment of different proteins, including many Ub E3s, comprising the TNF-R1-signaling complex. The adaptor protein TRADD bound to the TNF-R1 cytoplasmic domain enrolls the receptor-interacting serine/threonine-protein kinase 1 (RIPK1), the adaptor protein Sam68, the E3 ligase TNF receptor-associated factor 2 (TRAF2) and possibly also TRAF5 [33]. TRAF2 is a scaffold for two other Ub E3s, the cellular inhibitor of apoptosis protein-1 (c-IAP1) and c-IAP2, which are responsible of conjugation of K63-, K48- and K11-linked Ub chains to RIPK1 [35]. Polyubiquitylated RIPK1, particularly with K63 chains, recruits the linear Ub E3 complex (LUBAC) and the TAK1 kinase complex, which are important for the downstream signaling cascade. The stimulation of the IL-1R or toll-like receptors (TLRs) recruits a different set of adapter proteins that leads to the enrollment of TRAF6, an Ub E3 that catalyze the synthesis of K63-linked Ub chains with the help of the Ub E2 Ubc13-Uev1A. These K63 Ub chains are attached to the same TRAF6 (autoubiquitylation) that is necessary for the recruitment of the transforming growth factor- $\beta$ -activated kinase 1 (TAK1) kinase complex, which in turn phosphorylates and activates the IKK complex [36]. TRAF6 was shown to promote K63- and K27-linked polyubiquitination over TAK1 after IL-1 $\beta$  stimulation [37].

**Recruitment of the main kinases complexes.** Different stimuli as well as pattern recognition receptors (PRRs) can trigger receptor mediated-signaling transduction, which converges at TAK1 activation. TAK1 is a member of the MAPKKK kinases that exists as a heterotrimer in the complex with the accessory proteins TAK1 binding protein (TAB) 1, TAB2 or TAB3. The complex TAK1/TAB2/TAB3 is recruited to the site of ubiquitylated RIPK1 thanks to the ubiquitin associated (UBA) domain Np14 zinc finger (NZF), present in TAB2 and TAB3, that recognizes K63-linked chains [38]. Free K63-linked Ub chains synthesized by TRAF6 are also able to bind TAB2 [36]. The identification of the K377 site on RIPK1 was shown to be important for the recruitment of TAK1 and IKK complexes, even if other sites were later characterized [39].

On the other hand, the recruitment of the IKK complex is mediated through the ubiquitylated-RIPK1 recognition by NEMO. The N-terminal of NEMO is involved in the interaction with IKK $\alpha$  and IKK $\beta$ , while the C-terminal attaches Ub chains. The NEMO C-terminal harbor two different motifs which together form a UBD that specifically recognizes K63-linked Ub chains, the UBAN motif (AKA NOA) and the zinc finger (ZF) motif [40]. Recruited IKK complex is responsible of phosphorylation of I $\kappa$ B through the IKK $\beta$  subunit. There is evidence that also IKK $\beta$  undergoes ubiquitylation with K48- and K63-linked Ub chains. The role of ubiquitylation in the regulation of this kinase was shown by mutations at the K171 site, which leads to constitutive phosphorylation activity. This particular mutation was previously observed in Multiple Myeloma, Spleen Marginal Zone Lymphoma and Mantle Cell Lymphoma. *In vitro* experiments simulating the K171 mutation showed an increased K63-linked ubiquitylation of IKK $\beta$  on different sites and a persistent activation of STAT3 signaling [41]. Otherwise, IKK3 (AKA IKK $\epsilon$ ) is a non-canonical IKK family member that is activated by TLRs and intracellular

receptors able to recognize viral nucleic acids such as dsRNA. IKK3 plays a role in the phosphorylation and activation of other transcription factors as interferon response factors IRF3 and IRF7, but can also activate NF- $\kappa$ B. IKK3 and Tank-binding kinase 1 (TBK1) are involved in the activation of the NF- $\kappa$ B pathway through phosphorylation of I $\kappa$ B and p65 subunit [42].

LUBAC is currently the only E3 ligase complex that synthesizes linear chains. It is composed of 3 members: HOIL-1L (AKA RBCK1), HOIL-1L-interacting protein (HOIP) (AKA RNF31) and SHANK-associated RH domain interacting protein (SHARPIN). The assembled heterotrimer is required to generate M1 linear chains where HOIP is the catalytic subunit [43]. Once LUBAC is enrolled by ubiquitylated RIPK1, it attaches M1-linked chains to the IKK complex subunit NEMO and to the same RIPK1 leading for an efficient activation of the canonical IKK [44]. MS analyses of ubiquitylated NEMO and mutagenesis experiments showed that K285 and K309 are the acceptors of LUBAC-mediated ubiquitylation. LUBAC can also modify other substrates as TRADD and TNF-R1 and undergo autoubiquitylation. It can synthesize linear Ub conjugates with different E2s as UbcH5, E2-25, and UbcH7. Recruitment of LUBAC is key to stabilizing the TNF-R1-signaling complex since several components harbor UBDs. Indeed, linear Ub chains bind UBDs and retention of cIAP1, TRAF2, RIPK1, and TAK1 was shown to be dependent on LUBAC ligase activity, protecting the complex from falling apart [45]. There is also evidence that the UBAN motif of NEMO binds to M1-linked Ub chains with higher affinity than to K63 chains, and that this interaction is crucial for the TNF- $\alpha$ -induced activation of the IKK complex. Interestingly, hybrid chains were later observed in the pathway mediated by IL-1R stimulation. Emmerich and collaborators found that K63-ubiquitylated substrates, such as the IL-1R1 associated kinase (IRAK) 1, were also ubiquitylated by linear chains. The formation of K63-linked Ub chains was shown to be a prerequisite for the formation of M1-linked chains, with NEMO being able to capture these hybrid chains [46]. Regarding the identification of heterotypic chains, it has been shown that K48-K63 branching Ub chains are formed in substrates as TRAF6 or IRAK1 in cells stimulated with IL-1 $\beta$ . Ubiquitylated TRAF6 with K63-linked chains seems to be remodeled by the action of the E3 ligase HUWE1, which conjugates K48 Ub chains on the preformed chains. Interestingly, there is no interference in the recognition of these K48-K63 branched chains by TAB2; but otherwise, branched chains appear to be protected from cylindromatosis (CYLD)-mediated deubiquitylation, which could contribute to amplifying the NF $\kappa$ B activation [47]. Even if LUBAC has been extensively studied in the context of TNF signaling, it has also been recently associated with oncogenic signaling, antibacterial autophagy and protein quality control [13].

**Release of NF- $\kappa$ B dimer.** NF- $\kappa$ B dimers can be assembled with two of the five subunits: RelA (AKA p65), RelB, c-Rel, p50 (from precursor p105) and p52 (from p100). Under basal unstimulated conditions, the dimer is retained in the cytoplasm by action of its corresponding inhibitor (an I $\kappa$ B family member). Phosphorylation of I $\kappa$ B takes place once the IKK complex has been activated.

In addition to the p105 and p100 precursors that contain ankyrin repeats, three other proteins inhibit NF- $\kappa$ B activity: I $\kappa$ B $\alpha$ , I $\kappa$ B $\beta$  and I $\kappa$ B $\epsilon$ . Ankyrin repeats allow the interaction with the Rel domain of NF- $\kappa$ B. I $\kappa$ Bs share a conserved sequence DSGXXS motif that includes the two serine residues that are targeted by the IKK complex [33]. In the TNF-stimulated cells, phosphorylation at residues S32 and S36 on the N-terminal of I $\kappa$ B $\alpha$  is followed by K48-linked ubiquitylation that leads to its proteasomal degradation. The phosphorylation signal is recognized by a key E3 Ub ligase complex, the multisubunit Skp, Cullin, F-box, beta-transducin repeat containing protein (SCF <sup>$\beta$ -TRCP</sup>) E3 ligase, which polyubiquitinates I $\kappa$ B $\alpha$  mainly on K21 and K22, favors its degradation releasing the NF- $\kappa$ B dimer. Monoubiquitylated forms of I $\kappa$ B $\alpha$  can be found in basal state, in forms that are not degraded by the proteasome after TNF-stimulation, as usually occurs with polyubiquitylated I $\kappa$ B $\alpha$  [48]. The role of this pool of monoubiquitylated I $\kappa$ B $\alpha$  remains to be fully investigated. Furthermore, I $\kappa$ B $\alpha$  is subjected to multiple PTMs beyond phosphorylation and ubiquitylation since SUMOylation, acetylation, glutathionylation and hydroxylation have also been reported [49].



**Non-canonical pathway.** A non-canonical or alternative pathway has been described in the activation of specific NF- $\kappa$ B family members. The p50/RelA dimer is predominantly translocated to the nucleus in the canonical pathway, while the p52/RelB dimer is particularly activated in the non-canonical pathway. This alternative mechanism relies on the inducible processing of the p100 precursor instead of degradation of I $\kappa$ B $\alpha$ . P100 takes on the inhibiting role of I $\kappa$ B impeding the RelB nuclear translocation, so the p100 processing to p52 allows dimer release and translocation for target genes activation. Resembling to the phosphorylation sites of I $\kappa$ B $\alpha$ , p100 contains two serine residues S866 and S870, which are phosphorylated by the NF- $\kappa$ B-inducing kinase (NIK, AKA MAP3K14). Phosphorylated sites of p100 generate a binding site for  $\beta$ TrCP subunit of SCF $^{\beta$ -TrCP E3 ligase that induces p100 ubiquitylation. The p100 modified-site is a Lys residue, K856, which is analogous to the I- $\kappa$ B $\alpha$  K22 residue adjacent to the phosphorylation site S32. Phosphorylation-dependent ubiquitylation promotes p100 processing releasing the p52/RelB dimer to trigger the transcription of a different set of target genes compared than those of the canonical pathway. The stimuli responsible of non-canonical pathway activation are different from those to the canonical pathway. Examples of non-canonical receptors are such as CD40 ligand or lymphotoxin  $\beta$ , that lead to the IKK $\alpha$  activation, phosphorylating p100 at multiple sites to drive p52 processing [50]. IKK $\alpha$  activation in turn depends on kinase activity from the newly synthesized NIK. Under basal conditions, NIK is constantly degraded by the proteasome due to K48 ubiquitylation mediated by the complex TRAF3-TRAF2-cIAP1/cIAP2 [51]. When CD40, which is a TNFR member, is stimulated by its ligand, TRAF2 ubiquitylates cIAPs with K63 chains promoting their ligase activity and targeting TRAF3. Conjugated K48 chains drive TRAF3 to proteasomal degradation and prevent its association with NIK, allowing NIK accumulation and therefore IKK $\alpha$  phosphorylation [34]. The processing of p105 precursor to p50 can also be dependent on ubiquitylation. Recently, the KIP1 ubiquitylation-promoting complex 1 (KPC1 AKA RNF123) was identified to form a heterodimer with KPC2 (AKA UBAC1), acting together as a Ub E3 ligase to promote a limited proteasome-mediated proteolysis of p105 [52].

**Role of DUBs in the NF- $\kappa$ B pathway.** A strict control of all of these Ub modifications is key to avoid cellular alterations that could result in inflammatory disorders and other NF- $\kappa$ B related diseases. NF- $\kappa$ B is deregulated in numerous tumors. Negative regulation of the NF- $\kappa$ B activation is prompted by the editing deubiquitylating enzymes CYLD and USP19, which are Ub specific protease (USP) family members. Also contributing to the regulation of NF- $\kappa$ B, OTU (ovarian tumor domain) deubiquitinase family members A20 (AKA TNF- $\alpha$ -induced protein 3 (TNFAIP3)) and OTULIN (AKA Gumby or Fam105b), a DUB with linear linkage specificity. The K63- and M1-linked Ub chains can be removed from different ubiquitylated substrates by CYLD, a DUB enzyme that was first identified as a NEMO-interacting protein [53]. Deficiency of this DUB leads to a constitutive NF- $\kappa$ B activation causing proinflammatory gene expression [54]. CYLD is constitutively expressed, but after TNF stimulation it is recruited to LUBAC through the adapter protein spermatogenesis-associated protein 2 (SPATA2), which in turn binds to the subunit HOIP of LUBAC [54,55]. In the pathway triggered by NOD2 that is involved in inflammation by infectious diseases, the DUB activity of CYLD is necessary to regulate K63- and M1-linked ubiquitylation on RIPK2 [56]. The functional role of CYLD in different pathological processes was well documented prior to understanding its role as a DUB enzyme. Several skin disorders related to CYLD gene mutations were described such as multiple familial cylindromatosis, trichoepithelioma and the Brooke-Spiegler syndrome. In cancers such as multiple myeloma, mutations on CYLD and other genes involved in the NF- $\kappa$ B pathway, as c-IAP1/2, were also reported [57]. CYLD activity was shown to be regulated by IKK3 kinase, which targets CYLD by phosphorylation at S418 decreasing its deubiquitylating activity [58]. Besides CYLD, OTULIN is a DUB that specifically recognizes and deconjugates M1-linked Ub chains; it is currently the only DUB able to cleave exclusively linear chains [59]. OTULIN interacts directly with the subunit HOIP of LUBAC and regulates LUBAC-mediated processes. The OTULIN function is important to regulate angiogenesis, innate immunity and autoimmunity [60].

The deubiquitylating/E3 Ub ligase enzyme A20, plays an important role in the regulation of the NF- $\kappa$ B pathway [61]. A20 was first described as an inhibitor of TNF-induced apoptosis, but its role as a negative feedback regulator of NF- $\kappa$ B pathway was later well documented. The A20 gene is one of the several target genes that respond to NF- $\kappa$ B. The N-term of A20 harbour an OTU domain with DUB activity that hydrolyzes *in vitro* K48-, K11- and K63-linked Ub chains. The C-term contains a series of seven ZF motifs, among them, the ZF4 is critical to confer its E3 ligase activity. The NF- $\kappa$ B inhibitory effect of A20 was mainly attributed to its ability to disassemble K63-linked chains from different substrates, including RIPK1, TRAF6 and NEMO [54]. For instance, A20 was shown to remodel Ub chains on RIPK1 by removing K63-linked chains and conjugating K48-linked chains favoring its proteasomal degradation [62]. Although there is evidence that A20 as DUB or E3 ligase enzyme contributes to the regulation of the NF- $\kappa$ B pathway, later studies suggest that also its non-enzymatic features, like the ZF7 motif, play a significant role in the regulation of this pathway as it regulates cell death [63].

The DUB USP19 appears to be also a negative regulator of TNF- $\alpha$ - and IL-1 $\beta$ -triggered NF- $\kappa$ B activation by hydrolyzing K63- but also K27-linked Ub chains from TAK1. TAK1 was recently found to be a substrate of USP19. TRAF6 mediates K27-linked polyubiquitylation of TAK1, which is necessary for the recruitment of TAB2 and TAB3 to TAK1 to generate the TAK1-TABs complex. This deubiquitylation impairs TAK1 phosphorylation and therefore disrupts the TAK1-TAB complex, thus terminating the TAK1-mediated induction of NF- $\kappa$ B downstream genes [37]. The K27 ubiquitylation was first linked to NEMO regulation by the tripartite motif (TRIM) Ub ligase TRIM23, a step required for NF- $\kappa$ B and IRF3 induction [64]. Since then, several TRIM ligases have been shown to form K27 Ub chains that regulate intracellular signaling, transcription, autophagy, and carcinogenesis, among other important processes [65]. TRIM26 and TRIM21 regulate immune responses using distinct mechanisms synthesizing K27 Ub linkages. For instance, TRIM26 can be auto-ubiquitylated with K27 Ub chains to favour its interaction with NEMO while inducing the expression of autoinflammatory cytokines and type I IFNs [66]. The regulation of the NF- $\kappa$ B pathway is an example of complex Ub dynamics that will be regulated in a spatio-temporal manner depending on the physiologic or pathologic situation of the cell.

#### 4.2 Ubiquitin chains in the regulation of Akt pathway

The activity of many kinases is controlled by K63 chains, as we showed with RIPK1 that is implicated in the regulation of cell death besides inflammation [67]. Another example is the serine/threonine kinase Akt (AKA Protein kinase B) that plays key roles in multiple cellular processes such as glucose metabolism, apoptosis, cell proliferation, transcription, and cell migration. Although it is well known that PIP3 produced by PI3K is essential for the membrane recruitment and activation of Akt, K63-linked polyubiquitylation contributes to its hyperactivation and therefore to the regulation of tumorigenesis [68]. TRAF6 is an E3 ligase that ubiquitinates Akt upon stimulation with insulin-like growth factor 1 (IGF 1). CYLD acts on Akt permitting to keep Akt-stimulating activity in balance. By studying the molecular mechanisms of lung injury, Lim *et al.* discovered that loss of CYLD led to the development of lung fibrosis in mice infected with *Streptococcus pneumoniae*. They found that CYLD deubiquitinates and inactivates K63-polyubiquitylated Akt, leading to inhibition of transforming growth factor- $\beta$  (TGF- $\beta$ ) signaling by decreasing the stability of the TGF- $\beta$  effector protein Smad3 [69]. In line with this study, Yang *et al.* showed that CYLD and Akt are physically associated. They found that, in the absence of growth factors, CYLD keeps Akt hypo-ubiquitylated through direct association and deubiquitylation, whereas growth factor stimulation allows E3 ligases such as TRAF6 to replace CYLD from Akt, thereby promoting its ubiquitylation, membrane translocation, phosphorylation and activation [70]. The E3 ligase Skp2-Skp-cullin-F-box-containing (SCF) complex, also ubiquitylates Akt in response to the activation of the epidermal growth factor family of receptors (ErbB) [70]. These findings show that Akt

K63-linked ubiquitylation and activation can be induced by diverse signaling cascades using distinct E3 ligases.

#### 4.3 Atypical ubiquitin chains in signaling and immune related functions

Beyond K63 chains, there are atypical Ub linkages also having non-proteolytic roles in cells mediating signaling, intracellular trafficking, and autophagy among other functions [71,72]. K33 Ub chains regulate T-cell antigen receptor (TCR) and AMP-activated protein kinase (AMPK)-related kinases [4]. E3 Ub ligases such as Cbl-b and Itch synthesize K33 Ub chains on the T cell receptor-zeta (TCR-zeta). Deficient formation of K33 Ub chains increases T cell activation and spontaneous autoimmunity [71], demonstrating their role in regulation of cell surface receptor-mediated signal transduction. K33 Ub signals are also implicated in immune responses upon DNA or RNA virus infection. Viral infection leads to TBK1 K33 poly-ubiquitylation, promoting IRF3 activation and type I Interferon (IFN) signaling. This antiviral response can be reversed by the DUB USP38 that removes K33 Ub chains on K670 of TBK1 and favours the formation of K48 Ub chains at the same position to drive its proteasomal degradation. Lin *et al.* found that knockout of USP38 increased K33-linked ubiquitylation and decreased K48 Ub-mediated degradation of TBK1 kinase, thus enhancing type I IFN signaling. Conversely, overexpression of USP38 decreased K33-linked ubiquitylation of TBK1 and increased its K48-linked ubiquitylation [73]. Another example of the role of K33 chains is led by the Ub E3 RNF2, which generates K33 Ub chains on the STAT1 DNA binding domain allowing its dissociation from the promotor of several interferon stimulated genes (ISG) underlining the role of these chains in the regulation of IFN pathway [74].

K29-linked polyUb chains have been associated with the regulation of inflammation, antiviral immune response, and the regulation of the Wnt/ $\beta$ -catenin pathway at multiple levels. The Wnt/ $\beta$ -catenin pathway is essential to the regulation of events such as cell proliferation, organized migration, self-renewal, and tissue polarity. Various Ub E2s in coordination with E3s attach distinct Ub linkages to different K residues on  $\beta$ -catenin, causing context-specific functional consequences. For example, both the E2 Rad6B and the E3 Ub ligase EDD (identified by differential display) interact with  $\beta$ -catenin and enhance its ubiquitylation, stability and activity. While EDD catalyzes the formation of K29-linked chains on  $\beta$ -catenin, Rad6B adds K63-linked polyUb chains on the same target protein. Under basal conditions, a destruction complex comprising Axin and adenomatous polyposis coli (APC) promotes  $\beta$ -catenin degradation. Wnt ligands lead to the inactivation of this complex, allowing  $\beta$ -catenin to accumulate, and thus activate a transcriptional program [75].

Smad ubiquitylation regulatory factor 1 (Smurf1) commonly serves to regulate Ub-dependent protein degradation in several signaling pathways. It also drives non-proteolytic ubiquitylation through the formation of K29 Ub chains on Axin [76]. Axin ubiquitylation on K789 and K821 negatively regulates Wnt/ $\beta$ -catenin signaling by disrupting Axin interaction with the Wnt co-receptors LRP5/6 that together with Frizzled receptor initiate this signaling cascade.

K29 ubiquitylation has also been linked to the modification and activation of ASK1 (apoptosis signal-regulating kinase 1), promoting type I interferon production upon viral infection. The F-box only protein Fbxo21, a functionally unknown component of SCF (Skp1-Cul1-F-box protein) complex, mediates ASK1 ubiquitylation. Fbxo21 deficiency impairs the production of proinflammatory cytokines resulting in reduced antiviral response and enhanced virus replication and infection. The Ub Specific Peptidase 9 X-Linked (USP9X) is a C19-peptidase family protein with known DUB activity, which can cleave K48, K63 and K29 linkages. USP9X binds and stabilize ASK1 by removal of Ub chains and prevention of its degradation. Interestingly, ASK1 contains six amino acids identical to the Ub C-terminus (LRLRGG), of which the GG sequence was required for the USP9X-ASK1 interaction [77].

TRIM21 assembles K27 polyUb chains on mitochondrial antiviral signaling protein (MAVS), promoting the recruitment of TBK1 to mitochondrial adaptor molecules, or mitochondrial antiviral signaling proteins leading to the upregulation of innate immunity [78]. MAVS is also targeted by RNF34 Ub ligase, that mediates K27 and K29 Ub chains after viral infection, enhancing its autophagic degradation and negatively regulating antiviral immunity [79]. Another example of K27 implication was observed upon microbial DNA invasion, the endoplasmic reticulum (ER) protein AMFR is recruited to interact with stimulator of interferon genes (STING) in an insulin-induced gene 1 (INSIG1)-dependent manner. AMFR and INSIG1 form an E3 Ub ligase complex to catalyse K27 Ub-linked chains on STING. This modification served as a scaffold to recruit TANK-binding kinase 1 (TBK1) triggering the activation of the interferon regulatory factor-3 (IRF3) and/or NF- $\kappa$ B signaling pathway inducing the expression of type I IFNs and proinflammatory cytokines [4]. The DUB USP13 has been reported to remove the K27-linked Ub chains on STING and thereby inhibiting the recruitment of TBK1 to STING, negatively regulating IFN production during DNA virus infection [80]. Other ligases also synthesize K27 Ub chains to regulate immune responses. The TIR domain-containing adaptor inducing interferon- $\beta$  (TRIF), an essential adaptor protein required for innate immune responses, was shown to be regulated by K27 ubiquitylation. The RING E3 Ub ligase complex Cullin-3-Rbx1-KCTD10 assembles K27 polyUb chains on TRIF, and the reaction is reversed by USP19 [81]. Tumorigenesis can be also regulated by K27 Ub chains. For example, BRFA (key player in tumorigenesis and MEK/ERK pathway) is modified by K27 Ub chains by the ITCH Ub E3 ligase, regulating proliferation and invasion in melanoma cells [82]. Further investigations are required to evaluate full potential of K27 Ub chains to regulate other functions.

## 5. Ubiquitin chains dynamics in autophagy regulation

The Autophagy Lysosome System (ALS) is one of the main intracellular degradation systems, together with the UPS. Autophagy digests long-lived, protein aggregates, stress RNA granules, and abnormal cytoplasmic organelles, including among others, mitochondria. Chaperone-mediated autophagy, microautophagy and macroautophagy use distinct mechanisms to drive proteolysis. Chaperone-mediated autophagy uses HSC70 to sequester proteins containing a KFERQ motif into lysosomes. Microautophagy is a direct lysosomal uptake of substrates whereas in macroautophagy (here referred as autophagy), cytosolic cargos are sequestered by a double membrane vesicle including a fraction of the cytoplasm using a complex multistep process. Recent general autophagy reviews give more details on the whole process [83].

*Activation of autophagy by non-proteolytic ubiquitin chains.* Several Ub chains and Ub-like molecules play an important role in the regulation of autophagy and in the crosstalk between the UPS and ALS. Nevertheless, their role in distinct mechanisms including the regulation of signaling cascades and targeting cargoes to proteolysis has not been fully elucidated. It has been suggested that non-degradative Ub chains stimulate autophagy induction, whereas degradative Ub chains are needed to restore the basal autophagy flux once the stress situation is resolved [84]. UbE3s and DUBs determine K63 and K48 Ub chains formed on distinct regulatory complexes and define their role in a dynamic reversible manner (**Fig. 3**) [85]. The initiation of autophagy and autophagosome formation is regulated by ULK1 and PI3K-III kinases whose activity is regulated by mTORC1 and AMPK dependent phosphorylation [86]. Ubiquitylation mediated by the concerted action of various E3 ligases also contributes to regulating autophagy initiation and autophagosome formation [85]. For instance, the Ub E3 TRAF6 can regulate ULK1 and PI3K-III kinases by the formation of K63 chains. Upon starvation, TRAF6 uses unphosphorylated AMBRA1 (Autophagy and BECLIN 1 Regulator 1) to bind ULK1 which is stabilized and activated by K63 chains favoring autophagy (**Fig. 3A**). K63-activated ULK1 phosphorylates AMBRA1 to set a regulatory autophagy loop. Interestingly mTORC1 also phosphorylates AMBRA1 on distinct sites and controls ULK1 ubiquitylation self-association and function, which has a negative effect on autophagy [87].

The catalytic core of the PI3K–III complex is regulated by TRAF6-mediated K63 ubiquitylation of BECLIN 1 which abolishes its binding to BCL2 and induces autophagy (**Fig. 3A**). A20, a DUB that hydrolyzes the K63 chain of BECLIN 1, contributes to restore the binding to BCL2 [88,89]. BECLIN 1 phosphorylation by DAPK negatively regulates its association with BCL2, underlining an important interplay between these two PTMs to regulate autophagy [90]. Other Ub E3s such as Rbx1/Cul4-ligase can also form a complex with AMBRA1 to target BECLIN 1 under starvation. ULK1 can also phosphorylate BECLIN 1, increasing the PI3K–III complex activity [91]. Other members of the Ub family such as SUMO contribute to regulating the PI3K-III complex since VPS34, a component of the catalytic core of this complex, is targeted by the SUMO ligase KAP1. SUMOylated VPS34 increases its interactions within the PI3K–III complex and promotes phagophore formation [92].

***Autophagy termination is regulated by degradative ubiquitylation.*** Under normal growth conditions or when a specific stress situation is finished, autophagy is stopped to avoid the unnecessary destruction of cellular components that could compromise cell viability. Phosphorylation and ubiquitylation also contribute to switch off the activity of the ULK1 and PI3K–III complexes [84]. In particular, K11 and K48 Ub chains have been proposed to promote the proteasomal degradation of autophagy regulators to restore a new equilibrium compatible with the physiological basal autophagy flux. NEDD4, a Ub E3 of the HECT family, uses K11 Ub chains to target BECLIN1 for degradation, limiting autophagy [93] (**Fig. 3B**). Interestingly, an E3 ligase homologous to NEDD4, called NEDD4L, drives the proteasome degradation of ULK1 via K27 and K29 chains during autophagy [94] (**Fig. 3B**). The CULLIN3–KHLH20 ligase can also drive the K48 chains-driven degradation of ULK1, BECLIN 1, and VPS34 [95] highlighting the participation of several ligases in the control of both autophagy induction and termination. During starvation, AMBRA1 is another important factor targeted for proteasome-mediated proteolysis by various Ub ligases including CULLIN4–DDB1 [96] or RNF2 [97].

Protein de-ubiquitylation importantly contributes to regulate the degradation of autophagy regulators, like DUBs USP13 and USP10 that protect BECLIN 1 from ubiquitylation. Their chemical inhibition by Spautin-1 treatment drives a Ub-proteasome mediated degradation of BECLIN 1. The chemical separation of BECLIN 1 from its chaperone HSP90 by geldanamycin also results in the K48 ubiquitylation of BECLIN 1 [98] (**Fig. 3B**).

It is clear that the autophagy system uses multiple E3 ligases to regulate different steps inside this pathway like the activation, progression and the termination of the flux. We can speculate that a DUB will be counteracting their role for each ubiquitylation reaction. However, many of these enzymes are still uncharacterized.

## 5.1 Role of Ub in selective autophagy pathways

The concept of selective autophagy derives from the fact that distinct types of aggregated proteins and damaged organelles are targeted for degradation under distinct physiologic and pathologic situations, implying the existence of specialized pathways. However, some of the enzymes, adaptors and cofactors are shared under certain circumstances, complicating our understanding of the selectivity of proteolytic events. This includes the autophagy receptors that bring cargoes into autophagosomes for clearance. More than 30 autophagy receptors, also called Sequestosome-1-like receptors (SLRs) after the first described p62/SQSTM1 (Sequestosome 1) [99]. p62 carries a UBA, a UBD that binds ubiquitylated cargoes. The UBA domain of p62 can homodimerize, modulating its interaction with monoubiquitin. p62 contains LC3 Interacting Regions (LIR) that interact with ATG8 proteins that will be integrated into autophagosomes. Other SLRs with similar architecture include Next to BRCA1 gene 1 protein (NBR1), Optineurin (OPTN) or the nuclear dot protein 52 kDa (NDP52). The diversity of receptors underlines the complex regulation of selective autophagy mechanisms. Some of them show functional redundancy for cargo recognition and cooperate with multiple cofactors [100].

Selective autophagy events can be classified as Ub-dependent or -independent. The receptors involved in Ub-dependent autophagy bind Ub chains through a UBD which is absent in receptors implicated in Ub-independent autophagy. However, some cargos can be transported in a Ub-dependent and independent manner. It is most likely the reason why selective autophagy events are named according to the cargo they transport [101]. K63 Ub chains show preferential binding for the autophagy receptors [102], while K48, K27 and K11 Ub chains target for proteasomal degradation (**Fig. 3**). Nevertheless, in situations of defective autophagy, all Ub chains have been found enriched [103], most likely due to the fact that these chain types are also involved in the regulation of other cellular functions. The accumulation of Ub aggregates might also contribute to increasing the affinity of autophagy receptors for the cargoes. The role of Ub chains in the regulation of autophagy is still under investigation. Regardless of their Ub dependency, all autophagy receptors contain LC3-interacting regions (LIRs) that bind other members of the Ub family in mammals (LC3/GABARAP) or yeast (ATG8) (**Fig. 3**). These ATG8 molecules contribute to expanding the autophagosomes and providing specificity to selective autophagy events.

Around 20 types of selective autophagy have been reported, half of them driven by Ub [101]. Selective autophagy events include aggrephagy and mitophagy (that will be further developed in this review) but also xenophagy, lysophagy and ERphagy, to name a few. Characterized more recently, proteaphagy is quite intriguing since together with aggrephagy, it importantly contributes to the crosstalk between the UPS and ALS and is activated under similar stress stimuli. The nature of the aggrephagy-proteaphagy interconnection has not been totally explored (**Fig. 3**).

**Aggrephagy.** Heat-shock stress or proteotoxic insults generate protein aggregates. Part of these aggregated proteins are degraded by the proteasome via K48 chains while K63 chains activate the aggrephagy machinery to recruit the autophagy receptors p62, OPTN and NBR1 (**Fig. 3C**) [104]. These autophagy receptors and K63 Ub chains are essential to present protein aggregates for autophagosome inclusion. The aggregation process itself involves the participation of other UbL molecules such as SUMO-1 and FAT10 [105,106]. BAG3 also interacts with K48 chains and p62, favoring ALS degradation of ubiquitylated proteins. Interestingly, another member of the same family named BAG1 favors the presentation of misfolded proteins to the proteasome underlining the participation of BAG proteins in the balance between both proteolytic pathways [107] (**Fig. 3C**). Several DUBs are other important actors of the degradation of protein aggregates, including the RPN11 proteasome subunit that recruits HDAC6 to promote autophagy degradation [108,109]. The role of DUBs in the autophagy-dependent degradation of protein aggregates is still under investigation, but examples, like USP9X that acts on mono-ubiquitylated synuclein to favor aggrephagy rather than proteasomal degradation, illustrate the importance of these enzymes in selecting the proteolytic pathway to be used to eliminate these cargoes [110].

**Proteaphagy.** Upon nutrient starvation, autophagy works as a recycling machine to recover the nutrient pool from dysfunctional cellular components [111]. Under these circumstances, proteaphagy represents an efficient strategy to face nutrient limitation. Marshall *et al.* proved that proteaphagy occurs in both *Arabidopsis thaliana* and *Saccharomyces cerevisiae* under nitrogen starvation [112]. Nuclear proteasomes are also regulated by proteaphagy but the core particle (CP) and 19S subunit/regulatory particle (RP) have to be dissociated prior to their nuclear export in yeast. The autophagy of the CP depends on the DUB Ubp3, while RP export for cytosolic degradation relies on ATG7 and ATG17 factors [113].

In mammalian cells, proteaphagy is also activated in response to amino acid starvation. Proteaphagy induced by amino acid starvation involves the polyubiquitylation of proteasome subunits RPN1, RPN2, RPN10 and RPN13. The autophagy receptor p62 is required to drive the degradation of ubiquitylated proteasomes [114]. The participation of distinct autophagy receptors in distinct biological models and stress conditions suggest that the molecules involved can be distinct [115].

Proteaphagy can also be induced when the proteasome activity is impaired by point mutations or chemical inhibition by proteasome inhibitors [115]. After proteasome inhibition, ubiquitylated RP subunits are recognized by the autophagy receptor p62 [112,116,117] (**Fig. 3D**). In *Arabidopsis*, RPN10 acts as an autophagy receptor to promote the proteaphagy of inactive 26S proteasomes but does not participate in starvation-induced proteaphagy. In this process, USP2 plays a role as a regulator of RPN10 stability [112]. In yeast, Cue5 acts as an autophagy receptor to mediate proteaphagy after chemical inhibition and genetic mutation of 26S proteasomes [118]. The nature of the Ub chains implicated in proteaphagy must be better defined, but while K48 Ub chains are decreased, K63 Ub chains are enriched after proteasome inhibition in Bortezomib resistant cells [116,119]. The Ub ligases and DUBs implicated in proteaphagy remains to be identified.

**Mitophagy.** Mitochondrial degradation is a good example of collaboration between the autophagy and the proteasome, where Ub plays an important role (**Fig. 3E**). Mitophagy can also be achieved in a Ub-independent manner and involve the autophagy receptors FUNDC1, BNIP3, and NIX [120].

Ub-dependent mitophagy involves the E3 Ub ligase PARKIN, which is activated by PINK1-mediated phosphorylation, increasing the ubiquitylation of mitochondrial outer membrane (MOM) proteins. PINK1 also phosphorylates Ub chains on the mitochondrial surface, increasing mitophagy due to the higher affinity of PARKIN for phospho-Ub chains [121–123]. PARKIN ubiquitylation favors the binding of several autophagy receptors including p62, OPTN, NPD2, TAX1BP1, and NBR1. PARKIN interacts with AMBRA1 that could potentially recruit Ub E3s such as TRAF6 and CUL4–DDB1 to the mitochondria. It also regulates the interaction of BECLIN1 with BCL2 by controlling the monoubiquitylation levels of BCL2 and blocking BECLIN1 function in autophagy [124]. Therefore, PARKIN plays multiple roles by promoting autophagy through the formation of K63 and K48 chains on the mitochondria but also inhibits autophagy when it monoubiquitylates BCL2. The DUB USP30 controls PARKIN activity by removing preferentially K6 and K11 polyUb chains from the mitochondrial surface [125]. K6 Ub linkages have also been detected in MOM proteins. Using Ub linkage-specific affirmers for K6, Michel *et al.*, identified HUWE1 E3 as the major source of K6 Ub chain formation inside the cells. HUWE1 modifies mitofusin-2 (Mfn2), a known substrate required for mitochondrial membrane remodelling during mitophagy [126]. On the other side, a reduced membrane potential leads to the accumulation of the mitochondrial kinase PINK1 and subsequently the E3 Ub ligase PARKIN which predominantly assembles K6-linked Ub chains. Distinct Ub chains appear as key regulators of PARKIN-mediated mitophagy and can be disassembled by DUBs like USP8, USP15, USP30 and USP33 [126,127]. Ub-dependent or independent mitophagy must go through a fission process to be incorporated into autophagosomes.

Ub also plays an important role in mitochondrial fission and fusion since it modifies the MOM proteins MFN1/2, FIS1, DRP1, and OPA1, driving their K48 Ub chain-dependent proteasomal degradation with consequences in the mitochondrial network [128,129]. The degradation of MFN2 and OPA1 favors mitochondrial fission, which is required to isolate a single damaged organelle. Under starvation, healthy mitochondria escape proteasomal degradation by promoting their elongation to preserve ATP production and avoid mitophagy [130].

## 6. Ubiquitin chains dynamics in DNA repair

Cells possess different mechanisms to contend with exogenous or endogenous events that cause damage to DNA integrity. The DNA damage response encompasses a set of mechanisms involving detection, signaling and initiation of DNA repair. According to the type of damage, a particular pathway is triggered by the cell to repair the lesion. DNA adducts or ultraviolet (UV)-induced DNA lesions are repaired by the nucleotide excision repair (NER) mechanism. The Fanconi anemia pathway (FA) repairs DNA crosslinks, while base lesions are repaired by the base excision repair (BER) pathway [131]. DNA double-strand breaks (DSB) are low-frequency types of DNA damage producing very drastic effects when they are unrepaired or incorrectly repaired, leading to genome instability

promoting subsequent disorders such as cancer. Ubiquitylation regulates multiple steps of these mechanisms as will be reviewed below (Fig. 4).

When a DSB occurs, two major repair mechanisms can be initiated: nonhomologous end-joining (NHEJ) and homologous recombination (HR). Fidelity and template requirements are the main differences between the two ways. NHEJ modifies the broken DNA ends and ligates them together with no homology or reference template, therefore being considered as an error prone mechanism generating deletions or insertions. In contrast, HR requires a DNA template on the sister chromatid or homologous chromosome to repair the DSB and restore the original sequence [131]. After DSB, the ataxia telangiectasia mutated (ATM/ATR) signaling pathway takes place rendering a series of PTMs over histones and intermediate proteins as H2AX and mediator of DNA damage checkpoint protein 1 (MDC1). Ubiquitylation of histones is essential to select one of the two repairing ways. The E3 ligases RNF8 (RING finger protein 8) and RNF168 have a major role in promoting NHEJ mechanism, while the E3 ligase complex BRCA1/BARD1 (breast cancer susceptibility gene 1/BRCA1-associated RING-domain protein) promotes HR. In both cases, the main target is the histone H2A through ubiquitylation on specific sites. It was first thought that RNF8 had a priming role as E3 ligase, undertaking H2A ubiquitylation, followed by extension of K63-chains by RNF168. However, it became evident that RNF8 in coordination with the Ub E2 UBC13, is responsible of the observed K63 Ub chains generated at DSB sites even if the target substrate was not identified [132]. Thorslund T. and colleagues identified, by proteomics studies, that the isoforms of the linker histone H1 (H1.2 and H1.x) were substrates of the RNF8-UBC13 complex. The K63-polyubiquitylation of histone H1 at DSB sites is the signal for enrollment of RNF168 which initiates the ubiquitylation events on histone H2A [133]. RNF168, in combination with the E2 UBCH5, directs mono-ubiquitylation of the N-terminal region of histone H2A at K13 and K15 sites; specifically K15 ubiquitylation promotes recruiting of repairing protein p53-binding protein 1 (53BP1) towards nucleosomes near sites of broken DNA [134]. In 2015, Gatti *et al*, demonstrated that RNF168 also promotes non-canonical K27-linked chains on histones H2A/H2A.X upon DNA damage. This modification appears to be crucial in the DNA stress response, as mutations on Ub K27 prevent DNA damage response activation and the recruitment of mediators like 53BP1, and BRCA1 [4,135]. 53BP1 endorses DNA repair by NHEJ mechanism antagonizing DNA end resection that is the initial step in HR. To counteract H2A ubiquitylation at K13 and K15, the role of USP51 as a deubiquitylating enzyme has been demonstrated [136].

Once DSB triggers the ATM/ATR pathway, kinases guide phosphorylation of histones H2AX, MDC1 and L3MBTL2 (lethal (3) malignant brain tumor-like protein 2). L3MBTL2 is a polycomb group protein essential for embryonic development which is mutated in malignancies such as leukemia. ATM-mediated phosphorylation of L3MBTL2 promotes its interaction with MDC1 and localizes it to the DSB site. In addition to the H1 ubiquitylation by RNF8, this E3 can also conjugate K63-Ub chains into L3MBTL2 that allows its binding to the UDM1 domain of RNF168. UDM1 specifically recognizes K63-linked Ub chains and in this way RNF168 is attracted to the DSB site [137]. The same mechanism allows the UDM1 to interact with polyubiquitylated H1. Therefore, RNF8 and RNF168 are sequentially recruited at DSB sites and K63-linked chains generated by the complex RNF8-UBC13 are necessary for RNF168 participation. K63-Ub chains can be regulated by the deubiquitylating activity of BRCC36 (BRCC3), a DUB member belonging to the JAMM family that forms a heterodimeric complex with ABRAXAS1 (a metalloprotease-like with no activity) [138]. BRCC36 and ABRAXAS1 are scaffold subunits of a macro-multi-protein complex known as BRCA1-A complex, that is also recruited by K63-chains through the UIM domain of the RAP80 subunit [8,139]. Depletion of BRCC36 compromises the G2/M cell cycle checkpoint, inactivating BRCC36 DUB by RNAi and genome editing attenuates BRCA1-A functions at DSBs and leads to unrestrained DSB end resection and hyperactive DNA repair [140].

According to Kaiser S.E. *et al*, approximately 65% of total Ub (present in cell lines) are conjugated as mono Ub, and from this pool ~30% are present on the histones fraction. Histone H2A is probably the most abundant ubiquitylated protein in the cell [141]. Three E3s enable H2A monoubiquitylation



including RNF168 that was already mentioned. RING1B/ BMI1 is another E3 which is part of the polycomb repressive complex 1 (PRC1) and is responsible of the canonical K119 site modification. A third E3 is the complex BRCA1/BARD1 which modifies the K125, K127 and K129 sites located at the C-terminal of histone H2A [132]. Ubiquitylation by PRC1 takes part in transcriptional silencing that is more related to chromatin remodeling or epigenetics than DNA repair, while ubiquitylation by BRCA1/BARD1 inclines the balance for the HR mechanism.

BRCA1/BARD1 occupancy at DSBs antagonizes 53BP1. The BRCA1/BARD1 heterodimeric complex harbors a RING domain on each monomer through which it interacts with E2 enzymes [142]. Very recently, the structure of BRCA1/BARD1 heterodimer with the E2 enzyme UBCH5c bound to the nucleosome was resolved by cryo-EM. The H2A K-targeted by BRCA1/BARD1 (125/127/129) are nearby to those targeted by RING1B/BMI1 (118/119) but reside in the fully disordered flexible C-terminal tail of H2A. It is particularly interesting that the BRCA1/BARD1 complex selectively ubiquitylates only K125, K127 and K129 positions. Heterodimer arrangement effectively tilts the BRCA1 E2 interaction site, orienting UBCH5c active site away from the K119 position. This interface is also sensitive to H3 K79 methylation status and mutations found in individuals are associated with cancer [143]. USP48 deubiquitylating activity counters the BRCA1–BARD1 modifications and promotes the DNA-end resectioning [8].

DSB induced by ionizing radiation leads to enrollment of another E3 heterodimer, RNF20/RNF40 (BRE1 complex). This ligase operates with the Ub E2 UBE2B (RAD6) to promote monoubiquitylation on histone H2B at the K120 site. H2B ubiquitylation has a critical role in cellular function, and its impairment has been linked to various cancers [144]. H2B is deubiquitylated by the Spt-Ada-Gcn5-acetyltransferase (SAGA) complex deubiquitylation module, composed of ENY2, ATXN7, ATXN7L3, and the DUB Ub-specific protease 22 (Usp22). There is evidence of the RNF20/RNF40 role in HR as they are required for CtIP- and NBS1-dependent end resection. Observations also point out that BRE1 has a role in NHEJ for repair of I-SceI-induced DSBs which requires repression of resection [144]. A more accepted role for H2B monoubiquitylation is to allow access of repair proteins to DNA by promoting displacement of one H2A/H2B dimer from the nucleosome barrier for transcription activation [145].

If K63-linked chains and monoUb are important for recruiting of repairing factors, other Ub linkages participate in regulating other DNA damage events. It has been reported that RNF8 can conjugate K11-linked Ub chains in coordination with the E2 UBE2S [146]. The K11-linked ubiquitylation plays a critical role in inhibiting transcription at sites of damage and is essential for cellular resistance to ionizing radiation. The DUB enzyme Cezanne (OTUD7B), a member of OTU family, has been shown to catalyze hydrolysis of K11-linked Ub chains even if its role has been mainly described in the regulation of the NF- $\kappa$ B pathway [147]. In response to DSB, Cezanne antagonizes the activity of RNF8 and UBE2S [146].

Searching for K63-linked Ub chain-specific binders, Wu X. and collaborators developed a UBD microarray where UBA domains of Cezanne and Cezanne2 were identified as selective for K63 but not K48- or K11-linked chains. Cells depleted of Cezanne by siRNAs or knockout (KO) cells generated by CRISPR-Cas9, showed diminished ionizing radiation-induced foci of Abraxas and BRCA1 seemingly as occurs with depletion of Rap80, the subunit component of BRCA1-A complex responsible of recruiting towards K63-linked foci. Intriguingly, their experiments suggested that Cezanne promotes Rap80/Abraxas/BRCA1 recruitment to DNA damage sites through its UBA domain binding to K63-conjugates, and its DUB activity disassembled K11-linked Ub chains. A model has been proposed where Cezanne serves as a “reader” of the K63-linkage and “eraser” of the K11-linkage ubiquitylation for the regulation of DNA damage response [148].

#### **4 Conclusions and future perspectives:**

Over the last 20 years, Ub and UbL proteins have appeared as key regulators of the proteome and cellular plasticity, since these PTMs determine not only stability and localization of modified targets but also their function depending on the stimuli. The clinical relevance of these PTMs is based on the hundreds of reported diseases where deletions, translocations and mutations affect Ub chain synthesis, deconjugation, recognition and connection with effector functions. Understanding the meaning of the Ub language and its regulation will be crucial to better assess the possibilities for therapeutic intervention. Despite the technological progress on mass spectrometry analysis to unveil the Ub chain complexity, our community still faces a lack of tools to identify complex chains. Most of the available tools provide information on homotypic Ub chains. However, we know that distinct and complex Ub-chains exist and some of them include other PTM modifications related to the Ub family (eg. SUMO or NEDD8) or not (eg. phosphorylation or acetylation). Deciphering how Ub moieties are disposed within a complex Ub chain is still challenging [149]. Distinct tools such as molecular traps (eg. TUBEs), peptide aptamers (eg. nanobodies) or affimers, were recently developed to get information on specific chain types. One can expect that in the near future more tools recognising all Ub chain linkages or modified Ub will be developed and the identification and characterization of non-homotypic, mixed or branched chains will be possible [15]. The highly dynamic action of DUBs further complicates our understanding of Ub chains. With or without dedicated chain specificity, they indeed remodel Ub polymers depending on the stimuli, time, and cellular space where the substrate is present. It is also important to keep in mind that the concept of one enzyme for one target used, to easily explain or simplify our research paradigms is called into question. Since functional redundancy might exist between Ub enzymes to act on a single target and some targets could be modified by several members of the Ub family, cooperation or opposition could exist to control Ub-driven functions. The identification of enzymes participating in Ub chain synthesis and remodelling in physiology and pathology will be crucial to understand how cells remodel their proteome to face distinct situations. These efforts should allow the selection of the best candidate biomarkers and drug targets.

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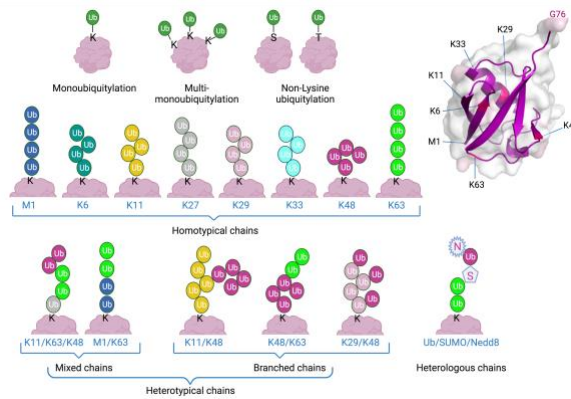


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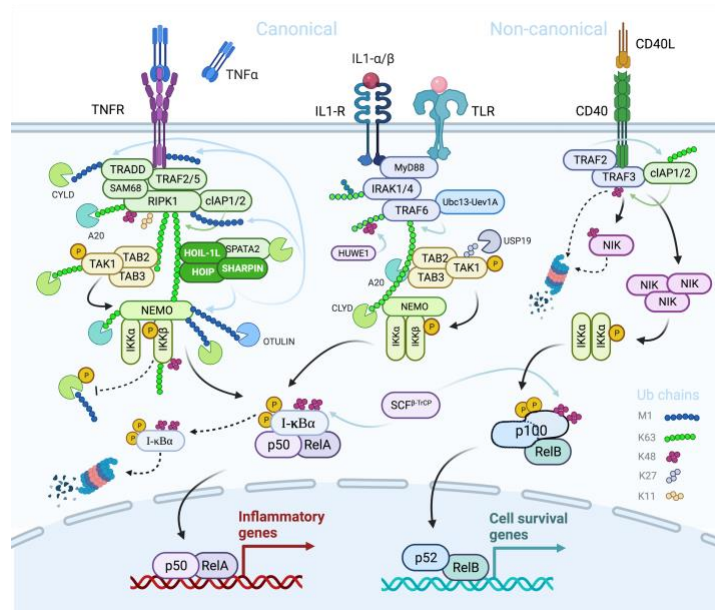
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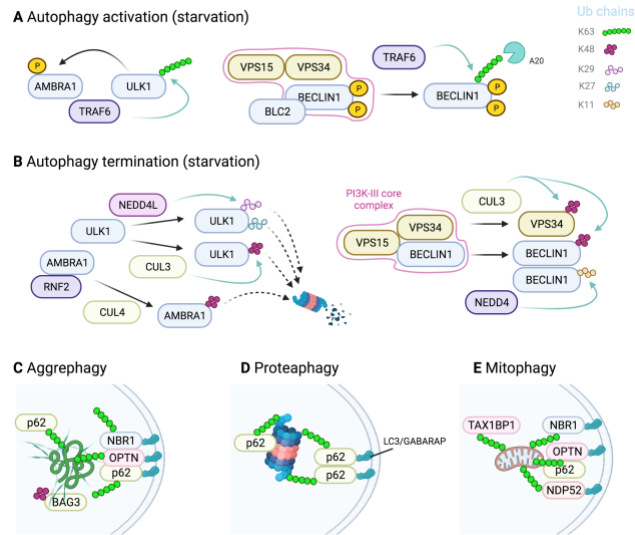
## Figures



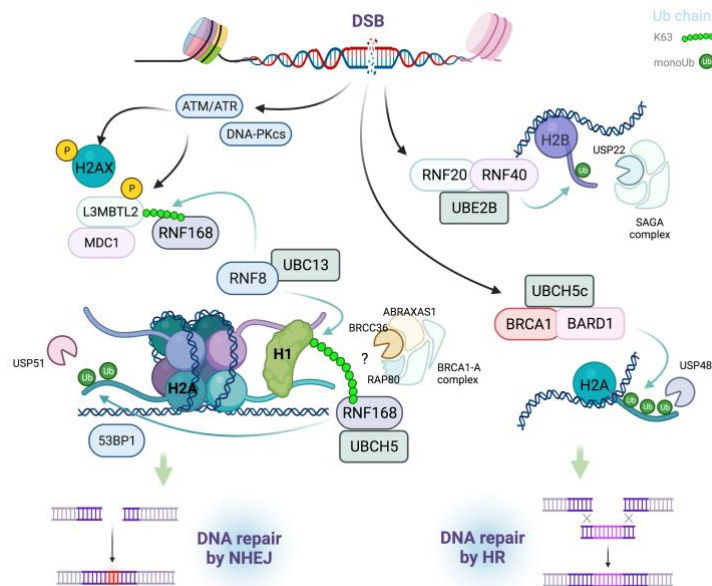
**Figure 1. Ub chains diversity.** Monoubiquitylation occurs when the Ub G76 residue is attached to a K residue on a target protein. Multimonomubiquitylation involves the attachment of several Ub molecules on multiple sites on the same target protein. Polyubiquitylation occurs when the first attached Ub is the target of another Ub moiety, which in turn, becomes the target for additional Ub molecules. The architecture and formation of Ub chains depend on the K residue that is used on the proximal Ub (which is the nearest to the substrate), to form the isopeptide link with the G76 residue of the adjacent or distal Ub. Structural diversity and complexity of chains can be appreciated and classified as it is shown in the cartoon representations. A structural view of Ub molecule (PDB:1ubq) is presented to illustrate the position of each K or M1 residues involved in the link formation.



**Figure 2. Highly dynamic regulation of the NF-κB pathway by distinct Ub chains.** The two canonical and the non-canonical pathways activating NF-κB are heavily regulated by M1, K63, K48, K27 and K11 Ub chain (illustrated with multiple colors). While the canonical pathway converges into phosphorylation of the inhibitor, IκBα, by a multi-subunit IκB kinase (IKK) complex to release the p50/RelA heterodimer, the non-canonical pathway activate the cleavage of p100 that will form the p52/RelB transcription dimer. Ubiquitylation and other posttranslational modifications participate in this fine regulation at the level of the recruitment of adaptor proteins after receptor-ligand binding, the activation of main kinase complexes and the NF-κB dimer releasing. The names and roles of regulated factors are well described in the main text.



**Figure 3. Multiple steps of autophagy are regulated by Ub chains.** Several Ub chains and Ub-like molecules play an important role in the regulation of autophagy and in the crosstalk of the UP and AL systems. While non-degradative Ub chains stimulate autophagy induction (A), degradative Ub chains are needed to terminate it (B). (A) TRAF6 form K63 chains that regulate ULK1 and PI3K-III kinases. TRAF6 also uses AMBRA1 to bind and stabilize ULK1 by forming K63 chains and activate autophagy. DUBs such as A20 regulate ubiquitin chains formed on distinct regulatory complexes and define their role in a dynamic reversible manner (see main text). (B) K11 and K48 Ub chains promote proteasomal degradation of crucial regulators to control autophagy. For example, NEDD4 targets BECLIN1 for degradation to inhibit autophagy and NEDD4L that induce proteasomal degradation of ULK1. Selective autophagy events such as aggrephagy (C), proteophagy (D) or Mitophagy (E) involve the participation of K63 Ub chains but also degradative chains. The type of chains involved depends in a large proportion in the stimuli activating these proteolytic events. The UbL proteins LC3/GABARAP contribute to the cargo recruitment into the phagophores, illustrated with a double membrane. Szveral autophagy receptors have been involved in these selective events such as p62, NBR1, OPTN or NDP52. For some of these selective events such as proteophagy, the information available is still limited (see main text).



**Figure 4. Regulation of DNA repair by multiple Ub chain types.** Protein ubiquitylation regulates the two major DNA repair mechanisms initiated by nonhomologous end-joining (NHEJ) and homologous

recombination (HR). The ataxia telangiectasia mutated (ATM/ATR) signaling pathway regulate H2AX and the mediator of DNA damage checkpoint protein 1 (MDC1). Ubiquitylation of histones determine the DNA repair mechanisms that will be used. While the E3 ligases RNF8 and RNF168 promote NHEJ mechanism, the E3 ligase complex BRCA1/BARD1 (breast cancer susceptibility gene 1/BRCA1-associated RING-domain protein) promotes HR. RNF8 and the Ub E2 UBC13 form K63 Ub chains at DSB sites. The K63-polyubiquitylation of histone H1 allows the recruitment of RNF168 and the E2 UBCH5 which initiates the ubiquitylation events on histone H2A at K13 and K15 sites recruiting the repairing protein p53-binding protein 1 (53BP1) towards nucleosomes near sites of broken DNA. RNF168 also promotes non-canonical K27-linked chains on histones H2A/H2A.X which is crucial in the DNA stress response since allow the recruitment of mediators like 53BP1, and BRCA1. The USP51 deubiquitylating enzyme regulate H2A ubiquitylation. DSB induced by ionizing radiation enrolls the E3 heterodimer, RNF20/RNF40 (BRE1 complex). Together with the Ub E2 UBE2B (RAD6), the BRE1 complex to promotes monoubiquitylation on histone H2B which has a critical role in cellular functions. H2B is deubiquitylated by the Spt-Ada-Gcn5-acetyltransferase (SAGA) complex which integrates the DUB ubiquitin-specific protease 22 (Usp22). The complex BRCA1/BARD1 modifies H2A favouring HR mechanism. The USP48 deubiquitylating activity regulates BRCA1–BARD1 modifications and promotes the DNA-end resectioning.

Type of Ub chain		Ub chain linkage-type	Main described functions	References
Homotypical	Canonical	K48	Proteasomal degradation	[18,22]
		K63	Cell signaling, DNA repair, autophagy, protein trafficking	[30,40,41,67,150]
	Non-canonical or atypical	M1	NF- $\kappa$ B signaling, cell death regulation, protein quality control	[13,43–45]
		K6	DNA repair, mitophagy	[6,126,126]
		K11	Proteasomal degradation, cell cycle, IFN signaling	[24,72]
		K27	Cell signaling, immune regulation, autophagy, DNA repair	[72,151]
		K29	Wnt/ $\beta$ -catenin signaling, immune regulation	[75,76,152,153]
K33	Cell signaling, immune regulation, protein trafficking, autophagy	[71,74,153–156]		
Heterotypical	Mixed	K6/K11	unknow	[157]
	Branched	K6/K48	unknow	[14]
	Branched	K11/K48	Proteasomal degradation, cell cycle, protein quality control	[24,25,25,157,158]
	Mixed	K11/K63	unknow	[157]
	Branched	K27/K29	unknow	[14]
	Branched	K29/K33	unknow	[14]
	Branched	K29/K48	ER-associated protein degradation (ERAD)	[159]
	Branched	K48/K63	NF- $\kappa$ B signaling	[47,160]
	Mixed	K63/M1	NF- $\kappa$ B signaling	[46]
	Mixed	K48/K63/K11	Cyclin B1 regulation	[161]

Table 1. Functions associated to different ubiquitin-chain linkages. Non exhaustive examples.