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Chapter

The Role of Lysine 63-Linked Ubiquitylation in Health and Disease

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Abstract

A specific subfamily within the E2 protein family is involved in the synthesis of noncanonical poly-ubiquitin chains, linked through lysine 63 residues. The role of lysine 63-linked polyubiquitylation in diseases has emerged only recently. Under physiological conditions, this process does not seem to be involved in the classical protein degradation by the proteasome, but it is involved in the regulation of intracellular signaling, DNA damage response, cellular trafficking, and lysosomal targeting. The alteration of this process has been described in a number of pathological conditions, including immune disorders, diabetes, and cancer. In this chapter, we will describe the role of lysine 63-linked ubiquitylation in the regulation of diverse signaling pathways involved in cell behavior. We will also describe some pathological conditions in which altered lysine 63-linked ubiquitylation has been referred to play an important role.

Keywords: lysine 63-linked ubiquitylation, immune system, diabetes complications, autophagy, cancer

1. Introduction

The ubiquitin signaling system, often referred to as "ubiquitin code", is very complex, although the ubiquitin moieties engaged in protein ubiquitylation are always identical. In order to understand the complexity of the ubiquitin code, we need to remember that the ubiquitin moiety contains seven lysine residues, all of which can be potentially engaged in the formation of polyubiquitin chains, and the protein fate depends upon the specific lysine residue involved in the polyubiquitin link as well as the length of the polyubiquitin chain [1, 2].

Several types of polyubiquitin chains exist in cells and the type of chain defines how ubiquitinated proteins are regulated. For instance, we know that ubiquitin chains generated via lysine (K)48 of ubiquitin (K48 chains) function as a signal for proteolysis, while chains generated via K63 (K63 chains) are involved in nonproteolytic functions, such as DNA repair, protein kinase activation, and membrane trafficking [3].

The ubiquitin signaling starts with the activation of the ubiquitin moiety by an ubiquitin-activating enzyme E1, and the energy to initiate this process is provided by an ATP molecule. Exploiting its active site containing a cysteine, E1s attack the ubiquitin-AMP intermediate, forming a thioester bond. The subsequent reaction

involves the transfer of the activated ubiquitin from the E1 to an E2 enzyme through a transthioesterification reaction; E2 enzymes also contain an active site that includes a cysteine residue. The final step is carried out by ubiquitin protein ligase E3 enzymes, which allows the transfer of ubiquitin to the lysine of the target protein [4, 5]. Although target specificity is given by E3 enzymes, not all E3s are able to ligate the ubiquitin molecule to their target directly, as this ability is dependent on the type of active site they are furnished with. There are two main types of catalytic domains on E3 enzymes. The E3s possessing a RING (really interesting new gene) domain in their active site do not contain a cysteine residue within the active-site; thus, they work by bringing the "charged" E2 in close proximity to the target protein, and ubiquitin is transferred by the E2. E3s that possess a HECT domain [homology to E6-AP carboxyl terminus] instead, contain a cysteine residue in their active site that allows the formation of a thioester intermediate and the subsequent transfer of ubiquitin to the target protein [6, 7].

In summary, the enzymatic cascade that leads to the formation of an isopeptide bond between ubiquitin and its target molecule involves E1, E2, and E3 enzymes, and the ubiquitin chain linkage specificity is generally conferred by E2s.

Among the E2s that participate in the specific formation of K63-linked ubiquitin chains, Ubc13 (also known as Ube2n) is probably the best characterized. To finalize the reaction, Ubc13 requires the concomitant presence of specific E2-like partner proteins, among which Ube2v1 (also known as Uev1A) is involved in the formation of K63-linked ubiquitin chains in the cytosol, while the protein Mms2 participates in the nuclear K63-linked chain formation. It was also shown that this enzymatic complex exerts its activity on previously mono-ubiquitinated substrates; thus, the priming of the substrate with the first ubiquitin molecule can likely be promoted by different E2s [8].

Overall, the complexity of the cellular responses elicited by ubiquitin is actually greater than previously foreseen; it was recently discovered that heterogeneous ubiquitin chains also exist, possessing both proteolytic and nonproteolytic functions. Also, ubiquitin itself can be modified through phosphorylation and/or acetylation [9, 10]. Finally, deubiquitinating enzymes (DUBs) exist which operate through either the editing or disassembly of ubiquitin chains, allowing the fine-tuning of the entire system [11].

Importantly, both the proteolytic and nonproteolytic functions of ubiquitin are crucial to regulate different intracellular signaling pathways involved in the modulation of immunity, inflammation, and cell survival [12]. Here we report an overview on the main pathways modulated by K63-linked ubiquitylation and the role of this post-translational modification in health and disease.

2. Physiological roles of lysine 63-linked ubiquitylation

2.1 Lysine 63-linked ubiquitylation in NF-κB signaling

NF-κB is a dimeric transcription factor that controls cytokine production and cell growth, ultimately modulating processes such as inflammation and immune responses. This protein complex is ubiquitously present in the cell cytoplasm in an inactive state but it can rapidly be processed into its active form by different regulatory mechanisms including ubiquitylation.

The canonical ubiquitylation trough K48-linked chains controls processing of the NF-κB precursor p100 and p105, as well as degradation of the NF-κB inhibitor IκB when it becomes phosphorylated by the IκB kinase (IKK) complex. The noncanonical ubiquitylation through K63-linked chains is instead involved in the

activation of the IκB kinase complex IKK [13]. These events are mediated, among others, by a class of E3 enzymes known as the TNF receptor-associated factor (TRAF). TRAF proteins mediate NF-κB activation from a number of receptors such as the TNF receptor (TNFR), IL-1 receptor (IL-1R), and Toll-like receptor (TLR). TRAF6 transduces signals from IL1-R/TLR while TRAF2 transduces signals from TNFR. Both TRAF-2 and TRAF-6 are able to form K63-linked ubiquitin chains on their specific targets [14].

2.1.1 IL1-R/TLR-induced NF-κB signaling

In the NF-κB signaling triggered by activation of the IL1-R/TLR, the E2 protein complex consisting of Ubc13-Uev1A, in concert with the E3 enzyme TRAF6, leads to the formation of K63-linked ubiquitin chains on several target proteins including Interleukin 1 Receptor Associated Kinase 1 (IRAK1), NF-kappa-B essential modulator (NEMO), and TRAF6 itself [15]. Once ubiquitinated, TRAF6 can be recognized by a specific ubiquitin binding domain (UBD) within the protein TGF-β Activated Kinase 1 (MAP3K7) Binding Protein 2 (TAB2) and this interaction activates the TAB2-associated TAK1 kinase, which in turn phosphorylates and activates IKK. Active IKK promotes degradation of IκB and ultimately releases inhibition on NF-κB (**Figure 1**). It was shown that TAK1 can activate IKK only in the presence of the NF-κB essential modulator NEMO. Importantly, this protein contains a C-terminal domain that binds preferentially to K63 ubiquitin chains. Thus, this type of ubiquitylation might be useful to provide a scaffold that facilitates protein interactions [14, 16, 17].

2.1.2 TNFR-induced NF-κB signaling

NF-κB activation can also be triggered by molecules that bind TNF-R1. As for IL-1/TLR, TNF-R1 signal transduction involves a cascade of reactions that are regulated at different levels by protein ubiquitylation.

The model proposed for NF-κB activation suggests that upon exposure to TNF-α, TNF-R1 undergoes a conformational change that allows recruitment of the adaptor

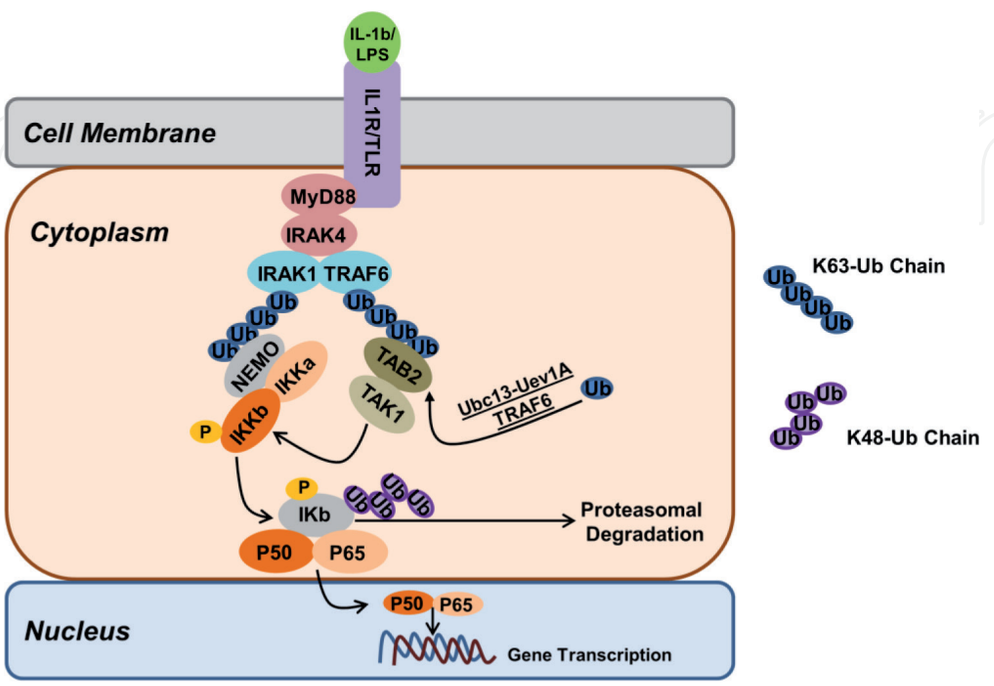


Figure 1.
IL1-R/TLR-induced NF-κB signaling.

tumor necrosis factor receptor type 1-associated death domain protein (TRADD) within the cytosol. This interaction elicits the enrollment of two additional proteins: TRAF2 and kinase receptor-interacting serine/threonine-protein kinase 1 (RIPK1). Unlike TRAF6, TRAF2 is unable to attach ubiquitin moieties to RIPK1 independently, but acts as a scaffold for the recruitment of two different E3 enzymes: cellular inhibitor of apoptosis protein-1 (c-IAP1) and c-IAP2. These proteins add, among others, K63-linked chains on RIPK1; thus, they are the actual effectors of RIPK1 ubiquitylation. The following events that lead to NF-κB activation involve the formation of a second E3 protein complex at the initial site of ubiquitylation known as linear ubiquitin chain assembly complex (LUBAC). In linear ubiquitin chains, the C-ter Gly76 of one ubiquitin is linked to the α-NH2 group of Met1 of another ubiquitin moiety. Thus, the LUBAC complex catalyzes the formation of linear (M1)-linked ubiquitin chains on RIPK1 proteins, c-IAP1 and c-IAP2. Once these linear chains are added, RIPK1 is structurally able to attract the kinase complexes TAK1 and IKK through their ubiquitin binding domain-containing subunits (TAB2/TAB3 and NEMO). This ultimately triggers IKK phosphorylation by TAK1 and NF-κB activation [18] (**Figure 2**). In summary, a variety of ubiquitin chain modifications seem to be required for NF-κB activation, and further studies will shed light on the many roles of each specific type of polyubiquitin chains.

2.2 Lysine 63-linked ubiquitylation in Wnt/β-catenin signaling

The Wnt/β-catenin signaling pathway is essential in the regulation of events such as cell proliferation, organized migration, self-renewal, and tissue polarity. Disruption of the Wnt/β-catenin signaling pathway has been linked with oncogenesis and other pathological conditions. Regulation of Wnt signaling is controlled by protein ubiquitylation at many levels, and K48- and K63-linked ubiquitin chains in particular have been shown to regulate this pathway through both proteolytic and nonproteolytic functions.

Within the canonical Wnt/β-catenin signaling, the absence of a Wnt ligand at the transmembrane receptor Frizzled (Fz) determines the rapid phosphorylation

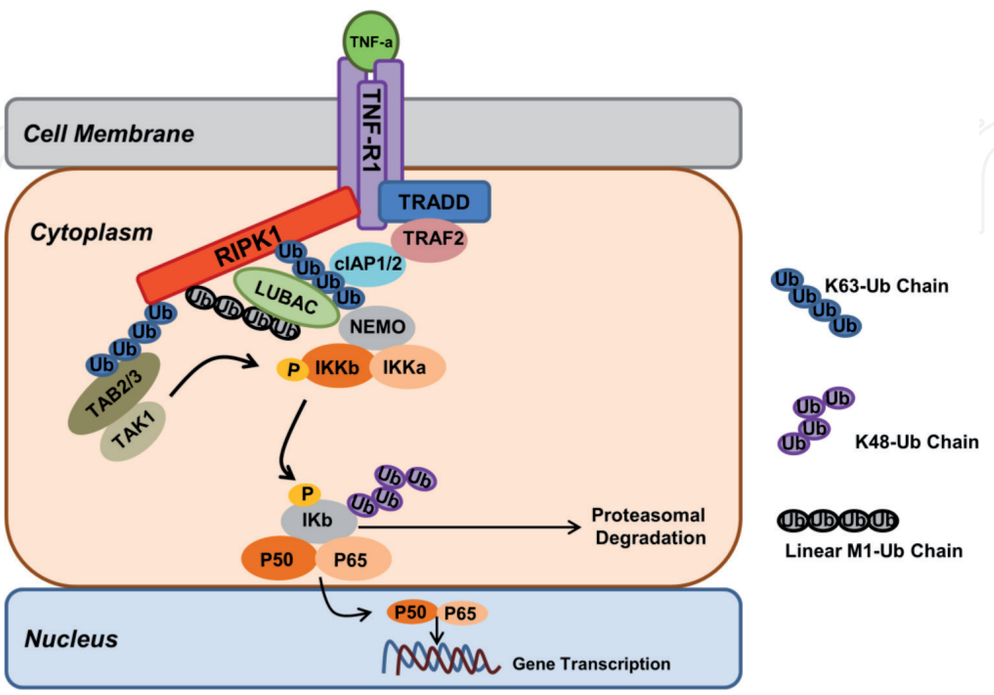


Figure 2.
TNFR1-induced NF-κB signaling.

of the free β-catenin in the cytosol. This reaction is catalyzed by a protein complex that includes the proteins axin, adenomatous polyposis coli (APC), casein kinase I (CK1), and glycogen synthase kinase 3 beta (GSK3β). When phosphorylated, β-catenin is recognized and ubiquitinated by a specific E3 ubiquitin ligase complex (SCF βTrCP), and degradation through the proteasome occurs (**Figure 3A**). Hence, one of the roles of the axin-APC complex is to maintain low cytosolic levels of β-catenin [19].

When a Wnt ligand binds a Fz receptor, in the presence of specific adapters known as low-density lipoprotein receptor-related proteins 5/6 (Lrp5/6), recruitment of the proteins Axin and Disheveled (DvI) occurs, and the formation of the protein complex that drives β-catenin degradation is inhibited. As a consequence, the levels of cytoplasmic β-catenin rise and the protein is transported into the nucleus where it forms a complex with the T cell factor (TCF) family of transcription factors, and activates transcription of its target genes [20] (**Figure 3B**).

A number of evidence demonstrate that K63-linked polyubiquitylation plays a role in the regulation of Wnt signaling although the precise molecular mechanisms that regulate these complex interactions have not been fully elucidated yet. We know that the formation of K63-linked polyubiquitin chains is promoted by the E2 protein complex Ubc13-Uev1a and recent evidence suggests that deletion of Ubc13 is associated with accumulation of β-catenin and increased transcription of Wnt target genes; however, the precise molecular mechanisms that drive these signaling events remain to be fully elucidated [21]. As largely known, deubiquitinating enzymes (DUBs) act in concert with ubiquitinating enzymes to precisely adjust the extent and duration of ubiquitin signals. Cellular experiments show that Trabid is a DUB protein able to reverse K63-linked hyperubiquitylation of the APC complex, thus acting as a positive regulator of the Wnt/β-catenin signaling [22].

Abnormal Wnt signaling underlies a wide range of pathological conditions in humans, including cancer. Hyperactivation of the Wnt pathway, for instance, is a characteristic of tumor cells from patients with cylindromatosis. These patients present mutations in the CYLD gene; this gene encodes for a DUB enzyme whose loss in human cells causes K63-linked hyperubiquitylation of the upstream Fz-binding effector protein Disheveled (Dvl) resulting in enhanced responsiveness to Wnt [23].

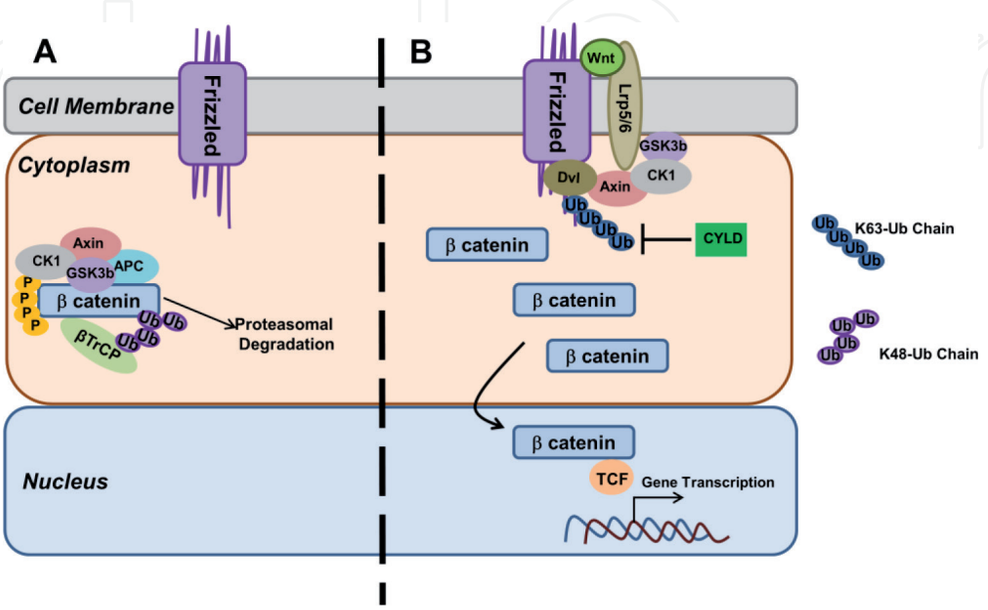


Figure 3.
Wnt/β-catenin signaling in the absence (A) or presence (B) of a Wnt ligand.

2.3 Lysine 63-linked ubiquitylation in membrane protein trafficking

Membrane proteins serve different functions: they allow cells to sense and/or interact with molecules in the extracellular space, confer a proper shape to the cell, regulate the osmotic pressure, and channel the passage of ions, endogenous compounds, xenobiotics, etc. Both the sorting and degradation of membrane-bound proteins are regulated at least in part by K63-linked protein ubiquitylation.

The synthesis of membrane protein takes place specifically on those ribosomes attached to the endoplasmic reticulum (ER). Once newly synthesized, proteins migrate from the inner lumen of the ER to the cis face of the Golgi apparatus (more proximal to the nucleus) where they undergo refinement and quality control to ensure proper folding. Within the Golgi apparatus, proteins are also sorted according to their predetermined cellular destination and finally secreted through the trans face (more distal to the nucleus). The migration of proteins through these cellular compartments is guided by lipid vesicles known as endosomes. Importantly, endosomes also coordinate the downregulation of cell-surface receptors through internalization of these proteins and subsequent degradation in the lysosomes.

A growing number of studies seem to suggest that K63-linked ubiquitin chains act as a signal for the internalization and intracellular sorting of integral membrane proteins [24]; more specifically, K63-linked ubiquitin chains have been shown to direct proteins to a specialized subclass of endosomes known as multivesicular bodies (MVBs). Once in the MVBs, proteins are either sent to the lysosomes for degradation, or secreted as exosomes via fusion with the plasma membrane; this process also serves to position membrane bound receptors to their specific location.

The first evidence that K63-linked ubiquitin chains could function as a signal to stimulate internalization of plasma membrane proteins through endocytosis and targeting into the lysosomal degradation pathway was acquired in *Saccharomyces cerevisiae* [25]. These preliminary observations led to the discovery of several mammalian proteins undergoing a similar regulatory mechanism, for example, the epidermal growth factor receptor (EGFR) [26], the human dopamine transporter (DAT) [27], the nerve growth factor receptor tyrosine receptor kinase A (TrkA) [28], major histocompatibility complex class I molecules [29], and the prolactin receptor [30] and possibly the low-density lipoprotein receptor (LDLR) [31].

3. Lysine 63-linked ubiquitylation in diseases

3.1 Lysine 63 ubiquitylation in immune disorders

Protein ubiquitylation has emerged as a key mechanism in the modulation of the immune system development and intensity of the immune responses [32, 33]. K63-linked ubiquitylation is involved in immune cell development since germline ablation of Ubc13 induces embryonic lethality [33]. Moreover, through the regulation of different intracellular signaling pathways, K63 ubiquitylation has emerged as critical for T cell differentiation [34]. K63 ubiquitylation chains have been described as fundamental for both the innate and adaptive immune systems given their involvement in master pathways controlling immune responses, such as NF-κB signaling [35] and MAPK activation [36], as previously described.

3.1.1 Lysine 63 ubiquitylation and adaptive immune response

The adaptive immune response, also called acquired immunity, refers to antigen-specific immune response; thus, antigen presentation induces the development

of effective T- and B-cell responses. B-cells can be divided into memory B cells that express membrane-bound antibodies, or plasma B cells that can secrete antibodies to identify free pathogens circulating into the body. All B cells express a B cell receptor involved in antigen binding, internalization, and processing of antigens, other than activation of intracellular signaling pathways. T cells instead mature into the thymus where they start to express T cell receptors (TCRs) and CD4 and CD8 receptors. T cell receptors, assisted by CD4 or CD8 receptors, recognize antigens bound to certain major histocompatibility complex class 1 (MHCI) and class 2 (MHCII), expressed by antigen presenting cells such as macrophages and dendritic cells. Mature T cells can be mainly divided into Helper T cells, CD4+ cells involved in the activation of other immune cells, cytotoxic T cells, CD8+ that removes pathogens and infected cells, and T regulatory cells. T regulatory cells (Treg) play a central role in the regulation of the adaptive immune response and represent a T lymphocyte subpopulation that maintains tolerance to self-antigens and prevents autoimmune disease [37]. Tregs are produced in the thymus as a subpopulation of T cells and express a transcription factor (Forkhead box protein 3) involved in Treg development and function [38]. Tregs can also be induced from naive T cells in the periphery in the presence of transforming growth factor b (TGF-β).

K63 ubiquitylation is specifically involved in the suppressive function of Treg cells, and it has been described as fundamental for the immunosuppressive function of Tregs in murine models *in vivo* [39]. It is also well known that NF-κB signaling can modulate Treg cell differentiation [40]. Chang et al. demonstrated that Ubc13 deficiency in Treg, with the subsequent reduction in K63 ubiquitylation, impaired the *in vivo* suppressive function of these cells. Ubc13 deficiency in $Ubc13^{Treg-KO}$ mice, in fact, was able to influence the IKK signaling axis normally required for the expression of specific Treg functional factors, such as IL-10 and SOCS1 [39]. Both IL-10 and SOCS1 can specifically regulate Treg stability and inhibition activity.

Defects in Treg cells have been described in several human immune disorders including systemic lupus erythematosus (SLE). Treg disorders in SLE patients are characterized by abnormal peripheral tolerance that has been linked to a deficiency in the E3 ubiquitin ligase Cbl-b [41], involved in the regulation of T cell receptor signaling, during the induction of peripheral tolerance. Interestingly SLE patients were also characterized by an altered pattern of K63 ubiquitinated proteins in Tregs, with a decreased expression of K63 ubiquitinated proteins, related to increased pSTAT-3 expression [42]. These processes could be responsible for the loss of Treg suppressive capacity in SLE patients.

K63 ubiquitylation can also influence B cell receptor, T cell receptor, and IL-1 receptor (IL-1R)-mediated immune responses. Murine Ubc13-deficient T cells showed altered proliferation in response to diverse stimuli and impaired intracellular signaling altering the activation of both NF-κB and MAP kinases into T cells [43]. Also, murine Ubc13-deficient (Ubc13−/−) B cells showed impaired activation of the B cell receptor and CD40-induced activation, as well as Toll-like receptor mediated activation [44]. All this evidence underlies the meaning of this process in the mammalian immune response, thus indicating the importance of investigating K63 ubiquitylation in immune disorders.

3.1.2 Lysine 63 ubiquitylation and innate immune response

Innate immune response is triggered upon infections with pathogens such as bacteria, parasites, and viruses. It includes several mechanisms consisting in the physical and chemical barrier to infectious agents, in the activation of the complement cascade, and in the recruitment to the sites of infection of immune cells [such as macrophages and neutrophils] able to produce cytokines, thus inducing the

inflammatory response. Innate immune response can also influence the adaptive immune system through antigen presentation.

It has been demonstrated that K63 ubiquitylation facilitates the innate immune signaling activated by diverse receptors such as Toll-like receptors, able to recognize pathogen components (lipopolysaccharide—LPS—from Gram-negative bacteria or lipoteichoic acid from Gram-positive bacteria), or cytokine receptors [45]. Also, in the regulation of the innate immune response, such as the adaptive immune response, the IKK-NF-κB pathway and MAPK activation plays a central role, since they are activated by the engagement of Toll-like receptors (TLRs).

An important component of the innate immune system is represented by natural killer (NK) cells, involved in the direct elimination of infected or transformed cells and able to secrete diverse cytokines, including IFN-γ, thus increasing the inflammatory response and the recruitment of immune cells. Also, IFN-γ production in NK cells is regulated by K63 ubiquitylation through its involvement in the NF-κB pathway [46], underlying once again the importance of this mechanism in the modulation of the immune response.

Ubiquitylation signaling has been described as specifically involved in the anti-viral innate immune response. In fact, the importance of the ubiquitylation pathway in the innate immune response has been validated by the discovery of some viruses encoding deubiquitinating proteases. Deubiquitinating enzymes (DUBs) catalyze the removal of ubiquitin from different cellular substrates, thus influencing several intracellular processes [47]. These deubiquitinating proteases produced by viruses, can lead to the suppression of the anti-viral immune response in order to promote viral replication [48].

Herpes Simplex Virus 1 (HSV1), a dsDNA virus belonging to the alpha-herpesvirus subfamily, can cause humans gingivostomatitis, cold sores, and herpetic keratitis. HSV1 dsDNA induces NF-κB signaling activation that promotes the anti-viral immune-response. However, to evade the innate immune system, these viruses encode for a DUB domain, called UL36USP, which is also similar to an open reading frame encoded by other viruses such as the human cytomegalovirus (HCMV) [49]. HCMV is a member of the beta-herpes virus subfamily, whose infection, normally asymptomatic, once reactivated can cause severe disease in immune-compromised and immune-suppressed individuals. Interestingly the UL36USP deubiquitinase activity inhibits NF-κB activation, by deubiquitinating IκBα, thus blocking its degradation and, consequently, finally quenching IFN production [50], a cytokine important for the anti-viral immune response.

Also, retroviral infections, including HIV-1, are mediated by modulation of the ubiquitylation system. In particular, the retrovirus factor TRIM5 (tripartite motif-containing protein 5) promotes innate immune signaling by activating, through K63 ubiquitin, MAP3K7 kinase complex with the subsequent stimulation of AP-1 and NF-κB signaling [51].

K63 ubiquitylation is also involved in bacterial cytoplasmic infections. These infections induce the cytosolic exposure of peptidoglycans and are characterized by the activation of the nuclear oligomerization domain 2 (NOD2) intracellular signaling. Polymorphisms in NOD2 have been associated with 15–30% of genetic Crohn's disease [52], an inflammatory bowel disease that may affect any part of the gastrointestinal tract, from mouth to anus, characterized by a dysfunctional immune response to normal microbiota [53]. It has been demonstrated that the activation by NOD2 of the K63-specific E3 ubiquitin ligase TRAF6, leading to NF-κB stimulation, is seriously compromised in Crohn's disease-patients with the NOD2 allele L1007insC [54]. In addition, NOD2 (nucleotide oligomerization domain 2) regulates the formation of K63-linked polyubiquitin chains on the I kappa kinase (IKK) scaffolding protein, NEMO.

Thus, ubiquitin-mediated regulation of the innate immune response could represent an important node in the management of pathogen infection and could symbolize a novel target for future therapies.

3.2 Lysine 63 ubiquitylation in diabetes and diabetic nephropathy

Diabetes mellitus is a metabolic disorder characterized by the reduction and altered function of pancreatic insulin-producing β-cells, and by organ damage [55]. Type 1 diabetes is induced by autoimmune destruction of β-cells responsible for insulin insufficiency, while type 2 diabetes is due to peripheral insulin resistance and subsequent β-cell expansion and hyperinsulinemia [56]. The number of diabetic patients is increasing decade by decade, and this high prevalence is registered worldwide with a projection of more than 438 million of diabetic patients with 7.8% prevalence by 2030 all over the world.

Chronic hyperglycemia and oxidative stress have been described as pro-apoptotic signals for pancreatic β-cell, thus influencing the metabolic state of these cells and the cell fate decisions [56]. Several intracellular signaling pathways can contribute to modulate β-cell function. Among these, also post-translational modification has been recognized to play a role. SUMOylation, a post-translational modification consisting in covalent attachment to target proteins of the small ubiquitin-like modifier (SUMO) peptides, has been recently described as a key event regulating β-cell survival and function [57]. Different cytokines can induce autoimmune destruction of pancreatic β-cell through the modulation of several intracellular signaling pathways characterized by the activation of phosphorylation and ubiquitylation events, including K63 ubiquitylation, in the cells. In fact, one of the factors involved into cytokine-mediated apoptosis of β-cell is represented by the mixed lineage kinase MLK3 [58], a pro-apoptotic factor involved in a cascade of events ultimately leading to mitochondrial outer membrane permeabilization, thus compromising mitochondrial integrity. Humphrey et al. demonstrated that IL-1β, one of the cytokines involved in the autoimmune destruction of pancreatic β-cells, stimulates K63-linked ubiquitylation of MLK3, thus promoting its activity and finally influencing the progression toward β-cell death [59]. Thus, K63 ubiquitylation could represent a potential target for therapeutic intervention in promoting β cell survival in diabetic patients.

Diabetes is also responsible for the insurgence of different complications such as retinopathy, cardiovascular diseases, and renal diseases. In the last years, the importance of the ubiquitylation pathway in diabetes and diabetic complications, such as cardiac diseases [60] and diabetic nephropathy [61–64], has emerged significantly [65].

Hyperglycemia, hypertension, and other hemodynamic changes intensify the filtration and reabsorption processes and this can lead to kidney failure progressing toward end-stage renal disease (ESRD). The incidence of ESRD due to diabetes varies among countries between 15 and 45%, with a mean value of 33%, which means that 33% of patients are starting renal replacement therapy.

Renal damage in type 2 diabetic patients can be characterized by different patterns including diabetic glomerulosclerosis, vascular and ischemic glomerular changes, and other glomerulonephritis in the presence or absence of diabetic lesions [66]. Pure diabetic nephropathy is characterized by mesangial proliferation, podocyte loss, glomerular basal membrane thickening, and nodular extracellular matrix accumulation with the classical Kimmelstiel-Wilson lesions.

The specific role of K63 ubiquitylation in diabetic nephropathy has been recently described [67]. Hyperglycemic conditions induce in tubular cells an increased expression of specifically K63-ubiquitinated proteins. Also, kidney biopsies from

diabetic nephropathy patients are characterized by increased K63 ubiquitylation at tubular cells when compared to diabetic patients without renal damage or patients with other nephritides such as membranous nephropathy. Interestingly, increased K63 ubiquitylation in glucose-stimulated tubular cells was able to promote epithelial to mesenchymal transition, a process already described as involved in diabetic nephropathy dysfunction [68]. Epithelial to mesenchymal transition represents a potential source of myofibroblasts involved in the progression of kidney fibrosis. Also, in *in vivo* kidney biopsies of diabetic nephropathy patients, tubular cells characterized by increased accumulation of K63 ubiquitinated proteins were also characterized by expression of mesenchymal markers [67], thus underlying the importance of K63 ubiquitylation in the progression of renal fibrosis in diabetic patients.

Other than in epithelial-to-mesenchymal transition, hyperglycemia-induced K63 ubiquitylation is also involved in the apoptotic death of tubular cells through the deregulation of autophagy. Autophagy is an intracellular process involved in degradation of damaged proteins/organelle or in the intracellular response to nutrient deprivation, stress, and extracellular environmental changes. In human glomerulopathies, changes in the ubiquitin-proteasome system have been correlated with autophagy [69]. Impaired autophagy has also been described as a characteristic feature of diabetics [70], and recently, the molecular mechanisms responsible for this alteration have been correlated to hyperglycemia-induced K63 ubiquitylation [71]. In diabetic nephropathy patients *in vivo*, those tubules characterized by increased expression of the autophagic factor LC3 were also characterized by increase in K63 ubiquitinated protein accumulation. Interestingly, accumulation of autophagic particles into tubular cells, due to K63 ubiquitinated protein accumulation, could be responsible for increased apoptosis of these cells, as observed both *in vitro* and *in vivo* in kidney biopsies from diabetic nephropathy patients [70]. Taken together, all this evidence support the role of K63 ubiquitylation in the progression of tubular damage in diabetic nephropathy patients, which could be responsible for the progression of kidney fibrosis and for the induction of apoptosis of tubular cells with the consequent reduction of renal function.

3.3 Lysine 63 ubiquitylation and neurodegenerative disorders

Neurodegenerative disorders like Parkinson's disease or dementia or Alzheimer's disease are, in some cases, characterized by the presence of insoluble deposits in neurons containing components of the ubiquitin-proteasome system. It has been reported, in fact, that the ubiquitylation pathways play an important role in the pathogenesis of these diseases.

The most common cause of familial Parkinson's disease is characterized by mutations in Parkin [72], a ubiquitin ligase whose loss of function leads to both toxic accumulation of its substrates [73], and impaired formation of Lewy bodies, fibrillary masses of molecules implicated in the formation and degradation of alpha-synuclein aggregates [74]. Alpha-synuclein is a protein expressed in neurons involved in the formation of synaptic vesicles in presynaptic terminals and in the release of the dopamine, one of the brain's neurotransmitters. It has been demonstrated that K63 ubiquitylation plays an important role in the generation of these aggregates. Lim et al. observed that parkin-mediated ubiquitylation of proteins within Lewy-body-like inclusions was augmented by K63 ubiquitylation and occurs mainly through K63 linkages [75]. Interestingly, it has been demonstrated that the ubiquitin hydrolase UCHL1 is able to promote also K63-linked ubiquitylation of alpha-synuclein [76]. Thus, K63 ubiquitylation could represent a mechanism by which protein inclusion can occur and by which proteins are stabilized [77] forming

aggregates observed in neurodegenerative disorders including Parkinson disease and dementia.

Interestingly, K63 ubiquitylation can contribute not only to inclusion biogenesis, but also to the clearance of inclusions. Intracellular alpha-synuclein can be ubiquitinated in K63 residues also by the E3 ubiquitin ligase Nedd4-1 [neural precursor cell expressed developmentally down-regulated protein 4-1], thus inducing its degradation by the endo-lysosomal pathway [78]. The first evidence of the role of K63 ubiquitylation in modulating autophagy clearance of aggregates in neurodegenerative disorders was described by Tan et al. in 2008 [79]. These authors demonstrated that K63 polyubiquitin chains linked to protein inclusions represent a target, driving aggregates to the clearance by autophagy.

Poly-ubiquitinated proteins can also be accumulated into the mitochondria during proteolytic stress and can be responsible for mitochondria-mediated cell death during proteasomal dysfunction, as demonstrated in an *in vitro* model of dopaminergic degeneration [80]. Interestingly, also monoamine oxidases (MAOs), located on the outer mitochondrial membrane and involved in the control of the neurotransmitters levels in the brain, can induce K63 ubiquitylation of mitochondrial proteins and promote autophagy of damaged organelles in neuroblastoma cells [81].

Taken together, these results evidenced the importance of K63 ubiquitylation in neurodegenerative disorders and open novel scenarios for the treatment of these diseases.

3.4 Lysine 63 ubiquitylation in cancer

The involvement of ubiquitin proteasome system dysregulation in the degradations of apoptotic proteins and subsequent induction of tumor formation has been well established, whereas, the finding that nonproteolytic ubiquitylation has a role in cancer and metastasis is recent.

With respect to K63-linked ubiquitylation, it was recently shown that both Ube2v1 and its partner Ubc13 are overexpressed in breast cancer, ovarian cancer, prostate cancer, and colorectal cancer, as well as in lymphoma [82, 83]. In breast cancer in particular, Ubc13 was identified as a key protein for metastasis spreading to the lung. This action appears to be mediated through TGF-β-induced activation of the TAK1-p38 MAP Kinase cascade. Notably, it was also demonstrated that *in vivo* inhibition of UBE2v1 through RNA interference can prevent breast tumor growth and metastasis formation [84, 85].

The role of Ubc13-Uev1A in tumorigenesis was also suggested by Pulvino et al., in accordance with the finding that small-molecule inhibitor of Ubc13-Uev1A interaction, known as NSC697923, can inhibit proliferation and survival of diffuse large B-cell lymphoma cells via inhibition of the Nf-KB signaling in these cells [86].

Additional proof that K63-linked ubiquitylation is involved in the regulation of tumorigenesis comes from the observation that several E3s and DUBs involved in this particular type of ubiquitylation/deubiquitylation are important regulators of proteins that guide cell cycle, DNA damage, and cell death [87–90]. For a complete review on the role of ubiquitylation on tumor formation and metastasis, refer to the work by Gallo et al. [83].

4. Conclusions

The role of post-translational modification in the regulation of cell behavior in response to diverse stimuli is emerging more and more and provides additional

information regarding layers of regulation in cells. In this scenario, K63 ubiquitylation is starting to play an increasingly important role, since several crucial mechanisms involved in cell-signaling are regulated by this type of post-translational modification.

A vast group of human disorders such as Alzheimer's disease, Parkinson's disease, Type II Diabetes, and cancer have been investigated. In this chapter, we focused on the description of the main intracellular signaling pathways regulated by post-translational modification with respect to K63-linked ubiquitylation. The goal of this overview was to summarize the main findings regarding the main regulatory mechanisms that contribute to the disease pathogenesis or progression.

A better understanding of the different layers of regulation within molecular pathways, including the ubiquitin code, will indeed clear the path to a more precise manipulation of those aberrant post-translational signaling events that cause disease, marking a new era in therapeutic management and personalized medicine.

The specific targets of these modifications, influencing intracellular signaling pathways and cellular behavior, represent the future of target therapy; thus, the investigation of these mechanisms should be further analyzed in depth.

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Conflict of interest

None.

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