Ubiquitylation Pathways In Insulin Signaling and Organismal Homeostasis

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The insulin/insulin-like growth factor-1 (IGF-1) signaling (IIS) pathway is a pivotal genetic program regulating cell growth, tissue development, metabolic physiology, and longevity of multicellular organisms. IIS integrates a fine-tuned cascade of signaling events induced by insulin/IGF-1, which is precisely controlled by post-translational modifications. The ubiquitin/proteasome-system (UPS) influences the functionality of IIS through inducible ubiquitylation pathways that regulate internalization of the insulin/IGF-1 receptor, the stability of downstream insulin/IGF-1 signaling targets, and activity of nuclear receptors for control of gene expression. An age-related decline in UPS activity is often associated with an impairment of IIS, contributing to pathologies such as cancer, diabetes, cardiovascular, and neurodegenerative disorders. Recent findings identified a key role of diverse ubiquitin modifications in insulin signaling decisions, which governs dynamic adaption upon environmental and physiological changes. In this review, we discuss the mutual crosstalk between ubiquitin and insulin signaling pathways in the context of cellular and organismal homeostasis.

1. Introduction

Sustaining a healthy proteome is not only a long-term challenge for individual cells but also for entire organisms since damaged proteins accumulate under proteotoxic conditions caused by environmental and physiological changes. Protein homeostasis or proteostasis is achieved via a conserved network of quality control pathways that support the generation of correctly folded proteins, prevent proteins from misfolding, and remove potentially harmful protein species.[1] Especially during aging, the proteostasis network has a limited capacity, and its impairment causes protein aggregation that deteriorates both cellular and organismal viability.[2] Recent studies identified cell non-autonomous regulation of proteotoxic stress response, suggesting the existence of intricately balanced proteostasis networks important for integration and maintenance of the organismal proteome. In humans, many diseases lead to the ultimate collapse of proteostasis, including cardiovascular, oncological, neurodegenerative, and metabolic disorders. However, disease-related proteostasis defects of therapeutic relevance remain largely unclear.[1,2] Notably, different longevity-promoting pathways provide increased stability of the proteome, delaying the onset of age-related diseases.[3,4]

The insulin/insulin-like growth factor-1 (IGF-1) signaling (IIS) pathway is an evolutionarily conserved genetic program that governs cell growth, tissue development, metabolic physiology, proteostasis, and longevity of multicellular organisms.[4,5] IIS is triggered by insulin, a peptide hormone regulating glucose homeostasis via synthesis, transport, absorption, and degradation systems. Circulating insulin reaches target tissues where it binds to the insulin receptor (INSR).[5] The INSR belongs to the family of cell surface receptors possessing intrinsic tyrosine kinase activity (RTK), including insulin-like growth factor-1 receptor (IGF-1R), and the INSR-related receptor (IRR).[5] The INSR gets activated upon insulin or insulin growth factor-1 (IGF-1) binding, which initiates a complex signaling cascade triggering metabolic and mitogenic effects.[5,6] Upon activation, INSR/IGF-1R stimulates the phosphorylation of insulin receptor substrates (IRS) that in turn recruit and activate phosphatidylinositol-3-kinase (PI3K). Activated PI3K induces an AKT protein kinase signaling cascade that controls the activity of various transcription factors, notably Forkhead box (FOXO) transcription factors, which coordinate cellular differentiation, proliferation, and survival by responsive gene expression programs.[6,7] Therefore, insulin-dependent signaling pathways require precisely balanced regulation to maintain cellular homeostasis. To this end, the activity of IIS components is modulated by a network of multi-layered control pathways including ubiquitin-dependent proteinolysis.[8]

Post-translational protein modification by ubiquitin is essential for cell cycle progression, genome maintenance,
protein homeostasis, and metabolic control. The covalent attachment of ubiquitin to internal lysine residues is a dynamic process mediated by the interplay of three classes of enzymes. The ubiquitin-activating enzyme (E1) utilizes ATP to catalyze the formation of a ubiquitin thioester on its active site followed by its transfer onto the ubiquitin-conjugating enzyme (E2). Subsequently, the E2 interacts with a specific ubiquitin ligase (E3), which provides the scaffold for substrate recognition (Figure 1). The ubiquitylation reaction is highly specific and could either provide individual attachment with one (mono-ubiquitylation) or several (multi-monoubiquitylation) ubiquitin moieties. In addition, polyubiquitylation of substrates is mediated by repetitive conjugation through one of its seven lysine residues (K6, K11, K27, K29, K33, K48, and K63) or via the N-terminal methionine (M1) of ubiquitin itself. The functional consequence of substrate conjugation is mainly defined by the ubiquitylation pattern and lysine-dependent linkage type (Figure 1). For example, chains of four to six ubiquitin moieties linked via K48 of ubiquitin usually promote substrate degradation by the 26S proteasome, while K63 chains are mainly involved in protein trafficking and DNA damage response pathways. Ubiquitylation is further regulated by deubiquitylation enzymes (DUBs), which modulate the size and topology of polyubiquitin chains, thereby affecting the fate of the substrate conjugate.

The ubiquitin/proteasome system (UPS) is a major proteolytic route that coordinates dynamic signal transduction networks of the IIS pathway. The UPS is associated with IIS in multiple ways by mediating degradation of regulatory factors involved in insulin signaling. Conversely, insulin directly influences UPS activity by modulation of ATP production and gene expression. Findings in human skeletal muscle demonstrated that insulin triggered expression of 17 E2 enzymes and 11 proteasomal subunits. Moreover, insulin also stimulates transcription of USP16, a DUB involved in deubiquitylation of the histones H2A and H2B, which could reprogram gene expression even further. Intriguingly, these studies reflect an intricate crosstalk between IIS and UPS important for integration and maintenance of a healthy proteome. In this review, we comprehensively discuss this complex coordination with a special emphasis on particular IIS targets and the regulatory function of E3 ligases.

Figure 1. Schematic representation of the UPS. The UPS targets specific substrates for degradation through the sequential activity of ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin ligases (E3). Substrate ubiquitylation is initiated by an E1 enzyme, which hydrolyzes ATP and forms a high-energy thioester bond between an internal cysteine residue and the C-terminus of ubiquitin. Activated ubiquitin is then passed on to an E2 enzyme, which forms similar thioester-linked complexes with ubiquitin. Finally, ubiquitin is covalently attached mainly to lysine (K) side chains of the substrate protein by target-specific E3 enzymes. Further ubiquitylation of ubiquitin via one of its' seven lysine residues (K6, K11, K27, K29, K33, K48, and K63) promotes the formation of different ubiquitin chains, regulating the fate of the conjugated substrates. For example, K48-linked ubiquitin chains target the protein substrate for degradation by the 26S proteasome, whereas K63-linked chains promote nuclear trafficking and DNA damage response.
2. Central Regulators of IIS are Targeted by Ubiquitylation

Despite several structurally unrelated substrates, ubiquitin conjugation is remarkably selective owing to a large number of specialized E3 enzymes. The detailed analysis of substrate selection and processing mediated by E3 enzymes identified regulatory mechanisms and specific degradation pathways.[10,17] Interestingly, some of the E3 ligases target one or more substrate proteins within the same signal transduction network such as IIS, moderating a prominent role in its spatiotemporal regulation.[18] Besides E3 ligases, insulin-signaling is also controlled by DUBs providing a finely tuned and responsive crosstalk between the UPS and IIS pathways. For instance, the ubiquitin hydrolase activity of ATX-3 (Ataxin-3) regulates the ubiquitylation status and stability of substrates involved in IIS and contributes to longevity in C. elegans.[19] Here, we provide an overview of central factors important for insulin/IGF-1 signaling, ranging from INSR/IGF-1R to downstream FOXO transcription factors, which are regulated by ubiquitylation.

2.1. INSR/IGF-1R

IIS signaling is activated upon ligand binding to the INSR/IGF-1R bound to the plasma membrane. Accordingly, the INSR/IGF-1R is the central regulator of the conserved IIS pathway, and its activity is tightly balanced.[7,20,21] Recent work identified several E3 ligases that regulate INSR/IGF-1R turnover in response to physiological changes. For example, the E3 ligase c-Cbl is recruited by adaptor protein with pleckstrin homology and Src homology 2 domains (AP5) to the INSR and induces its ubiquitin-mediated internalization.[6,22] c-Cbl also associates with and polyubiquitylates IGF-1R through K48-linked chains. However, c-Cbl-mediated ubiquitylation occurred only when cells were stimulated with a high concentration (50–100 ng mL⁻¹) of IGF-1, leading to IGF-1R internalization through the caveolin-dependent route.[23] Independent of insulin-mediated induction, the INSR level is also regulated under basal conditions. It was recently recognized that the E3 ligase MARCH1 ubiquitylates the INSR at the cell surface, which induces its degradation. In turn, insulin-receptor binding downregulates MARCH1 transcription through the FOXO1 transcription factor, which provides an effective feedback loop to trigger IIS in the postprandial state by increased INSR level.[24] Ubiquitylation modulates IIS components distinctively, depending on the tissue type. For instance, the E3 ligase MG53 is reported to target the INSR specifically in muscle cells for ubiquitin-mediated degradation. Defects in this regulatory mechanism could initiate systemic effects such as metabolic and cardiovascular complications.[18]

2.2. IRS Proteins

IRS proteins perform an essential role in transmitting signals from the INSR and IGF-1R to PI3K/AKT kinases.[25–27] Besides the INSR, MG53 also initiates ubiquitin-dependent degradation of IRS-1 in the muscle.[18] In addition to tissue-specific effects, the UPS rewires IIS in response to cellular stressors. For example, oxidative stress is recognized as a major risk factor for inflammation, cancer, and neurodegenerative disorders.[28] Inflammation induces expression of suppressors of cytokine signaling (SOCS) 1 and 3 adaptor proteins, which support the ubiquitin-mediated degradation of IRS-1 and 2 by the Elongin-BC complex.[29] This observation indicates a mechanism of inflammation-associated insulin resistance, abrogating the cellular response and downstream signaling even upon high insulin level.[30] This misregulation is linked to the pathogenesis of type 2 diabetes (T2D).[27–29] Similarly, mammalian target of rapamycin complex 2 (mTORC2) is an important nutrient sensor, and its disruption impairs insulin-induced AKT activation leading to insulin resistance.[31] Upon insulin stimulation, mTORC2 stabilizes Fbw8, the substrate-targeting subunit of the CUL7 based E3 ligase complex, which ubiquitylates IRS-1 for proteasomal turnover. Thus, mTORC2 signaling serves to sense nutrient availability and prevents chronic activation of IIS, which otherwise might cause hyperproliferation and carcinogenesis.[32] In addition to cellular stressors, environmental cues also affect the UPS-dependent regulation of IIS. Removal of gravitational stress leads to muscle wasting induced by weightlessness, which activates ubiquitylation and degradation of IRS-1 by the E3 ligase Cbl-b. Consequently, reduced IRS-1 level results in the activation of FOXO transcription factors, triggering expression of the E3 ligase Atrogin-1/MAFbx. Atrogin-1/MAFbx initiates degradation of prominent proteins such as MyoD, calcineurin, and troponin-I involved in the regulation of striated muscle growth.[33]

2.3. FOXO Proteins

FOXOs represent a well-conserved group of transcription factors regulated by the PI3K/AKT pathway.[20] FOXO activity can be modulated through its non-canonical ubiquitylation by the ubiquitin ligase Atrogin-1. Atrogin-1 modifies FOXO1 and FOXO3a with K63-linked ubiquitin chains in the cardiac tissue, which in turn enhances nuclear translocation and transcriptional activity of FOXO1 and FOXO3a.[34] Another example of stress-related IIS regulation highlights the role of the ubiquitin-specific protease USP7/HAUSP. USP7 performs deubiquitylation of monoubiquitylated FOXO4 inhibiting its nuclear translocation.[35] This activity has been suggested to compensate the oxidative stress induced monoubiquitylation, mediated by the E3 ligase Mouse/Murine Double Minute 2 protein (MDM2).[36] In addition to FOXO4, USP7 also deubiquitylates activated FOXO1 and thereby specifically abrogates the transcription of gluconeogenic genes.[37] Under physiological conditions FOXO1 stimulates gluconeogenesis, however, increased nutrient availability strongly restricts the activity of this metabolic pathway to prevent a toxic increase of glucose level. Therefore, insulin stimulates the expression of the E3 ligase COP1, which ubiquitylates FOXO1 for proteasomal turnover and inhibits gluconeogenesis. This degradation pathway plays a prominent role in the regulation of glucose metabolism specifically in liver.[38] Besides regulating gluconeogenesis, FOXO1 acts as tumor suppressor by inducing growth arrest and apoptosis in malignant cells. The E3 ligase SKP2, a subunit...
of the Skp1/Cul1/F-box protein ubiquitin complex, ubiquitylates phosphorylated FOXO1 and promotes its degradation, thereby inhibiting the tumor suppression function of FOXO1 leading to cell transformation.

A recent study on *C. elegans* shows that E3 ligase RLE-1, a homolog of mammalian Roquin, catalyzes the ubiquitin-mediated degradation of DAF-16/FOXO. Consequently, removal of RLE-1 extends lifespan in worms. In contrast, the deubiquitylating enzyme MATH-33, a close ortholog of mammalian USP7, associates with ubiquitin-conjugated DAF-16 and counteracts the role of RLE-1, promoting longevity via DAF-16 stability in response to decreased IIS. On the other hand, DAF-16 contributes to the regulation of proteasome activity. DAF-16 was shown to affect the expression of the deubiquitylating enzyme UBH-4, which functions as a tissue-specific inhibitor of the 26S proteasome. Consecutively, depletion of UBH-4 leads to proteasome activation and increased lifespan in *C. elegans*. The role of UBH-4 seems to be well-conserved, as downregulation of its human ortholog, UCHL-5, boosts proteasome operations and degradation of damaged proteins. Although numerous studies point to the roles of UPS and DAF-16 in lifespan regulation, additional studies are required to understand the conservation of crosstalk between UPS and FOXOs in promoting longevity.

### 2.4. PTEN

Phosphatase and tensin homolog (PTEN), a well-studied tyrosine phosphatase is a key negative regulator of PI3K/AKT signaling and another element of the IIS tightly controlled by the UPS. WWP2, an E3 ligase belonging to the NEDD4-like protein family, ubiquitylates PTEN and promotes its proteasomal turnover. PTEN degradation is counter-balanced by phosphorylation, which extends its cellular half-life by protecting against WWP2. PTEN is also under the control of other ligases, and the details regarding this relationship are described in the paragraphs dedicated to the main E3s modulating the IIS.

### 2.5. AKT

Besides regulating PI3K/AKT signaling by PTEN degradation, the UPS also supports the recruitment of AKT for IIS pathway function. Upon IGF-1 stimulation, the E3 ligase TRAF6 promotes K63-linked ubiquitylation of AKT, which induces its plasma membrane sorting and subsequent phosphorylation by PDK1 and mTORC2, resulting in full activation of AKT function. UPS activity also modulates the level of nuclear AKT. For instance, the E3 ligase TUC3 recognizes phosphorylated AKT in the nucleus and targets it for proteasomal degradation. Dysregulation of this process has been linked to the development of clinical manifestations of Down syndrome.

### 2.6. Nrf2/SKN-1

Nrf2 is the transcription factor downstream of IIS that mainly induces oxidative stress protection programs. The Nrf2 level is under the tight control of the adaptor protein Keap1 associated with a Cullin3-type E3 ligase complex, which promotes ubiquitylation and degradation of cytoplasmic Nrf2. Nrf2 could also be negatively regulated in the nucleus independent of Keap1. A recent study in *C. elegans* sheds light on this mechanism. Under stress conditions, SKN-1, a member of the Nrf family, translocates and accumulates in the nucleus, where it stimulates expression of anti-oxidant and stress response genes, promoting proteostasis and longevity. In the absence of stress, however, the SKN-1 level is controlled by the E3 ligase WDR-23, which together with the CUL-4/DDB-1 E3 ligase complex target the transcription factor for proteasomal degradation. Loss of WDR-23 function causes elevated SKN-1 protein levels and its accumulation in the intestinal nucleus, which in turn results in increased stress resistance and longevity.

### 3. E3 Ligases Trigger the Crosstalk Between Ubiquitin and Insulin Signaling

So far we discussed the regulation of central nodes within the IIS pathway governed by ubiquitin. Interestingly, the main E3 ligases involved in this coordination usually target more than one substrate. These data suggest the existence of a multi-layered network of ubiquitylation pathways guided by E3 ligases, which rewires insulin-signaling decisions for spatiotemporal adaptation in response to environmental and physiological cues (Figure 2).

#### 3.1. MDM2

E3 ligase MDM2 is well known for its role in p53 regulation, a prominent tumor suppressor protein. MDM2 is also one key factor modulating IIS activity. MDM2 triggers ubiquitylation and degradation of the IGF-1R by entailing β-arrestin-1 as an adaptor protein under both basal and IGF-1 stimulated conditions. The concentration of MDM2 in the cytosol can be influenced by PI3K/AKT signaling and its interaction with p53. Accordingly, degradation of the IGF-1R is antagonized by an increased level of p53 in the nucleus, which may determine the amount of cytoplasmic MDM2 that is available for the IGF-1R.

Besides IGF-1R, MDM2 also regulates the cellular amount of IRS-1. Chronic insulin binding to the INSR leads to desensitization of insulin signaling in part by stimulating MDM2-dependent ubiquitylation and degradation of IRS-1. Similar to MDM2/β-arrestin-1-mediated turnover of IGF-1R, IRS-1 processing also involves β-arrestin-1. However, in this case, β-arrestin-1 interrupts the process of IRS-1 degradation through competitive binding to MDM2 for becoming its substrate. Insulin stimulation leads to constitutive phosphorylation of β-arrestin-1, which prevents MDM2 binding. Therefore, chronic insulin treatment triggers constitutive β-arrestin-1 phosphorylation, causing IRS-1 degradation and decreased insulin sensitivity.

Along with upstream components of the IIS, MDM2 is also reported to serve as a general E3 ligase for various FOXO proteins including FOXO1, FOXO3a, and FOXO4. MDM2
promotes both mono- and polyubiquitylation, which initiates nuclear sorting or degradation of FOXO proteins, respectively. The regulation of FOXO proteins by MDM2 varies depending on the proteins’ phosphorylation state. Hence, AKT phosphorylation of FOXO induces MDM2 mediated polyubiquitylation and degradation. Conversely, in response to oxidative stress, MDM2 monoubiquitylates non-phosphorylated FOXO, thereby activating and promoting its nuclear localization. Taken together, MDM2 appears to control the switch between the growth arrest and apoptotic signals of p53 and the metabolic and anti-apoptotic signals of IIS.

3.2. NEDD4

Neural precursor cell-Expressed Developmentally Downregulated gene 4 (NEDD4) is another critical E3 ligase promoting the coordination of IIS. Although NEDD4 has three WW domains for substrate binding and a C2 domain that associates with phospholipids for translocation to subcellular membranes, it requires Grb10 to interact with the IGF-1R. Grb10 is a prominent adaptor protein that binds the INSR and IGF-1R upon insulin/IGF-1 stimulation. Studies on mouse embryonic fibroblasts (MEFs) reported that IGF-1R is subjected to NEDD4-dependent ubiquitylation and internalization with the help of Grb10. On the other hand, NEDD4 knockout MEFs displayed an increased abundance of Grb10 and reduced levels of IGF-1R at the cell surface. This effect is rescued by depletion of Grb10, demonstrating that Grb10 is a negative regulator of IGF-1R when overexpressed. In the absence of NEDD4, Grb10 might be associated with another E3 ligase, resulting in the ubiquitin-mediated turnover of IGF-1R. However, the mechanism of Grb10 regulation by NEDD4 and the identity of a yet unknown E3 ligase remains to be elucidated.

Phosphorylation of IRS-1/2 by IGF-1R is vital for IGF signaling. Fukushima and colleagues have identified NEDD4 as a positive regulator of IRS-2. NEDD4 induces IRS-2 monoubiquitylation, which promotes its association with Epsin1, a ubiquitin-binding protein of the endocytosis machinery. Epsin1 allows efficient recruitment of IRS-2 to the plasma membrane, where it can be phosphorylated by the IGF-1R, thereby enhancing IRS-2-mediated signaling and cell proliferation. Further data support the positive role of NEDD4 in IIS modulation. Namely, NEDD4 enhances IGF-1-induced IIS by antagonizing PTEN. Besides polyubiquitylation, NEDD4 monoubiquitylates PTEN and promotes its nuclear localization and stabilization rather than turnover by the proteasome. This alternative conjugation state is essential for the PTEN tumor suppressor

Figure 2. Multilayered regulation of IIS by the E3 ligases CHIP, MDM2, and NEDD4. CHIP initiates the turnover of INSR, PTEN, AKT, and FOXO1. Similarly, MDM2 promotes degradation of IGF-1R, IRS-1, and FOXO proteins. Likewise, NEDD4 induces IGF-1R, IRS-2, PTEN, and AKT degradation or modulates their activity. The regulation of insulin signaling is indicated by black arrows, protein degradation pathways with red arrows, and ubiquitin-mediated activation with green arrows. Black and red circles represent ubiquitin and phosphate molecules, respectively.
activity.\(^{[71,72]}\) Contrary to these results, PTEN displayed regular stability and nuclear sorting in NEDD4 depleted MEFs, which questions whether NEDD4 is the only regulator of PTEN function.\(^{[63,73]}\) Different model systems and experimental procedures might cause these apparent discrepancies. A recent study on the mammalian nervous system reports that PTEN can modulate NEDD4 translation by antagonizing mTORC1 activity during neurite outgrowth.\(^{[74]}\) Due to the controversy over the role of NEDD4 in PTEN regulation, additional explanatory studies are needed to understand the cellular cues modulating the tissue-specific cross-talk between NEDD4 and PTEN. In addition to IGF-1R and IRS, NEDD4 is also found to regulate AKT function. NEDD4 conjugates the plasma membrane-bound p-AKT with K63-linked polyubiquitin chains, which stabilizes the kinase and induces its nuclear sorting, promoting cell growth and mitogenic activity.\(^{[47,75]}\)

### 3.3. CHIP

Proteostasis is supported by coordination of folding and removal of damaged proteins.\(^{[76,77]}\) The conserved E3 ligase C-terminus of Hsc70-interacting protein (CHIP/STUB1) is central to this cross-talk, acting along with molecular chaperones and ubiquitin-dependent degradation pathways to maintain a healthy proteome.\(^{[78]}\) CHIP provokes the ubiquitylation of non-native proteins consigned by chaperone partners to induce disposal through endocytic-lysosomal pathways,\(^{[79,80]}\) proteasomal degradation,\(^{[81,82]}\) autophagy,\(^{[83]}\) and CHIP boosts the proteotoxic stress response by activating the transcription factor HSF-1 (heat shock factor 1) and regulating the level and activity of general chaperones.\(^{[78,84]}\) In addition to its quality control function, CHIP is also demonstrated to control a myriad of proteins in various signaling pathways.\(^{[85]}\)

Recently, we revealed an essential role of CHIP-mediated proteolysis in the regulation of IIS, which determines lifespan, stress responses, and metabolism in metazoan organisms.\(^{[86]}\) We identified the INSR as a direct target of CHIP, which mediates monoubiquitylation and subsequent endocytic-lysosomal turnover of the receptor. Accordingly, INSR is stabilized either by mutations in the ubiquitylation sites of the receptor or depletion of the CHIP activity. CHIP recognizes and ubiquitylates INSR independent of the chaperones, thus distinguishing INSR regulation from chaperone-assisted quality control. CHIP deficiency results in increased INSR level that leads to premature aging in various organisms supporting an evolutionarily conserved regulation mechanism.\(^{[86]}\)

The detrimental effects of the increased INSR level are mainly due to a PI3K/AKT signaling.\(^{[87,88]}\) As described earlier, PI3K/AKT signaling suppresses FOXY-mediated transcription, which consequently leads to a reduced expression of longevity genes.\(^{[4]}\) PI3K/AKT dysregulation is involved in cellular transformation,\(^{[89]}\) suggesting that this signaling pathway is controlled by the tumor suppressor PTEN.\(^{[44]}\) However, in some cases, somatic mutations of PTEN lead to continuous and uninterrupted PI3K/AKT signaling causing tumorigenesis.\(^{[90,91]}\) Thus, PTEN represents a key factor in governing IIS, making it a prime candidate for tight regulation. Essentially, CHIP is shown to maintain a physiological level of PTEN by regulating its chaperone-mediated ubiquitylation and proteasomal degradation.\(^{[92]}\) Accordingly, CHIP overexpression activates the PI3K/AKT pathway, which promotes cancer cell growth.\(^{[93]}\)

Although, CHIP indirectly influences AKT signaling through its direct targets such as the INSR and PTEN. AKT is also a substrate of the CHIP E3 ligase, which marks AKT for ubiquitinated degradation by the 26S proteasome.\(^{[94]}\) CHIP ubiquitylates AKT independent of its phosphorylation state, although activated p-AKT has increased affinity towards CHIP and thus higher degradation rate.\(^{[95]}\) Remarkably, transcription of CHIP is also modulated in response to changes in AKT level,\(^{[94]}\) showing a tight feedback regulation between CHIP activity and insulin signaling. Similar to AKT regulation, CHIP indirectly impacts on FOXOs function on various levels through modulation of upstream IIS substrates. CHIP also promotes ubiquitin-mediated degradation of Ser-256 phosphorylated FOXO1 in smooth muscle cells (SMCs) supporting the growth and proliferation of SMCs.\(^{[96]}\)

### 4. Insulin Signaling Decisions are Regulated by Substrate Selection and Processing

Given the growing list of IIS pathway components regulated by ubiquitylation, common principles of substrate recognition and processing largely remain unclear. Selectivity by E3 enzymes depends on various properties including protein level, structural motifs, and post-translational modifications of substrate proteins. Structural studies have revealed binding sites and catalytic domains that facilitate E3 ligase function, especially in IIS regulation.\(^{[97]}\) Some IIS substrates are recognized by substrate-specific adaptor molecules similar to the regulation of COP1, which ubiquitylates transcription factors such as FOXO1, c-Jun, and ETS for degradation.\(^{[38,56,99]}\) The substrate binding region of COP1 resides within a WD40-repeat-containing domain and binds to Trib proteins, which act as adaptors for proteasomal degradation of the C/EBPa transcription factor.\(^{[100]}\) This adaptor-ligase system has also been identified for NEDD4-Grb10, MDM2-β-arrestin-1, and the E3 ligase c-Cbl-APS.\(^{[22,54,61]}\) Here, the adaptor proteins effectively assist in the selection process of dynamically changing substrates. Future research should further characterize additional adaptor proteins involved in IIS regulation, particularly specific to the cell-type and physiological status of an organism.

Besides the role of substrate-targeting adaptors, some E3 ligases directly target substrate proteins for degradation by recognition of specific degradation signals or degrons, which could be short amino acid sequences or structural motifs. The E3 ligase NEDD4 contains WW domains, which bind to proline-rich regions or the UBR-box family of E3 ligases recognize N-degrons specified by the N-terminal amino acid residues of substrate proteins.\(^{[101,102]}\) In fact, the E3 ligase CRL7 was shown to ubiquitylate a hyper-phosphorylated form of IRS1.\(^{[103]}\) Since many enzymes and receptors of the IIS pathway are activated/deactivated by phosphorylation, it is intriguing to speculate that these signaling events modulate phospho-degrons and substrate stability. Therefore, elucidation of specific E3/degron interactions would help to further characterize spatiotemporal regulation events important for IIS integrity. On the other hand, insulin-signaling influences the activity of different E3 ligases...
including CHIP, NEDD4, and Atrogin-1, which are regulated by AKT, PTEN, and FOXO3a providing a tight feedback loop.\(^{33,74,94}\)

Different modes of ubiquitylation can offer various ubiquitin signals defined by the ubiquitylation pattern and lysine-dependent linkage type leading to different substrate fates (Figure 1). Monoubiquitylation of the FOXO transcription factors triggers stabilization and nuclear localization, whereas polyubiquitylation is mainly responsible for FOXOs degradation. Likewise, conjugation of FOXO1 and FOXO3 with K63-linked ubiquitin chains by Atrogin-1 promotes transcriptional coactivation ultimately reducing pathological cardiac hypertrophy.\(^{34}\) A similar mode of regulation also applies to other IIS substrates. For example, AKT ubiquitylation by CHIP triggers proteasomal degradation whereas the conjugation with K63-linked ubiquitin chains by NEDD4 promotes nuclear translocation of AKT.\(^{75,95}\) Similarly, IRS proteins are regulated by the E3 ligases MDM2 and NEDD4. While NEDD4 enhances IRS activation, MDM2 promotes its turnover via polyubiquitylation.\(^{56,68}\) One should also underline the importance of E2s for ubiquitin chain assembly. Recent reports point to the key contribution of E2 enzymes in substrate processing defining the length and topology of the ubiquitin chain.\(^{104}\) However, more studies are needed to understand the regulatory role of E2 proteins in insulin signaling.

Some ubiquitin ligases are responsible for the regulation of IIS exclusively in specialized tissues. For example, the mammalian ubiquitin ligase MG53 is highly expressed in both cardiac and skeletal muscle.\(^{105}\) In muscles, MG53 modulates myogenesis through ubiquitylation and degradation of IRS-1, negatively regulating insulin-like growth factor and insulin signaling.\(^{105}\) MG53 also induces myocardial insulin receptor and IRS-1 degradation, and subsequently causes insulin resistance and metabolic syndrome,\(^{18,105}\) leading to diabetic cardiomyopathy.\(^{106}\) Furthermore, the E3 ubiquitin ligase MARCH1 influences cellular insulin sensitivity in hepatocytes and white adipocytes by controlling insulin receptor levels, whereas the FOXO1-targeting E3 ligase COP1 plays a role in the regulation of hepatic glucose metabolism.\(^{24,38}\) Since the list of IIS related substrates is continuously increasing, further characterization of ubiquitin-dependent insulin signaling events will be indispensable to delineate common regulatory principles.

### Table 1. List of E3 ligases that regulate IIS proteins and their functional impact.

| E3 ligases               | IIS substrate | Effect on lifespan | Functional impact            | References |
|-------------------------|---------------|--------------------|-------------------------------|------------|
| CHIP                    | INSR          | Loss of function   | Lysosomal degradation         | [84]       |
|                         | PTEN          | Loss of function   | Proteasomal degradation       | [92]       |
|                         | AKT           | Loss of function   | Proteasomal degradation       | [93]       |
|                         | FOXO1         | Loss of function   | Proteasomal degradation       | [94]       |
| MDM2                    | IGF-1R        | Unknown            | Proteasomal degradation       | [76]       |
|                         | IRS-1         | Unknown            | Proteasomal degradation       | [74]       |
| FOXP1-FOXO3a-FOXO4      |               | Unknown            | Protonasomal degradation/ Nuclear trafficking | [58,59]   |
| NEDD4                   | IGF-1R        | Unknown            | Proteasomal degradation       | [62,66]   |
|                         | IRS-2         | Unknown            | Plasma membrane recruitment   | [68]       |
|                         | PTEN          | Unknown            | Protonasomal degradation/ Nuclear trafficking | [71,72]   |
|                         | AKT           | Unknown            | Nuclear trafficking           | [73]       |
| APS and c-Cbl           | INSR          | Unknown            | Receptor internalization      | [22]       |
|                         | IGF-1R        | Unknown            | Receptor internalization      | [23]       |
| MARCH-1                 | INSR          | Unknown            | Polyubiquitin mediated degradation | [24]       |
| MG53                    | INSR          | Unknown            | Proteasomal degradation       | [18]       |
|                         | IRS-1         | Unknown            | Proteasomal degradation       | [20]       |
| SOCS1/3 and elongin BC  | IRS-1/2       | Unknown            | Proteasomal degradation       | [29]       |
| CUL7                    | IRS-1         | Unknown            | Proteasomal degradation       | [29]       |
| Cbl-b                   | IRS-1         | Unknown            | Proteasomal degradation       | [29]       |
| MAFbx/Atrogin-1         | FOXP1-FOXO3a  | Unknown            | Nuclear trafficking           | [54]       |
| SKP2                    | FOXP1         | Unknown            | Proteasomal degradation       | [30]       |
| COP1                    | FOXP1         | Unknown            | Proteasomal degradation       | [30]       |
| RLE-1                   | DAF-16        | Loss of function   | Proteasomal degradation       | [40]       |
| WWF2                    | PTEN          | Unknown            | Proteasomal degradation       | [41]       |
| TRAF6                   | AKT           | Unknown            | Plasma membrane recruitment   | [46]       |
| TTC3                    | AKT           | Unknown            | Proteasomal degradation       | [47]       |
| CUL3-KEAP1              | NRF-2         | Unknown            | Proteasomal degradation       | [48]       |
| CUL4-DDB-1 and WDR23    | SKN-1         | Loss of function   | Proteasomal degradation       | [48]       |
5. Conclusion and Outlook

Insulin-signaling decisions require precise control of central factors through regulation of gene expression, spatiotemporal activity, and protein turnover. Recent studies in the field have identified a central role of the UPS in directing insulin/IGF-1 inducible programs to maintain metabolism, proteostasis, and longevity upon environmental and physiological changes. In this context, IIS function is modulated through the balanced interplay between E3 ubiquitin ligases and deubiquitylation enzymes, which regulate the abundance and/or activity of the insulin/IGF-1 receptors, downstream effector proteins, and nuclear receptors (Table 1). The regulation of IIS by different E3 ligases provides highly dynamic response mechanisms depending on the type of ubiquitylation signals (Figure 1),[9,11] cellular environment,[29] tissue-specific expression,[38] and stress-inducible conditions.[33] IIS and UPS are intricately intertwined, and dysregulation triggered by elevated stress conditions, aging, or other environmental insults often results in pathological consequences such as cancer, neurodegeneration, muscle wasting, T2D, and obesity.[1,107] Accordingly, aberrant activation of AKT signaling is observed in several cancer types.[80,108] CHIP-dependent ubiquitylation modulates AKT levels and thus provides tumor suppressive functions.[109] Therefore, CHIP could be a potential drug-target for cancer interventions. Although the impact of ubiquitylation in controlling IIS pathway components is compelling, the intricate crosstalk between IIS and UPS complicates the design of therapeutic implications. Remarkably, recent technological advances helped to develop small molecules, modified ubiquitin variants, and highly selective inhibitors targeting E2s, E3s, and DUBs.[110,111] Further studies on substrate selection mechanisms, substrate processivity, and tissue specificity of individual E3 ligases will help to provide valuable insights into the IIS-UPS interaction network and might lead to the development of novel therapeutic strategies.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

aging, CHIP, diabetes, DUBs, E3 ligase, insulin signaling, oncogenesis, proteostasis, ubiquitin

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