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From Trash Collectors to Guardians of Cell Signaling and Immune Homeostasis

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Ubiquitination, the covalent attachment of ubiquitin molecules to target proteins, occurs rapidly and often reversibly, and regulates a vast array of protein-protein interactions that underlie cellular immune responses (1). Ubiquitination is best known for mediating protein interactions with degradative proteasomes, thereby dictating protein stability. This critical function was recognized by the Nobel Prize in Chemistry in 2004. Recent studies indicate that ubiquitination also mediates a broad array of non-proteolytic interactions between proteins that regulate protein trafficking, transcription factor complexes, and signal transduction (2). In this volume, the review articles utilize the lens of individual ubiquitin ligase enzymes that regulate ubiquitination of signaling proteins in immune cells.

While other post-translational modifications typically alter proteins in a binary fashion (e.g. phosphorylated vs. non-phosphorylated), ubiquitination occurs in multiple biochemical conformations, thereby affording greater specification of protein fate. Ubiquitination involves chains of ubiquitin molecules linked via one of the seven lysines or the N-terminal methionine on ubiquitin. All eight conformations of ubiquitin chains exist in cells, and attachment of single ubiquitin molecules to target proteins has also been described. Importantly, the biochemical complexity of ubiquitination confers diverse biological consequences upon modified targets. Distinct types of polyubiquitin chains are recognized by different ubiquitin binding proteins, or ubiquitin sensors. For example, conjugation of proteins with K48-linked ubiquitin chains recruits degradative proteasomes, while proteins conjugated with K63-linked or M1-linked chains typically recruit other signaling proteins with the capacity to bind these chains (3, 4) (*Fig. 1*).

The complex biology of ubiquitinated signaling complexes is further enhanced by recent evidence that individual proteins can be ubiquitinated with different types of polyubiquitin chains. The receptor-interacting protein-1 (RIP1), implicated in tumor necrosis factor (TNF) and Toll-interleukin-1 receptor (TIR)-domain-containing adapter-inducing interferon- β (TRIF)-dependent Toll-like receptor (TLR) signaling, may undergo initial ubiquitination with K63-linked chains followed by ubiquitination with K48-linked chains (5). TNF receptor-associated factor (TRAF) proteins, discussed in this volume, can also undergo conjugation with both K48-linked and K63-linked polyubiquitin chains (6). In addition, recent data suggest that myeloid differentiation factor 88 (MyD88), interleukin-1 receptor-

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associated kinase 1 (IRAK1), and IRAK4 undergo ubiquitination with K63-linked chains that in turn trigger subsequent ubiquitination with MI-linked (so-called 'linear' ubiquitin chains) (7). These observations raise the notion that individual signaling proteins can be modified with combinations of ubiquitin chains that may facilitate segregation of ubiquitinated targets toward distinct signaling complexes as well as affording temporal control. In this regard, one can visualize ubiquitinated signaling complexes as branched signaling nodes rather than simply steps in linear pathways.

Building specific types of ubiquitin chains on target proteins requires collaboration between a common E1 enzyme, specific E2 ubiquitin ligase enzymes, and E3 ligases. E2 enzymes, of which ~35 have been identified in higher eukaryotic genomes, have been shown to preferentially build certain types of polyubiquitin chains. By contrast, over 600 E3 ligases are predicted in the human genome, and these enzymes play major roles in conferring target specificity. Studies of genetically engineered mice lacking specific E2 or E3 enzymes have revealed immune consequences ranging from lethal inflammation to profound immunodeficiency, affecting innate and adaptive immune cells. The specific roles of some of these enzymes are highlighted in the articles of this volume. In addition, deubiquitinating enzymes (DUBs) perform critical functions in restricting ubiquitination, and ubiquitin binding proteins (or ubiquitin sensors) play critical roles in recognizing ubiquitinated substrates. While these latter two classes of proteins are not reviewed in this volume, their contributions to the regulation and specification of ubiquitin-dependent signaling are critical.

One of the earliest links between ubiquitination and T-cell functions was the recognition that the Itch E3 ligase restricts T-helper 2 (Th2) cell functions *in vivo* (8). Subsequent studies expanded Itch functions to supporting degradative ubiquitination of a number of T-cell signaling molecules. This topic is discussed by Aki *et al.* (9) in this volume. Itch targets include JunB and Forkhead transcription factors (e.g. Foxo1), as well as proximate T-cell receptor (TCR) signaling proteins such as TCR ζ . This diversity of targets raises one of the central unsolved questions in ubiquitination: how E3 ligases recognize their physiological substrates. This general question has not yielded to primary sequence analyses, and no clear consensus ubiquitination sites have been defined on target proteins. Studies of Itch-deficient T cells have suggested that Itch supports K48-linked (degradative) ubiquitination of proteins that propagate immune signals. Hence, Itch appears to restrict immune signals in these cells.

The spontaneous phenotype of mutant mice lacking the Itch protein, *itchy* mice, demonstrates the importance of this E3 ligase in basal immune homeostasis as well as explicit immune responses. Indeed, studies with mice lacking other E3 ligases indicate that maintaining physiological immune homeostasis is a function shared by multiple E3 ligases. As ubiquitination of signaling proteins regulates the activity of signaling cascades and transcriptional responses, these observations indicate that E3 ligases are actively involved in regulating immune signaling during basal conditions *in vivo*. Itch is expressed in multiple cell types, and the cell type-specific functions of Itch have been studied in several lines of Itch^{Flox} mice. These studies reveal a variety of Itch dependent cellular functions in T cells, regulatory T cells (Tregs), follicular T helper cells (Tfh), B cells, and myeloid cells. Some of these functions may explain how Itch functions to regulate human autoimmune diseases such as asthma.

The importance of studying ubiquitination in cell type specific contexts are also highlighted by studies of Tregs (10). Tregs may exhibit distinct dependence on ubiquitin-dependent TCR and proliferation signals. In addition, ubiquitination may regulate stability of FoxP3, a key transcription factor maintaining Treg functions. Given the potent immunoregulatory roles of Tregs, modulating ubiquitin-dependent signals in these cells can have dominant physiological consequences.

TRAFs are a family of six proteins including several E3 ligases. They were originally described as adaptors associated with TNF receptor family members (e.g. TNFR, CD40) and have subsequently been associated with TLRs, IL-1 receptor, TCRs and B-cell antigen receptors (BCRs), and others. TRAFs have complex roles in ubiquitination. TRAFs regulate cellular activation signaling cascades via nuclear factor κ B (NF κ B) and mitogen-associated protein kinases (MAPKs), and also regulate cell survival signals. However, the mechanisms by which TRAFs perform these functions are incompletely understood and may vary considerably between TRAF family members. For example, it remains unclear which TRAFs regulate signaling by directly performing E3 ligase activity, whether and when TRAFs homo- or hetero-oligomerize with other TRAFs to modulate their ligase activities, and/or whether TRAFs perform ligase-independent functions. In addition, some TRAFs become ubiquitinated during cell signaling, implying a complex interplay between TRAF-dependent ubiquitination and ubiquitin-dependent TRAF function.

Lin *et al.* (11) describe the roles of TRAF3 in B-cell activation and tumorigenesis, and the roles of TRAF3 and TRAF2 are discussed in the context of non-canonical NF κ B signaling by Yang *et al.* (12). TRAF3 mediates signaling downstream of lymphotoxin- β receptor, CD40, B-cell activating factor belonging to the TNF family (BAFF), and BCRs in B cells. It is unclear whether TRAF3 regulates signaling by directly performing E3 ligase activity, whether TRAF3 hetero-oligomerize with TRAF2 or other TRAFs to modulate their ligase activities, and whether TRAF3 performs other critical signaling functions that are independent of ubiquitination. In addition, TRAF3 itself can undergo ubiquitination with at least two types of polyubiquitin chains: K48- and K63-linked chains. These two types of modifications are proposed to facilitate distinct signaling outcomes for TRAF3 signaling complexes. These proteins regulate NIK signaling in dendritic cells and T cells, in addition to B cells. Negative regulatory functions for TRAF3 in TLR signaling are apparent in innate immune cells, and these dichotomous functions raise the issue of differential functions of non-canonical and canonical NF κ B signaling.

Choi *et al.* (13) review the diverse functions for another TRAF family member, TRAF6, in several immune cell types. While TRAF6 broadly resembles TRAF3 in supporting the propagation of NF κ B and MAPK signals, the phenotypes of mice and of immune cells lacking TRAF6 differ considerably from those lacking TRAF3. These differences suggest that TRAF family proteins, like other protein families, have diverged to perform more specialized functions in immune cells. Studies with mice bearing lineage specific deletions of TRAF6 have confirmed key roles for TRAF6 in supporting TLR, IL1R and other receptor triggered signaling cascades. These activities are thought to be due to TRAF6's ability to collaborate with Ubc13/Uev1A to generate K63-linked polyubiquitin chains and activate TAK1, NF κ B, and MAPK signaling (2). These studies also revealed surprising phenotypes

that were not entirely predicted from *in vitro* signaling studies. These include hyper-activated phenotypes in the very immune cells that bear TRAF6 deficiency. Hence, TRAF6 appears to play immunosuppressive as well as immunostimulatory roles in a cell autonomous fashion. How TRAF6-dependent signaling complexes perform these diverse functions is one of several important open questions.

Pellinos are a family of three E3 ligases that, like TRAFs, exhibit both immunostimulatory and immunosuppressive functions. Moynagh (14) discusses the roles of Pellinos in TLR signaling, and Li *et al.* (15) review the functions of this protein family in several immune cell types. During TLR signaling, Pellinos appear to interact with IRAK-1, IRAK-4, TRAF6, and TAK-1. Pellinos exhibit E3 ligase activity *in vitro* as well as *in vivo*. Interestingly, mice expressing a ligase-dead mutant form of Pellino1 as a knockin allele reveal that some but not all Pellino1 functions appear to require its E3 ligase activity. This observation highlights the capacity of *bona fide* E3 ligases to perform ligase independent biochemical actions. These genetic tools should prove useful in dissecting these functions moving forward and may shed insights into how other E3 ligases regulate signaling. Pellinos appear to restrict antigen receptor signals in B and T lymphocytes and to support innate immune signals in myeloid cells. Thus, Pellino targeted therapies could lead to complex immunomodulation.

Suppressor of cytokine signaling (SOCS) proteins include a family of eight E3 ligases that inhibit intracellular JAK/STAT signals triggered by cytokines and growth factors. Lessons from studies of these proteins are discussed by Linossi and Nicholson (16). Physiological consequences of SOCS deficiency range from profound inflammation to subtle perturbations in immune homeostasis. SOCS proteins share a SOCS box motif that facilitates binding to ElonginC and Cullin5. Together with Rbx2, this complex supports degradative (K48 chain linked) ubiquitination of substrates such as phosphorylated signal transducer and activator of transcription (STAT) proteins. SOCS proteins contain SH2 domains that help recognize phosphopeptides on target STAT proteins. SOCS proteins also contain kinase inhibitory regions (KIRs) that can block JAK substrate binding to JAKs. How these motifs mediate the biochemical mechanisms of inhibiting JAK/STAT signaling vary between SOCS family members. Hence, like TRAFs and Pellinos, SOCS proteins may regulate signaling via both E3 ligase-dependent and -independent mechanisms. How SOCS proteins are recruited to specific substrates is also incompletely understood. Nevertheless, the recruitment of E3 ligase complexes to phosphorylated substrates highlights the interplay between phosphorylation and ubiquitination—a theme that recurs several signaling cascades. In addition, SOCS proteins extend the ubiquitin-dependent regulation of immune signaling from NFκB and MAPKs to JAK/STAT cascades.

In addition to regulating cell signaling, ubiquitination regulates protein trafficking and receptor recycling. Oh and Shin (17) discuss the ubiquitin-dependent regulation of major histocompatibility complex class II (MHCII) expression on cell surfaces. MHCII molecules are ubiquitinated by membrane-associated RING-CH1 (MARCH1), an E3 ubiquitin ligase that belongs to a family of MARCH proteins. MHCII ubiquitination at a single lysine exhibits strong effects on MHCII surface expression. Other MARCH proteins can also

ubiquitinate MHCII, and MARCH-dependent ubiquitination of transmembrane receptors may play a broader role in immune cells.

In addition to regulating cellular activation signals, ubiquitination regulates cell death signaling. Given the importance of lymphocyte selection during primary differentiation and after antigen exposure, cell death is an important component of immune homeostasis. In addition, the susceptibility of non-lymphoid cells to die in the setting of inflammation may be a significant factor in disease. Justus and Ting (18) discuss RIP1 ubiquitination, a molecular node that influences NF κ B signaling versus death signaling downstream of the TNF receptor. The cellular IAP ligases (cIAP1, cIAP2) are thought to mediate RIP1 ubiquitination, and the deubiquitinating enzyme CYLD restricts RIP1 ubiquitination. RIP1 ubiquitination appears to prevent RIP1 recruitment to caspase 8 as well as RIP1 kinase-dependent activation of RIP3. Ubiquitination also regulates other steps in death signaling (19). These studies highlight the importance of ubiquitination in regulating the fate of signaling complexes and cellular outcomes.

K63-linked chains were the first non-K48 linked (and non-degradative) polyubiquitin chains to be defined in cell signaling (2), and Ubc13/Uev1a was the heterodimeric E2 enzyme associated with this K63 chain modified protein complex. Among the E2 enzymes, Ubc13 tends to build K63 linked polyubiquitin chains. Wu and Karin (20) discuss the physiological functions of Ubc13. Questions that remain to be answered include how Ubc13/Uev1a selectively build K63 chains, and how E2 enzymes selectively interact with some but not all E3 ligases. As E2 ubiquitin ligase enzymes are far less numerous (~35) than E3 ligases, E2 enzymes typically collaborate with dozens of E3 ligases to build polyubiquitin chains on target proteins.

As noted earlier, the conformation of linkages within polyubiquitin chains (e.g. K48, K63, M1 linkages) dictates the ubiquitin-binding proteins, or ubiquitin sensors, that are recruited to ubiquitinated substrates. A number of recent studies has identified a linear ubiquitin assembly complex (LUBAC), comprised of HOIL, HOIP, and SHARPIN proteins, as being the critical E3 ligase complex that assembles polyubiquitin chains linked via the N-terminal methionine of ubiquitin (M1-linked, or 'linear' ubiquitin chains) (4). Articles by Sasaki and Iwai (21), Shimizu *et al.* (22), Elton *et al.* (23), and Ikeda (24) review the biochemical and physiological aspects of M1 or linear ubiquitin chain biology. Linear ubiquitin chains appear to be formed predominantly if not exclusively by the LUBAC complex, rendering them genetically accessible. Intriguingly, HOIP interacts with K63-linked ubiquitin chains, and linear chains are built on K63 ubiquitin modified IRAK1, MyD88, and IRAK4 proteins (7). Proteins modified with both K63-linked and linear ubiquitin chains—both non-degradative signals—could provide a biochemical substrate for signaling nodes that coordinate downstream signaling events. Linear chain specific DUBs, e.g. Otulin, have also been identified. This DUB binds to LUBAC complexes, potentially limiting the formation of linear ubiquitin chains. Phenotypes of mice lacking distinct LUBAC components appear to differ slightly but significantly, providing possible avenues to understanding how these proteins collaborate to build linear chains. Proteins involved in the formation of linear ubiquitin chains are also associated with human diseases.

Ubiquitination in signaling provides remarkable biochemical complexity and cellular plasticity for the immune system. The reviews in this volume provide insights into the cellular and physiological functions of ubiquitin ligases. The field is now poised to tackle a number of important questions: how E3 ligases recognize their substrates, how E2 enzymes collaborate with E3 ligases to build specific types of chains on substrates, and how ubiquitinated signaling complexes with multiple chain conformations function as signaling nodes and coordinate downstream signaling complexes (and why ubiquitin chains in signaling complexes are so long). Answers to these questions may be facilitated by newer technical approaches, including mass spectrometric approaches.

Two general classes of proteins not covered in this volume, de-ubiquitinating enzymes (DUBs) and ubiquitin-binding proteins, collaborate with ubiquitin ligases to edit and sense ubiquitination in immune signaling. Ubiquitin sensors are capable of recognizing distinct ubiquitin chain types by utilizing spatially separated ubiquitin-binding motifs. Some of these sensors may recognize ubiquitinated substrates via simultaneous interactions with target proteins and ubiquitin chains. Greater definition of all three classes of proteins will help us understand how signals are coordinated to attain appropriate cellular responses. They should also provide insights into how genetic polymorphisms in the ubiquitin system affect human diseases. Ultimately, although pharmacologic approaches to ubiquitin modifying and sensing proteins present distinct challenges than more established approaches utilized for kinases and phosphatases, the ubiquitin system has already been targeted via the proteasome and is likely to provide potent opportunities for therapeutic intervention.

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UBIQUITINATION	UBIQUITIN SENSORS	CELLULAR LOCALIZATION	CELLULAR ROLES	DISEASE ASSOCIATION
monoUb	mono-Ub Sensor	Lysosome	Sorting Degradation	Cancer
K48 polyUb	K48 Ub Sensor	K48 Ub Sensor	K48 Ub Sensor Cell cycle	Cancer Metabolic Syndrome Muscle wasting
K63 polyUb	K63 Ub Sensor	Intracellular trafficking Signaling complex formation	DNA repair Autophagy Endocytosis Immune signaling Cell death	Cancer Huntington's Parkinson's Alzheimer's Crohn's & other Inflammatory diseases
Linear (M1) polyUb	Linear (M1) Ub Sensor	Signaling complex formation	Innate Immune signals	Autoinflammation Muscular amylopectiosis Immunodeficiency
K11 polyUb	K11 Ub Sensor	Proteasome ERAD	Cell Cycle	Cancer Neurodegenerative

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'Proteins conjugated with diverse ubiquitin chains are recognized by ubiquitin sensors that lead to different protein complexes and different protein and cell fates'.