Variations On A Theme: Intricacies Of Unanchored Poly-Ubiquitin Signaling And Toxicity

Jessica Renee Blount-Pacheco
Wayne State University

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VARIATIONS ON A THEME: INTRICACIES OF UNANCHORED POLY-UBIQUITIN SIGNALING AND TOXICITY

by

JESSICA BLOUNT-PACHECO

DISSERTATION

Submitted to the Graduate School

of Wayne State University,

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Approved by:

Advisor

Date

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DEDICATION

All my work is dedicated to Estlin, Emmett, Vivian, Eleanor, and Ruby.
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LIST OF ABBREVIATIONS

Ub: ubiquitin
E1: Ub activating enzyme
E2: Ub conjugating enzyme
E3: Ub ligase enzyme
RING: Really Interesting New Gene
HECT: Homologous to the E6AP Carboxyl Terminus
RBR: RING-BetweenRING-RING
DUB: deubiquitinating enzyme
USP: Ub-specific protease
UCH: Ub C-terminal hydrolase
OTU: ovarian tumor protease
JAMM: jAB1/MPN/MOV34 metalloenzyme
MINDY: motif interacting with Ub-containing novel DUBs
ZUFSP: zinc finger with UFM1-specific peptidase domain proteins
ZnF-UBP: zinc-finger Ub-binding domain
IL-1R: IL-1 receptor
TLR: Toll-like receptor
IRAK-1: IL-1R-associated kinase 1
TRAF6: TNFR-associated factor 6
TAK1: transforming growth factor β-activated kinase 1
TAB1: TAK1-binding protein 1
TAB2: TAK1-binding protein 2
IKK: IκB kinase
NEMO: NF-κB essential modulator
UBAN: Ub binding in ABIN and NEMO
MEFs: mouse embryonic fibroblasts
TNFR: tumor necrosis factor receptor
RLR: RIG-I-like receptor
IFN: interferons
IRF3: interferon regulatory factor 3
IRF7: interferon regulatory factor 7
TBK1: TANK-binding kinase 1
CARD: caspase activation and recruitment domain
MAVS: mitochondrial activatory of virus signaling
STAT1: signal transducer and activator of transcription 1
UAS: upstream activating sequence
RNAi: RNA-interference
NMJ: neuromuscular junction
RIN: RNA integrity number
DAVID: Database for Annotation, Visualization and Integrated Discovery
IPA: Ingenuity Pathway Analysis
HMW: high molecular weight
UBD: Ub-binding domain
LUBAC: linear Ub chain assembly complex
CHAPTER 1: UNANCHORED UBIQUITIN CHAINS, REVISITED

1.1. Introduction

Ubiquitin (Ub) is a highly conserved 76-residue protein that facilitates the complex post-translational modification of protein function, fate, and binding partners within the eukaryotic cell [1-3]. “Ubiquitination” refers to the ATP-dependent conjugation of a Ub molecule onto a substrate protein via an isopeptide bond between the C-terminal carboxylic group of a Ub and the ε-amine of a lysine residue within the substrate [1]. The cellular machinery that brings about this conjugation usually consists of three main components: a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase (E3). A protein that has accepted a single Ub molecule is said to be mono-ubiquitinated; additional ubiquitination can result in a multi-ubiquitinated protein decorated with individual Ub molecules (figure 1.1A).

Following a mono-ubiquitination event, the conjugated Ub can also be ubiquitinated itself, forming a polymeric chain (figures 1.1A, B). A single Ub molecule harbors seven lysine residues (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48, and Lys63) that serve as Ub acceptor sites, spread over the surface of the protein and pointing in distinct directions. In addition, an eighth ubiquitination site exists at the N-terminal methionine (Met1) [1]. Poly-Ub chains are typically characterized by the Lys or Met residue used for chain elongation and can be composed of homogenous linkages (e.g., a tri-Ub chain with only Lys48 linkages; figure 1.1B) or multiple linkage types can exist in mixed-linkage (multiple linkage types within a continuous chain; figure 1.1C) or branched chains (a Ub chain attached to a second, previously formed chain, resembling branches in a tree; figure 1.1D). Linkage type also determines the conformation and flexibility of the chain – while Met1- and Lys63-linked chains adopt open conformations with more space between Ub molecules, Lys6-, Lys11-, and Lys48-linked poly-Ub are more compact. The Lys48- and Lys63-linked chains diagrammed in figure 1.1B illustrate the basic difference between compact and open conformations. The shape of a Ub chain affects its binding partners, as some
proteins are attracted by the pockets created by specific linkage types [4, 5]. This binding landscape gains even greater complexity through mixed-linkage and branched chains, and through additional post-translational modifications to Ub, including phosphorylation [6, 7]. Two poly-Ub chains that contain the same linkage types can behave in unique ways, depending on the arrangement of those linkages, the chains’ proximity to post-translational modifiers that act upon them, and accessibility to Ub-binding proteins attracted to certain chain types [3].

The composition of a poly-Ub chain and the context of its attachment to a substrate can trigger different outcomes in the cell. Several Ub chains function as signals to target proteins to the 26S proteasome. Lys48-linked chains are the most abundant linkage with this role, although Lys11-, Lys29-, and in some cases Lys63-linked chains, have also been implicated in this functional degradation pathway [8-10]. Branched Lys11/Lys48-linked Ub chains have recently
emerged as an enhanced degradation signal vital to both cell-cycle regulation and quality control of aggregation-prone proteins [11, 12], and Lys48/Lys63-linked, branched chains are an additional degradation signal that associates with the proteasome [13]. In addition to proteasomal degradation, mono-Ub and Lys63-linked chains have been shown to target plasma membrane proteins to lysosomes for degradation [14].

Outside of degradation, ubiquitination has been shown to play a role in recruiting proteins to participate in signaling pathways, altering localization by attracting trafficking factors, and even controlling the conformation and activity of the substrate itself [1-3, 15]. These non-degradation pathway functions are typically associated with mono-Ub, Met1- and Lys63-linked chains. Furthermore, many chains induce non-degradative regulation of protein activity through more indirect means. Met1-, mixed Lys11/Lys63-, Lys63-, and Lys48-linked chains can regulate signaling through inhibitor degradation, proteasomal processing, allosteric activation, and recruitment of upstream activating enzymes [1-3, 15]. Table 1.1 in the appendix describes functions of poly-Ub chains of each linkage type and the ubiquitinating and deubiquitinating enzymes reported to build and disassemble them.

Decades of research have been dedicated to the study of ubiquitination, yet we have only begun to understand its countless functions in the cell and the players that direct Ub’s roles. What determines which proteins are ubiquitinated, and with what type of chain? One determinant of substrate specificity is the particular E2 and E3 involved. A mono-ubiquitination event is initiated when the E1 activating enzyme hydrolyzes ATP and forms a thioester bond with Ub. The Ub is then passed to an E2 conjugase through a transthioleation reaction. An E3 ligase then facilitates the formation of an isopeptide bond between Ub and a lysine in its target protein. How this final step happens depends on the type of E3, which is classified into one of three families based on its domains and transfer mechanisms: RING E3s, HECT E3s, and RBR E3s. RING (Really Interesting New Gene) E3s, the most abundant type, serve as a scaffold to enable the direct transfer of Ub from the E2 to the target protein. HECT (Homologous to the E6AP Carboxyl
Terminus) E3s transfer Ub in two steps: first, a transthiolation reaction moves the Ub molecule from the E2 to the E3, before passing it to the substrate. RBR (RING-BetweenRING-RING) E3s also work in multiple steps: a RING domain recruits a Ub-charged E2, a RING-like domain forms a thioester bond with the Ub, and the Ub is subsequently transferred to the substrate. While the human genome contains only two E1 Ub-activating enzymes, there are ~40 E2 conjugases and more than 600 E3 ligases that can confer substrate and linkage specificity. HECT and RBR E3s each determine the types of Ub linkages they create, while RING E3s depend on their cooperating E2 to impart that specificity. [1, 2, 6, 16, 17].

Once a Ub chain has been formed on a substrate, it can be removed or edited by deubiquitinating enzymes (DUB). Humans have ~100 DUBs, some of which have preferences for chain type, length, and location, while others are promiscuous in those regards. DUBs are divided into seven families: Ub-specific proteases (USPs), Ub C-terminal hydrolases (UCHs), ovarian tumor proteases (OTUs), Josephins, JAB1/MPN/MOV34 metalloenzymes (JAMMs), the motif interacting with Ub-containing novel DUBs (MINDYs), and zinc finger with UFM1-specific peptidase domain proteins (ZUFSPs). USPs, UCHs, OTUs, Josephins, MINDYs, and ZUFSPs are cysteine proteases, whereas JAMMs are zinc metalloproteases [15, 18-20]. The significance of DUBs is evident from the beginning of a Ub molecule’s existence, as Ub genes are transcribed as tandem repeats (UBB and UBC) or as ribosomal fusion proteins (UBA52 and RPS7A) that require processing by DUBs before they can be used as mono-Ub for substrate ubiquitination [1, 20]. DUBs also serve as negative regulators of Ub signaling; for example, a protein with a Lys48-linked tetra-Ub chain attached as a proteasomal targeting signal can be spared of degradation by a DUB that removes that chain [15, 21]. The 26S proteasome itself contains and is closely associated with DUBs that recognize Ub chains and remove them from degradation-bound proteins to be reused for novel ubiquitination events [15, 22, 23]. Finally, DUBs participate in poly-Ub editing to change the length or composition of the chain, thus modifying the substrate protein’s fate or participation in specific pathways. Some DUBs partner with E3 ligases to attach new Ub
molecules, and there is even a DUBs that has E3 ligase domains itself, the NF-κB modulator A20 [24].

DUBs can disassemble chains one molecule at a time (e.g., UCH37 [25]), or they can remove the entire chain at once (figure 1.1A, bottom left), resulting in unanchored, or free, poly-Ub that is not attached to a substrate protein. An example of the latter type, USP14, removes en bloc poly-Ub chains from multi-poly-ubiquitinated cyclin B (a cell cycle regulator) until only one chain remains attached, reducing cyclin B’s interaction with the proteasome and thus effectively inhibiting its degradation in vitro [26]. Another DUB, the proteasome resident Poh1, is a zinc-dependent metalloprotease that cleaves poly-Ub from substrates as they are being degraded, saving the chain from degradation, and yielding an unanchored poly-Ub [27].

Unanchored Ub chains make their first appearance in the cell at the time of Ub gene transcription, as human UBB and UBC encode three and nine tandem repeats Ub, respectively. Transcription of these genes results in linear, unanchored poly-Ub that is processed by DUBs that have zinc-finger ubiquitin binding domains (Znf-UBPs) that specifically recognize the chains’ free C-termini, including USP3, USP4, and USP16 [15]. In mammals, UBB and UBC transcription is upregulated during cellular stress when increased signaling requires an ample supply of Ub. Unanchored poly-Ub can also be assembled anew by specialized E2/E3 pairs in vitro and in cells. This type of production is seen when the E3 TRIM6 and the E2 UbE2K generate unanchored, Lys48-linked Ub chains that activate an IFN signaling component [28].

To date, not much is known about free Ub chains. They are commonly thought of as toxic, and their rapid disassembly is thought to be essential to cellular health and Ub homeostasis, based on findings from the studies described below. However, untethered Ub chains are also directly implicated in specific pathways, including NF-κB, so their presence in the cell is clearly important. Yeast and in vitro studies suggested that unanchored poly-Ub inhibits the proteasome and is toxic, while Drosophila studies more recently indicated that they are tolerated in an intact,
multi-cellular organism. For the remainder of this review, we examine the details and complexities of the unanchored Ub chain.

1.2. Toxicity of unanchored chains

Much of our knowledge of unanchored poly-Ub arose from research on the best-known DUB that processes it, USP5. Mammalian USP5 (also known as isopeptidase T) was first purified from reticulocytes in 1985 [29] and later characterized as an enzyme that preferentially disassembles poly-Ub species after they are removed from ubiquitinated substrates at the 26S proteasome [30]. It specifically recognizes the free C-terminal diglycine motif of untethered poly-Ub and removes Ub monomers sequentially, from the chain’s proximal end [31]. Kinetic assays revealed that Ub, itself, can modulate USP5 activity \textit{in vitro}: low Ub concentrations activate USP5, while partial inhibition is observed at higher Ub concentrations [32]. It has since been established that USP5 contains four Ub-binding domains that cooperate to recognize and process multiple types of unanchored poly-Ub, with a preference for Lys48-linked chains [33]. Its ZnF-UBP domain governs its specificity for untethered chains, with a specialized pocket that recognizes the unencumbered C-terminus of the proximal Ub [34]. In the fruit fly, \textit{Drosophila melanogaster} USP5 knockdown or null mutation is developmentally lethal [35-38]. One proposed purpose for USP5 is to maintain a pool of available mono-Ub by cleaving untethered poly-Ub; when USP5 cannot perform this function, perhaps the cell lacks the building blocks necessary for normal ubiquitination. However, toxicity from RNAi knockdown of USP5 is not alleviated by over-expression of mono-Ub in \textit{Drosophila} [38]; thus, toxicity arising from reduced or absent activity of USP5 cannot be explained solely by a disruption of mono-Ub supply, and may involve dysregulation of specific ubiquitinated substrates [1, 5, 39-41].

When Ub is properly folded, several hydrophobic amino acid residues converge to form a hydrophobic patch that serves as the interaction site for many Ub-binding domains [39, 42]. It is through this hydrophobic patch that substrate-conjugated poly-Ub binds S5a, a subunit of the regulatory compartment of the 26S proteasome, allowing proteasomal DUBs to detach the chain.
and enhancing the degradation of the targeted protein [39, 42]. Could unanchored Ub chains directly cause toxicity by binding to the proteasome in lieu of ubiquitinated substrates, interrupting normal proteolysis? Using reconstituted reticulocyte proteasome complexes to study USP5 function in vitro, Hadari et al. determined that USP5 stimulates the proteolysis of poly-ubiquitinated substrates [30]. If unanchored Ub chains can outcompete ubiquitinated substrates at the proteasome and hinder normal proteolysis, the authors suggested that USP5 prevents this by quickly disassembling chains—as soon as they are removed from their substrates—thus enhancing proteolysis by removing competition [30]. In this study, the authors did not perform binding assays to assess competition between unanchored chains and ubiquitinated substrates at the proteasome, instead focusing on the effect of USP5 on in vitro proteolysis and inferring a corresponding effect from unprocessed poly-Ub.

In follow-up studies, Piotrowski et al. synthesized Lys48-linked, untethered poly-Ub chains of various lengths (from Ub² to Ub⁸) and examined their effects on in vitro proteasomal function [43]. They found that Ub chain length dictates the magnitude of proteasomal inhibition and the chain’s affinity for the proteasome: longer chains are more likely to bind and more strongly inhibit the proteasome [43]. Later, Thrower et al. identified Ub⁴ as the minimum signal for efficient proteasomal degradation in vitro and showed that unanchored chains compete with ubiquitinated substrates to bind to purified, mammalian proteasome, again in an in vitro setting [8].

Yeast studies support the notion that unanchored poly-Ub may inhibit proteasomal activity. When Amerik et al. deleted the UBP14 gene encoding the USP5 orthologue in S. cerevisiae (baker’s yeast) cells, they observed accumulation of unanchored Ub chains and inhibition of the Ub-dependent, proteasomal degradation of MATα2, L-βgal, and Ub–P-βgal, reporter proteins used to study Ub-dependent protein turnover [44]. Reasoning that free Ub chains may be the culprits hampering proteasomal degradation, they expressed in wild-type yeast cells a Ub mutant lacking the C-terminal glycine residues required for Ub’s conjugation onto other proteins and its recognition by UBP14. This mutant, UbΔGG, can be ubiquitinated, allowing for the creation of an
unanchored, UBP14-resistant poly-Ub chain with the mutant Ub molecule at the end [44]. (Similar mutants that lack an intact C-terminal “GG” motif are used in studies described below; although the precise amino acid mutations vary, the effect is the same, and all such mutants are referred to as “Ub∆GG” throughout this manuscript.) Yeast cells expressing Ub∆GG mutants suffer growth defects, sensitivity to environmental stressors, and a reduction in overall protein degradation in pulse chases [45, 46]. The presence of unanchored, Ub∆GG chains coincided with reduced proteolysis of MATα2 and Ub–P-βgal, again indicating proteasomal dysfunction, in this case in vivo, in a single cellular organism [44].

Moving beyond purified proteasomes and yeast studies, Dayal et al. linked USP5 to unanchored chain disassembly in a mammalian cellular system [47]. From a screen of DUBs affecting the activity of the tumor suppressor p53 in cultured, ARN8 human melanoma cells, they found that knockdown of USP5 stabilizes and activates p53. They also observed by Western blotting an increase in low molecular weight Ub species that comigrate with purified, free Lys48-linked chains and concluded that these are unanchored Ub species induced by USP5 suppression. Expression of Ub∆GG recapitulated the effects of USP5 knockdown in ARN8 cells, leading to increased p53 activity. USP5 siRNA, or Ub∆GG expression also caused increased ubiquitination of p53. Since this ubiquitination, which could be a degradation signal, counter-intuitively coincides with slowed p53 turnover, the authors argued that the effects of USP5 suppression on p53 activity are mediated by unanchored Ub chains that outcompete ubiquitinated p53 at the proteasome and extend its half-life [47]; this study did not directly examine whether the observed p53 ubiquitination is consistent with a degradation signal, or whether its binding to the proteasome is impaired in the presence of mutant Ub.

Ultimately, various early studies on unanchored poly-Ub pointed toward a toxic effect, often linked to inhibition of the proteasome and buildup of proteins that were destined to be degraded. It is important to note that these studies were performed using in vitro systems or a single-cell organism, yeast; there remains a potential for free Ub chains to behave differently in
cells in vivo and in multicellular organisms. Although Ub itself is highly conserved and enzymes that dictate its use, editing and recycling are also conserved at several levels, there is also a significant expansion in the number and types of Ub-related proteins and enzymes with evolutionary progression, leaving open the possibility that free Ub chains may have different roles and be regulated differently among species.

1.3. Unanchored chains and innate immunity

Beyond their potential for toxicity, untethered Ub chains have been implicated as participants in specific cellular pathways, discussed below. Their inclusion in these pathways is evidence that they are not exclusively toxic, but that they also have important physiological roles in regulating innate immune pathways that guard against invading pathogens and control inflammation.

1.3.1. TAK1 and IKK Activation

Just as USP5 studies uncovered the potential for untethered poly-Ub to inhibit the proteasome in some systems, much of our knowledge of the normal functions of free Ub chains came to us originally from investigations into Ubc13/Uev1a, an E2 complex that generates unanchored poly-Ub. Ubc13/Uev1a is important for innate immunity, as it is required for intracellular NF-κB signaling that originates with interleukin-1 receptors (IL-1Rs) and Toll-like receptors (TLRs) [48, 49]. IL-1 cytokines in humans, including IL-1β, are small, proinflammatory proteins that bind IL-1 receptors (IL-1Rs) on the cell’s surface (figure 1.2). IL-1β binding to IL-1R causes the receptor to form a complex that includes IL-1R-associated kinase 1 (IRAK-1) and the E3 ligase TNFR-associated factor 6 (TRAF6). IRAK-1 and TRAF6 dissociate from IL-1R and TRAF6 joins transforming growth factor β-activated kinase 1 (TAK1), TAK1-binding protein 1 (TAB1) and TAB2 in a new complex in the cytoplasm. Activated TAK1 phosphorylates another cytoplasmic complex, the IκB kinase (IKK) complex, consisting of two catalytic subunits (IKKα and IKKβ) and a regulatory subunit (IKKγ/NF-κB essential modulator (NEMO)). The IKK complex then
phosphorylates IκBα, resulting in its proteasomal degradation and the nuclear translocation of the freed NF-κB proteins (figure 1.2) [50, 51].

In 2009, Xia et al. directly implicated unanchored, Lys63-linked Ub chains in the activation of TAK1 – and, by extension, in the activation of a canonical NF-κB pathway [52]. At the time, it was known that Ubc13/Uev1a is required for TAK1 to phosphorylate the IKK complex [48, 49], but the mechanism was unclear. The authors reconstituted TAK1 activation using purified components and found that unanchored, Lys63-linked poly-Ub generated by Ubc13/Uev1a and TRAF6 triggered the phosphorylation and activation of TAK1 in vitro [52]. Moving to cultured cells, the authors used IL-1β to stimulate HEK-293 cells that stably express IL-1R (termed stable IL-1R
cells) and immunoprecipitated the TAK1 complex using TAB2 antibody. The kinase complex co-immunoprecipitated with endogenous, USP5-sensitive (suggesting unanchored) poly-Ub, which they then purified for in vitro experiments. The presence of these poly-Ub species activated TAK1 in vitro by binding TAB2 at its NZF Ub-binding domain, inducing TAK1 phosphorylation. Furthermore, USP5-sensitive poly-Ub species generated by Ubc13/Uev1a and Ubc5, which can make various Ub chain types beyond Lys63-linked species, activated the IKK complex via NEMO’s Ub-binding domain. The authors proposed a model by which unanchored, Lys63-linked chains bind TAB2 and draw two TAK1 complexes together for them to mutually phosphorylate and activate one another, while other types of untethered chains bind and activate NEMO in the IKK complex (figure 1.2).

What type of poly-Ub activates NEMO has been a complicated question to address. NEMO contains a Ub-binding domain, the Ub binding in ABIN and NEMO (UBAN) domain, that is required for NF-κB activation in mouse embryonic fibroblasts (MEFs) [53]. UBAN can interact with both anchored and unanchored Ub chains, and although it preferentially binds linear poly-Ub with as few as two Ub moieties, longer chains with different linkage types also bind NEMO in vitro and in cultured cells [53-56]. In stable, IL-1R HEK-293T cells, IL-1β treatment stimulates the production of Lys63-linked poly-Ub decorated with Met1-linked poly-Ub, i.e., branched Ub chains [57]. NEMO can bind these hybrid chains, based on immunoprecipitation experiments, but it was unclear whether any of the associated poly-Ub species were unanchored. Other in vitro studies showed that covalent, Met1-linked di-ubiquitination of NEMO activates the IKK complex more potently than unanchored, Met1-linked Ub\(^2\), but longer poly-Ub chains were not tested. Spectroscopy studies suggested that NEMO’s interactions with other proteins are mediated by long, linear poly-Ub chains: binding of Met1-linked Ub\(^{10}\) induces a conformational change in NEMO that promotes its association with IKKβ and IκBa and in vitro [58]. It seems that Ub-dependent NEMO activity can be mediated by both substrate-conjugated and untethered poly-
Ub, and the magnitude of the effect may depend on chain length, linkage composition, and the type of interaction or bond.

Untethered Ub chains have also been linked to the negative regulation of NF-κB through their interaction with A20, a dual function enzyme with an N-terminal OTU DUB domain and seven ZnF domains, with E3 activity at its C-terminus [24]. It suppresses NF-κB activity in the tumor necrosis factor receptor (TNFR) and TLR pathways by editing poly-Ub attached to various mediators and by disrupting the assembly of E2/E3 Ub enzyme complexes [24, 59]. Skaug et al. discovered an additional, noncatalytic mechanism for A20 suppression of NF-κB activity that depends on unanchored Ub chains [60]. Through in vitro experiments including GST pulldowns, Ub-binding assays, and cell-free IKK activation systems, the authors showed that A20 can form a complex with NEMO and long (six or more), untethered, Lys63-linked Ub chains, which then prevents IKK activation. Formation of this complex in vitro is aided by long, unanchored chains. Lys63-, Lys48-, and Met1-linked Ub had no effect, and long chains with linkage types other than Lys63 were not tested. Overexpression and RNAi studies using HeLa S100 cell extracts confirmed the formation of an A20-NEMO complex, dependent on TRAF6, Ubc13, and A20’s ZnF7 Ub-binding domain. The authors concluded that IL-1β binding to IL1R promotes the assembly of unanchored, Lys63-linked Ub chains by TRAF6 and Ubc13/Uev1a, which then recruit the TAK1 and IKK complexes via Ub-binding domains in TAB2 and NEMO, respectively. If A20 is present, it outcompetes TAB2 for poly-Ub binding, and poly-Ub becomes the scaffold in a complex with A20 and NEMO. Formation of this complex inhibits IKK phosphorylation by TAK1, thereby blocking NF-κB signaling (figure 1.2).

The studies summarized above provide evidence that unanchored poly-Ub species contribute to innate immune signaling by modulating at least two steps within NF-κB pathways: 1) the activation of the IKK complex (via TAK1) to phosphorylate IkB to free NF-κB transcription factors, and 2) the termination of TNFR- and TLR-regulated NF-κB signaling by A20 (figure 1.2).
Collectively, they underscore important physiological roles for free poly-Ub in normal physiological functions of eukaryotic cells.

1.3.2. RIG-I Activation

Innate immunity also protects organisms against viruses. In the cytosol of a virus-infected cell, RIG-I-like receptors (RLRs) detect viral RNA and initiate a signaling cascade that culminates in the production of antiviral molecules including type-1 interferons (IFNs) (figure 1.3). Transcription factors involved in this antiviral response include NF-κB, interferon regulatory factor 3 (IRF3) and IRF7. In contrast to NF-κB proteins, which are activated by the degradation of their inhibitor IκBα, IRF3 and IRF7 are activated by direct phosphorylation by the noncanonical IKKs, IKKε and TANK-binding kinase 1 (TBK1), which causes the transcription factors to dimerize and translocate to the nucleus [61, 62]. RIG-I is an RLR expressed at low levels in the cytoplasm of most human cells. When RIG-I detects double-stranded viral RNA, an ATP-dependent dimerization and conformation change is triggered, exposing two tandem, N-terminal caspase activation and recruitment domains (CARDs). These CARDs interact with a CARD on the N-terminus of the signaling adaptor protein mitochondrial activator of virus signaling (MAVS), which in turn activates IKKε and TBK1 to phosphorylate IRF3 and IRF7 (figure 1.3) [61, 62].

To study ubiquitin-dependent mechanisms involved in RIG-I signaling, Zeng et al. developed a cell-free model of viral infection that combines purified RIG-I protein, mitochondrial and cytosolic extracts, RNA, and ubiquitination enzymes, using IRF3 dimerization as a reporter for RIG-I and MAVS activity [63]. Viral or engineered RNA was used to activate RIG-I in cytosolic extracts, which stimulates MAVS in mitochondrial extracts to promote the dimerization of IRF3. The authors determined that Lys63-linked, unanchored Ub chains are potent, direct activators of RIG-I in vitro, binding its tandem CARD domains after RIG-1 detects viral RNA (figure 1.3). The authors also verified the existence of this Ub chain type in a human cell line by devising a method to immunoprecipitate endogenous, free poly-Ub from HEK-293T cells using recombinant RIG-I N-terminus (GST-RIG-I(N)). Some poly-Ub species that bind GST-RIG-I(N) formed β-
mercaptoethanol-sensitive thioester bonds with E1 Ub-activating enzyme, indicating a free C-terminus, and were sensitive to both USP5 and the Lys63-specific DUB CYLD, leading the authors to identify them as unanchored, Lys63-linked chains. The endogenous poly-Ub isolated by this method potently activated IRF3 dimerization in the cell-free system, and their expression was induced by viral infection in HEK-293T cells. TRIM25 is at least partially responsible for producing these Ub chains, as siRNA targeting this E3 ligase diminished Lys63-linked, free poly-Ub levels in HEK-293T cells; conversely, CYLD siRNA led to higher levels, indicating a negative regulatory role for the DUB.

In later studies, Jiang et al. expanded on these findings by using the reconstituted RIG-I activation assay to demonstrate that noncovalent binding of free, Lys63-linked Ub chains to RIG-I...
I's CARD domains promotes RIG-I's oligomerization (figure 1.3) [64]. Sedimentation velocity analytical ultracentrifugation indicated that RIG-I specifically forms tetramers in complex with four unanchored poly-Ub molecules; the 4:4 ratio remained constant with all Ub chain lengths tested, from Ub³ to Ub⁶. RIG-I formed high molecular weight complexes in response to viral infection in HEK-293T cells and MEFs, but not in Ubc13 knockout MEFs, or in RIG-I knockout MEFs expressing RIG-I that cannot bind Ub, leading the authors to conclude that RIG-1 oligomerization depends on binding to Lys63-linked poly-Ub generated by Ubc13. When RIG-I was isolated from both types of knockout MEFs to use in in vitro IRF3 dimerization assays, only the higher molecular weight RIG-I aggregates were active, suggesting that Lys63-linked poly-Ub-dependent oligomerization of RIG-I is necessary for its activity. Another RLR with a CARD domain, melanoma differentiation-associated protein 5, behaves similarly to RIG-I in vitro, with Lys63-linked Ub⁶ inducing its oligomerization and enhancing its activation of IRF3 in dimerization assays.

Hou et al. also used the cell-free RIG-I activation system to elucidate the details of MAVS activation. Performing biochemical assays using isolated, crude mitochondria from virus-infected or uninfected HEK-293T cells, they determined that activated MAVS forms large, prion-like fibrils that induce IRF3 dimerization. MAVS aggregation was induced by RIG-I in the presence of RNA and Lys63-linked, free Ub⁴. Based on these in vitro studies and the previous work from Zeng et al., the authors constructed a model for MAVS activation in the viral response that is dependent on binding of unanchored, Lys63-linked poly-Ub to the CARD domains of RIG-I to trigger MAVS aggregation on the mitochondrial membrane (figure 1.3) [65]. Crystal structure studies examining covalent and noncovalent binding between RIG-I and poly-Ub revealed that Lys63-linked free Ub² binds RIG-I CARD tetramers, stabilizing them as a scaffold to recruit and activate MAVS to form fibrils in vitro [66]. Covalent poly-ubiquitination of RIG-I by TRIM25 also induced MAVS aggregation in vitro, indicating a potential for multiple Ub-dependent mechanisms to activate this important antiviral pathway. As mentioned above, TRIM25 is a Lys63-specific E3 that also produces at least some of the free poly-Ub chains that activate RIG-I in cultured human cells [63].
In vitro, the RING domain of TRIM25 can partner with distinct E2s like Ubc13/Uev1a or Ubc5 to produce unanchored or substrate-conjugated Ub chains, respectively [67].

Another TRIM E3, TRIM6, is also involved in viral immunity through RIG-I signaling [28]. As mentioned above, IFN IRF3’s phosphorylation and activation is mediated by IKKε. This action depends on signal transducer and activator of transcription 1 (STAT1), which is phosphorylated by IKKε (figure 1.3). Co-immunoprecipitation experiments using HEK-293T cells and primary human monocyte-derived dendritic cells showed an interaction between TRIM6 and IKKε, and knockdown of TRIM6 depleted IFN-mediated antiviral activity in human lung epithelial A549 cells. In vitro, IKKε interacted with free, Lys48-linked poly-Ub synthesized by TRIM6 and the E2 UBE2K, and USP5-sensitive poly-Ub interacted noncovalently with IKKε in HEK-293T cells, based on co-immunoprecipitation studies. By confocal microscopy, TRIM6 and IKKε co-localize with Ub-rich, cytoplasmic puncta in HeLa cells, and the formation of these puncta was disrupted by the introduction of USP5, which the authors interpreted to indicate the presence of free poly-Ub in the observed foci. In vitro STAT1 phosphorylation assays revealed that untethered, Lys48-linked chains with two to sixteen Ub moieties induce IKKε phosphorylation of both itself and STAT1, inducing IKKε oligomerization and IRF3 activation. Taken together, these studies highlight the importance of two types of unanchored poly-Ub in innate immune pathways (figure 1.3).

The participation of free poly-Ub in various immune responses is evidence that these chains – especially open-conformation Met1- and Lys63-linked chains – have direct, physiological effects that are not limited to the proteasomal toxicity previously seen in yeast and in vitro systems (figures 1.2 and 1.3). As the pool of research on unanchored Ub chains has grown, there is increasing evidence that chain length and the type of linkage within the chain dictates its specific role in immune signaling (e.g., Lys63-linked chains interact with RIG-I, while Lys48-linked chains activate IKKε [28, 63, 64]). The roles of other linkage types remain to be studied in immune signaling and in other physiological processes; thus, it is possible that distinct species of free poly-Ub have yet-to-be discovered cellular functions.
1.4. Other physiological purposes of unanchored Ub chains

Other potential regulatory roles for free poly-Ub species have emerged, beyond their involvement in immune pathways. Braten et al. expressed a Ub∆GG mutant in yeast and performed a gene deletion screen to determine which E2(s) and E3(s) are responsible for constructing Ub∆GG-terminal chains. By exposing these yeast strains to stressors, they showed that free Ub chains are upregulated during certain types of stress, including heat shock, DNA damage, and oxidative stress, and that the yeast E3, UFD4 generates unanchored chains under basal conditions, while the E3, HUL5 is responsible for most of the ones generated in response to a DNA alkylation agent [68]. This study did not determine a physiological role for the free chains generated, but their stress-induced upregulation and the identification of two E3s responsible for them is notable and warrants further examination in this model organism and beyond.

Heat shock also induces the reversible formation of cytoplasmic stress granules, which are small, dense aggregations of proteins and mRNA. In cultured HeLa cells, USP5 is recruited to heat shock-induced stress granules, and its knockdown prevents their disassembly, leading Xie et al. to investigate the potential involvement of unanchored poly-Ub [69]. Heat shocking cells expressing Ub∆GG led to the formation of stress granules at the same rate as it did in cells expressing wild-type Ub, but more than twice as many Ub∆GG cells (76%) were unable to clear the newly formed granules [69]. The authors concluded that untethered poly-Ub interferes with the process of disassembling stress granules; the specifics of this action remain to be determined.

Thus far, not many physiological functions have been ascribed to unanchored poly-Ub; but, it is important to note that they indeed have innate roles and do not appear to simply be byproducts of chains assembly sand disassembly. Clearly, additional investigations are needed in intact organisms and in specific cell types and tissues to explore and understand their physiological implications.
1.5. Reduced or absent toxicity from unanchored poly-Ub in *Drosophila melanogaster*

The inclusion of free poly-Ub in several cellular pathways casts their previously described toxicity in a new, more complex light. In an effort to further understand the potential toxicity of unanchored chains, our laboratory conducted a series of studies to determine their impact *in vivo* and understand how they might be regulated in an intact, multi-cellular organism. Our collective results point to a non-toxic role for unanchored poly-Ub chains and mild transcriptomic response to their presence, indicating lower toxicity from free Ub chains than has been previously thought.

Our investigations began by focusing on unanchored chain disassembly and whether DUBs are necessary for their control *in vivo* [70]. This study set the stage for the investigations discussed in this section by introducing two types of unanchored poly-Ub transgenes that were expressed in *Drosophila melanogaster*. Both types of chains consist of six Ub in tandem and lack internal “GG” motifs that are necessary for isopeptide bond formation and disassembly by DUBs [1, 15, 22, 70]. Whereas one type of chain also lacks a terminal “GG,” and thus cannot be conjugated onto other proteins (referred to as Ub⁶-Stop), the other chain type contains a “GG” motif at its end to allow for protein conjugation (referred to as Ub⁶-GG; figure 1.4). Their expression was enabled by the binary, Gal4-UAS system, which allows for tissue-specific as well as timed expression through specific promoters and compounds delivered in fly media [71-74].

Biochemical analyses of the *Drosophila* lines generated with these transgenic free Ub chains showed robust expression. Intriguingly, the chains were amply modified by endogenous Ub, effectively creating branched Ub chains with various linkage combinations [70]. When expressed in all cells, Ub⁶ did not impact *Drosophila* development or adult fly longevity under both normal and heat-stressed conditions. Tissue-specific expression in muscles, glia, and neurons showed once again that there were no developmental differences between experimental chain expressing flies and controls, but expression in glia and neurons resulted in a mild decrease in adult fly longevity, whereas muscle-specific expression did not result in a significant change in
overall lifespan compared to controls [70]. Overall, longevity results indicated that unanchored chains are not necessarily universally toxic.

Because of studies indicating an inhibitory role for unanchored poly-Ub at the proteasome in vitro and in yeast, proteasomal activity was assessed in flies expressing, or not, Ub\(^6\)-Stop in all cells. Ub\(^6\)-Stop expression did not affect protein levels of proteasomal components, nor did it hinder the turnover of the proteasomal substrates GFP-CL1 or cyclin A [70]. Ub\(^6\)-Stop is itself degraded by the proteasome: RNAi against both 20S and 19S proteasome components led to high levels of these unanchored chains. Knockdown of certain proteasome-associated proteins

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**Figure 1.4. Summary of findings from transgenic, unanchored poly- ubiquitin in Drosophila.** Overview of the composition, processing and physiological effects of unanchored ubiquitin chains used in *Drosophila melanogaster*. Red arrows: worsening of phenotypes and outcomes as free poly-ubiquitin is mutated to become resistant to its own ubiquitination. Green arrows: improvement of phenotypes and outcomes as ubiquitination-resistant, free poly-ubiquitin is further modified to inactivate its Ile44 hydrophobic patch.
that help target ubiquitinated substrates to this degradative machinery resulted in markedly increased levels of Ub⁶-Stop (VCP, p47, and Ufd1-like) [70]. Additional genetic and biochemical experiments suggested a model whereby the VCP-associated protein, p47 is important for the association of unanchored, Ub⁶-Stop with VCP and the proteasome, and highlighted the proteasome as a key regulator of the stability of these Ub species in the fly.

Generally, ubiquitination is considered to occur at via serial addition of mono-Ub onto an extending chain or substrate. Intriguingly, the Ub⁶ constructs showed that the conjugation-capable Ub⁶-GG could be attached as a single unit onto other proteins in flies and in cultured mammalian cells [70]. These results indicated that Ub recycling need not only occur at the level of a monomer; in fact, a whole chain could be removed from one substrate and attached onto another en bloc. While the notion of en bloc transfer of an intact chain, without its disassembly into mono-Ub, has been purported before and has been observed in vitro [75-78], insofar as we know, it had not been shown before in a cell or intact, multi-cellular organism. These findings led to the possibility of free poly-Ub regulation beyond their disassembly into mono-Ub: they can be degraded by the proteasome, or become conjugated onto other proteins whole-sale, painting a wider tableau of possibilities for unanchored chain regulation in the cell.

Because of the surprising finding that untethered, linear poly-Ub species are not toxic [70], we wondered whether Drosophila is mounting a response towards these chains. We examined changes at the transcriptional level through RNA-seq, using flies expressing everywhere Ub⁶-Stop, Ub⁶-GG, or no transgene with the same genetic background. The presence of each type of Ub⁶ chain led to significant changes in the expression of approximately 90 fly genes, but with no clear coordinated cellular response to indicate the induction of any particular pathways [79]. Only 30% of the altered Drosophila transcripts observed have assigned gene names, indicating that most of the affected genes have not garnered enough attention to be ascribed function or genetic background in the fly. Additionally, 27% of the genes identified had no predicted function, opening up the potential for the future identification of proteins that interact with untethered poly-Ub. It
appears that the expression of unanchored poly-Ub in *Drosophila* translates to minimal transcriptomic or organismal response, judging by the low number of altered transcripts and a lack of specificity for Ub-related pathways in those transcripts that were altered [79]; we interpreted these results as further evidence that free poly-Ub species are not inherently toxic.

This lack of observable toxicity from linear hexa-Ub chains in *Drosophila* does not dovetail with earlier studies that strongly indicated that unanchored chains are toxic in yeast [8, 30, 43, 44], leading us to wonder what makes the difference between an unanchored Ub chain that causes proteasomal toxicity and one that is essentially innocuous in flies. As previously mentioned, the Ub⁶-Stop is ubiquitinated in flies, introducing endogenous Ub that could potentially change the way these chains are handled and explain why they are innocuous [70]. To assess the influence of endogenous ubiquitination on the toxicity of unanchored chains, we mutated all lysine residues in Ub⁶-Stop to alanine, creating Ub⁶-Stop-K⁰, a ubiquitination-resistant, linear hexa-Ub chain that cannot be cleaved by DUBs or conjugated onto a substrate (figure 1.4). By mirroring the longevity assays we used to characterize Ub⁶-Stop in various fly tissues, we observed that Ub⁶-Stop-K⁰ is significantly more toxic than its ubiquitination-prone counterpart: it is developmentally lethal when expressed in all cells, and it significantly reduces fly lifespan when expressed in neurons, glia, or muscles [80]. Compared to the ubiquitination-prone Ub⁶, turnover of Ub⁶-Stop-K⁰ is slowed but not halted, indicating that ubiquitination aids in the turnover of linear Ub⁶ but is not essential to its eventual clearance [80].

We then sought to elucidate a mechanism for the enhanced toxicity of ubiquitination-resistant Ub⁶. Since unanchored poly-Ub species are known to participate in NF-κB pathways, and some of them may be linear poly-Ub, we wondered whether Ub⁶-Stop-K⁰ can influence NF-κB signaling in *Drosophila* [50, 51, 81-83]. We co-expressed in glial cells Ub⁶-Stop-K⁰ and RNAi targeting several proteins involved in NF-κB signaling. We found that knockdown of NF-κB components extends the lifespan of Ub⁶-Stop-K⁰ flies, although not to the extent of controls that are genetically similar but without a Ub⁶ transgene [80]. RT-qPCR revealed that Ub⁶-Stop-K⁰
expression causes an increase in mRNA levels of several of these NF-κB components [80]. Based on these results, we concluded that ubiquitination-resistant, linear Ub chains induce aberrant NF-κB signaling, accounting for at least some of its toxicity in flies.

Because of the importance of Ub’s ile44-centered hydrophobic patch in its binding to Ub-binding proteins [1, 5, 39-41], including some NF-κB regulatory proteins, we examined ile44’s role in NF-κB-mediated toxicity from Ub-Stop-K0. We reasoned that Ub-Stop-K0 may not be capable of interacting with NF-κB without its hydrophobic patches; thus, we mutated ile44 to alanine in each Ub moiety to create the binding-deficient mutant Ub-Stop-K0-ile44a (figure 1.4). These mutations reversed most of the toxicity as well as aberrant NF-κB signaling in flies and also in cultured, HEK-293T mammalian cells [80]. These results indicated a role for free, linear, ubiquitination-resistant chains in NF-κB signaling that depends on an intact ile44 hydrophobic patch; without ile44, ubiquitination-resistant Ub likely is unable to interact with Ub-binding NF-κB components, prohibiting much of its toxicity in vivo.

Taken together, these studies add yet more complexity to the general understanding of unanchored poly-Ub. Clearly, free Ub chains are not always toxic. In flies, linear hexa-Ub chains are well tolerated, as long as they can be ubiquitinated or conjugated onto other proteins. Due to the current unavailability of genetic techniques to stably express in the fly chains of different topologies and linkages, we were restricted to using linear, head-to-tail chains. However, it bears highlighting that these linear chains quickly and abundantly become decorated with endogenous ubiquitin, and are thus transformed into a pool of free Ub species that comprises linear as well as branched chains consisting of M1, K27, K48, K63 [70] and, based on more recent proteomic assays, also K29 and K33 linkages (Blount and Todi, unpublished observations). Consequently, the findings summarized in this section pertain to various types of free poly-Ub in vivo and, with the above caveats in mind, may be extrapolated to apply more widely to other types of chains. Additionally, the fact that all of these Ub mutants are based on the same backbone – Met1-linked
hexa-Ub – but behave so differently in *Drosophila* is evidence of the complicated nature of unanchored poly-Ub *in vivo*.

### 1.6. Conclusions

The studies described in this review provide a complex picture of unanchored poly-Ub handling and function. It is now apparent that the previous understanding of these chains – that they only exist briefly before being disassembled by DUBs to prevent toxicity and allow mono-Ub recycling – is only part of the picture. These members of the Ub family have clear physiological roles and seem to be controlled in ways that have not garnered much attention so far. It appears that untethered Ub chains are regulated through various mechanisms. Not all of the possibilities we present next need to exist and operate at the same time in each cell. They may be cell type- and cell condition-dependent. Also, evolutionary differences may place more weight on some such pathways compared to others.

We propose the following routes of unanchored poly-Ub control and recycling (figure 1.5). The first, and in all likelihood major route of regulation and recycling remains the disassembly of free chains into mono-Ub so that they can be reused to modify other proteins. A second route may be that of unanchored poly-Ub degradation, either as they are or through additional decoration by endogenous Ub. A third mechanism of free poly-Ub use and control may be their conjugation onto another substrate, essentially removing them from the unanchored population. Additionally, free chains may associate with Ub-binding proteins in a type of “reserve” pool until they can be re-utilized, which may function in conjunction with the other proposed routes of unanchored chain management. In an example of the latter route--and under special conditions where they may not be able to be dispensed off or controlled in other ways--free Ub chains can interact with NF-kB signaling components, causing abnormal signaling and toxicity. Free poly-Ub is also involved in normal immune signaling, at least through the IL-1R and RLR signaling pathways, and also appears to be involved with stress granule clearance after cellular stress.
What we present is not an exhaustive tableau of potential outcomes; further studies may lead to the discovery of additional functions and cellular responses.

It may seem that more recent studies revealing unanchored Ub’s physiological roles and lack of toxicity contradict earlier studies that characterized free poly-Ub as harmful, but the seeming contradiction only highlights the importance of Ub chain type and context. Some of the previous *in vitro* and yeast studies that suggest proteasomal inhibition by untethered poly-Ub focused on Lys48-linked chains [8, 43, 47], while the Ub^6^ chains expressed in *Drosophila* are head-to-tail [70, 79]. In principle, it makes sense that unanchored chains that resemble poly-Ub...
attached to proteasome-targeted proteins – which are often Lys48-linked – could outcompete proteasomal substrates in a manner not observed with linear chains that are not traditionally associated with proteasomal degradation [1, 8, 43]. This out-competition most likely depends on chain abundance compared to endogenous substrates. (We should note here that in the fly, linear chains that could become ubiquitinated, including with Lys48 linkages, did not impact proteasome activity and both ubiquitination-resistant and -restricted free Ub⁶ were turned over in the fly, with the latter being a little delayed in the earlier stages of its degradation. [70]) Proximity to the proteasome is likely key: in vitro proteasome assays allow close association between Ub and the proteasome, with few proteins present to interfere, while Ub⁶ expression in transgenic flies does not guarantee such proximity. It is also clear that free poly-Ub toxicity is dependent on post-translational modification of the chain and its interactions with other proteins. Linear Ub⁶ species did not become particularly toxic until they could no longer be used or modified, at which point they were highly toxic through NF-kB in a manner dependent on their ability to interact with other proteins’ Ub-binding domains. Future studies need to take into account post-translational modifications and interactions that affect free poly-Ub function and processing.

It also stands to reason that there could be evolutionary differences between single-cell organisms like yeast, in which free poly-Ub is toxic, and higher order organisms that may have evolved compensatory mechanisms or pathways that utilize these chains. One function for untethered Ub chains that may be conserved between yeast and mammals involves stress granules: in cultured HeLa cells, free poly-Ub interferes with the clearance of stress granules [69]. Yeast also form stress granules [84], and they produce free poly-Ub species in response to stressors like heat shock and DNA alkylation [68], but any interplay between stress granules and unanchored Ub chains has not been established in yeast. In contrast to the conservation of stress granules, this review describes several studies linking unanchored Ub chains to signaling pathways that are not present in yeast [85]. Unanchored Ub chains can both positively and negatively regulate NF-kB pathways and viral response in multi-cellular organisms [28, 52, 57,
Continued studies will be essential to the discovery of additional physiological functions for free poly-Ub that may not be present in single-cell organisms.

Ultimately, the fact that unanchored Ub chains are not exclusively toxic presents exciting research opportunities. Different species of free poly-Ub have unique effects; it will be important to distinguish the type of chain responsible for specific actions within the cell, and under what circumstances these actions take place. Markers that recognize unanchored poly-Ub with specific linkage types could be developed to aid in the identification of chain types that are upregulated during certain stress responses, or in the mapping of the cellular distribution of different untethered Ub species. Since poly-Ub chains can be transferred en bloc to substrates in vivo, it will be interesting to investigate whether there are situations in which cells may prefer to use pre-formed chains for ubiquitination – perhaps when ATP is quickly consumed by other processes, such as during exercise. Proteomics, genetic and biochemical studies will be needed to identify E2s and E3s that can transfer Ub chains en bloc, and they can provide additional clues about pathways that involve free chains by identifying signaling proteins that bind or interact with them. Even within the pathways that are already known to involve unanchored poly-Ub, there are details that remain to be clarified; for instance, it is unclear why clearance of stress granules is impaired in cells that express free Ub chains. Do the chains interfere with the recruitment of proteins that normally disassemble stress granules? Do they serve a function, or are they just meant to be disassembled by USP5? In sum, this collection of studies highlights that unanchored poly-Ub species are multi-faceted entities that necessitate further study to comprehensively understand the consequences of their presence in the cell.

The next three chapters comprise the evidence that I collected regarding the expression, regulation, response and roles of free poly-Ub in Drosophila. Included verbatim are the following published manuscripts:


CHAPTER 2: EXPRESSION AND REGULATION OF DEUBIQUITINASE-RESISTANT, UNANCHORED UBIQUITIN CHAINS IN DROSOPHILA

2.1. Introduction

Posttranslational modification of proteins by ubiquitin (Ub) controls most cellular processes [1, 2, 20]. This modification adjusts how a protein interacts with cellular constituents, because Ub presents additional interaction interfaces [1, 21, 86]. Ubiquitination regulates protein localization, activity, function and half-life [1, 87]. Addition and removal of Ub from specific proteins is vital and is implicated in human diseases [1, 2, 20]. Ubiquitination involves the covalent attachment of Ub to—most commonly—lysine residues of substrate proteins through the coordinated action of a Ub activating enzyme (E1), a Ub conjugating enzyme (E2) and a Ub ligase (E3). Since Ub itself contains seven lysines, different Ub chains can be formed by the attachment of one Ub to another at different lysine residues; head-to-tail/linear chains, chains with mixed-linkages and branched chains are also generated [1, 6, 12]. Arguably the best known chain is K48-linked poly-Ub, which targets proteins for proteasomal degradation [8]. Other types of chains also exist in cells [1, 2, 57, 88-90].

Similar to other types of posttranslational modification, ubiquitination is reversible. Ub removal is accomplished by deubiquitinases (DUBs) and serves multiple roles: 1) editing chains to reduce errors; 2) bringing a molecular process to an end by, for example, disassembling a protein complex that merged as a result of a member’s ubiquitination; 3) recycling mono-Ub; and 4) disassembling unanchored/free poly-Ub [2, 21, 87, 91]. Unanchored poly-Ub results from en bloc cleavage of chains from substrates. Free chains function in autophagy-dependent processes, immune system-related steps and during DNA stress; unanchored poly-Ub chains are also synthesized anew [2, 21, 26, 33, 57, 68, 87, 88, 92-94].

Unanchored poly-Ub is thought to be quickly disassembled by DUBs [1, 21, 22, 87]. Accumulation of unanchored chains is proposed to be toxic by perturbing Ub-dependent processes. For instance, they may titrate out binding of ubiquitinated proteins to the proteasome.
[20, 33, 43, 92, 93, 95-97]. To the best of our knowledge, there are no published reports that directly examine unanchored poly-Ub species in an intact organism to address questions such as: what happens to unanchored chains if DUBs cannot dismantle them? Are unanchored poly-Ub species inherently toxic in vivo?

We generated poly-Ub transgenes that are not cleavable by DUBs and function as unanchored species. Their expression in Drosophila melanogaster is not lethal during development and adult flies tolerate the poly-Ub species well. These unanchored, non-cleavable poly-Ub are themselves decorated with Ub and regulated by the proteasome. We also found that these chains can be utilized en bloc without the need to be dismantled into mono-Ub. We propose that unanchored poly-Ub can be regulated independently of DUB-based disassembly and suggest a need to re-evaluate the extent of toxicity from free chains. Additionally, the new tools that we developed should help future work to pursue regulation of unanchored poly-Ub in non-canonical ways.

2.2. Materials and Methods

2.2.1. Antibodies

Anti-ataxin-3 (1:15000; MJD, rabbit polyclonal, [98]); anti-HA (1:1000; rabbit monoclonal; Cell Signaling Technology, #3724); anti-Ub (1:500; rabbit polyclonal; DAKO, #Z0458); anti-Tubulin (1:10000, mouse monoclonal, Sigma-Aldrich, #T5168); anti-Rpn10 (1:2000; rabbit polyclonal; AbCam, #ab18512); anti-20Sα (1:100; mouse monoclonal, Santa Cruz Biotech, #sc-65755); anti-Rpn9 (1:100; mouse monoclonal, Santa Cruz Biotech, #sc-65754); anti-Rpt6 (1:1000; rabbit polyclonal; Cell Signaling Technology, #13392); anti-VCP (1:1000; rabbit polyclonal; LSBio, #LS-C313248); anti-K63 (1:1000; rabbit monoclonal; Cell Signaling Technology, #5621); anti-K48 (1:1000; rabbit monoclonal; Cell Signaling Technology, #8081); anti-K27 (1:2000; rabbit polyclonal, Advanced Biomart, #FPA-21344M); anti-CycA (1:200; mouse monoclonal, Developmental Studies Hybridoma Bank at the University of Iowa, #A12); anti-actin (1:200; mouse monoclonal, Developmental Studies Hybridoma Bank at the University of Iowa,
anti-Lamin (1:200; mouse monoclonal; Developmental Studies Hybridoma Bank at the University of Iowa, ADL84.12); anti-GFP (1:1000; mouse monoclonal, Millipore, #MAB3580); goat anti-mouse, peroxidase conjugated secondary (1:5000; Jackson Immunoresearch); goat anti-rabbit, peroxidase conjugated secondary (1:5000; Jackson Immunoresearch). Antibodies against K27, K48 and K63 linkages were the only antibodies we were able to obtain.

2.2.2. Construct generation

Ub<sup>6</sup> transgenes (figure 2.1A) were synthesized by GenScript and cloned into pWalium10.moe. Purified plasmid was injected into yw; attP40 by Duke University Model Systems. The transformed chromosome was migrated onto w<sup>1118</sup> parental line. The Ub transgenes were subcloned into pcDNA3.1-HA vector for mammalian expression and into pGEX-6P1 for recombinant production in bacteria.

2.2.3. Drosophila-related procedures and stocks

In all panels and figures, flies were heterozygous for driver and transgenes. Flies were maintained in diurnal incubators at 25 °C and ~60% humidity, in conventional cornmeal media. Where noted, RU486 was used in the same media, as previously described [99]. Where noted, adults were maintained at 30 °C and ~60% humidity. Tubulin-Gal4-GS was a generous gift of Dr. R. J. Wessells, Wayne State University; sqh-Gal4 was originally gifted by Dr. Daniel Kiehart, Duke University; Mef2-Gal4 (#27390), elav-Gal4 (#458) and GMR-Gal4 (#8121) were from Bloomington Drosophila Stock Center; repo-Gal4 was gifted by Dr. Daniel Eberl, University of Iowa. The ataxin-3 lines have been described before [100-102]. The following RNAi lines were from the Bloomington Drosophila Stock Center: VCP (#32869, #35608), Rad23 (#44031, #44465), Rpt5 (#53886), Rpn1 (#34348), Ufd1-like (#41823), prosalpha2 (#36898), prosalpha3 (#55217), prosbeta5 (#34810), Rpn9 (#34034). The following RNAi lines were from the Vienna Drosophila RNAi Center: Rad23 (#30498), Rpn11 (#19272), Sem1 (#31787, #31789, #49152, #49153), Ufd1-like (#24700, #10473), p47 (#17529, #10748). For fly longevity, male and female adults were collected after eclosion and aged in conventional cornmeal fly media at 25 °C,
unless otherwise noted. The total number of flies per vial was ~20. Flies were transferred to new vials every 2–3 days, until all were dead.

2.2.4. Western blotting

Five whole adults flies, or ten dissected fly heads per group were homogenized in hot lysis buffer (50 mM Tris pH 6.8, 2% SDS, 10% glycerol, 100 mM dithiothreitol), sonicated, boiled for 10 min, and centrifuged at top speed at room temperature for 10 min. Western blots were developed and quantified using a CCD-equipped VersaDoc 5000MP system and Quantity One software (Bio-Rad), as described previously [103-105]. For transfected cells, media was removed, cells were rinsed with ice-cold PBS and lysed in hot lysis buffer, boiled for 10 minutes and spun for 10 minutes at max speed at RT.

2.2.5. Immunoprecipitation and subcellular fractionation

For stringent precipitation of HA-Ub from flies, 30 flies per group were homogenized in RIPA lysis buffer (50 mM Tris, 150 mM NaCl, 0.1% SDS, 0.5% deoxycholic acid, 1% NP40, pH 7.4) supplemented with complete protease inhibitor cocktail (PI; Sigma-Aldrich), sonicated, centrifuged at 15000 × g for 20 minutes at 4 ºC. Supernatant was denatured for 30 min with 1% final SDS at RT, renatured for 30 min with final 4.5% TritonX-100 at RT, and then incubated with anti-HA antibody-bound beads (Sigma-Aldrich) for 4 hours tumbling at 4 ºC. Beads were rinsed 5 × with RIPA, twice at 4 ºC for 5 minutes, and bead-bound complexes were eluted with SDS loading buffer and boiling for 5 minutes. For co-IPs under gentler conditions from the same flies, NETN lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, and 0.5% IPEGAL ca-630) was used instead, and the supernatant was incubated with beads without a denature/renature step, rinsed 4 × with NETN and bead-bound complexes were eluted with SDS loading buffer and heat. Precipitations for HIS-tagged Ub were conducted differently. Flies were homogenized in Buffer 1 (50 mM Tris pH 8, 6 M guanidine HCl, 10 mM imidazole) supplemented with PI, sonicated, centrifuged as above and the supernatant was incubated with Ni-NTA beads (Qiagen) for 2 hrs at 4 ºC. Afterwards, beads were rinsed 6 × each with Buffer 1, Buffer 2 (50 mM Tris pH 8, 150 mM
NaCl, 8 M urea, 20 mM imidazole), and Buffer 3 (50 mM Tris pH 8, 500 mM NaCl, 20 mM imidazole). Complexes were eluted with final 250 mM imidazole in Buffer 3. For stringent, HA-based purification from mammalian cells, pelleted cells (in ice-cold PBS) were lysed in RIPA buffer + PI, sonicated, centrifuged (15000 × g, 20 minutes, 4 °C) then denatured for 30 min at RT with 1% final SDS, renatured at RT with final 4.5 × TritonX-100 and incubated with anti-HA bead-bound antibody for 4 hours at 4 °C. Beads were then rinsed 10 × with RIPA + PI and protein was eluted with SDS loading buffer and heat. In all precipitation experiments, controls included bead-bound antibodies (for HIS$_6$ pulldowns, Ni-NTA resin) with lysate lacking the protein targeted by the antibody/resin. For subcellular fractionation, 5 flies per group were used with the ReadyPrep Protein Extraction Kit (Cytoplasmic/Nuclear; Bio-Rad). Flies were lysed in cytoplasmic extraction buffer and nuclei were resuspended in protein solubilization buffer. Samples were analyzed by western blotting.

2.2.6. Recombinant protein preparation and in vitro deubiquitination

Recombinant Ub$_6$ was produced in bacteria using previously published protocols, and eluted from glutathione sepharose beads using PreScission Protease (GE Healthcare) [38, 103, 104, 106-111]. For in vitro deubiquitination assays with recombinant Ub$_6$ and USP5, we utilized methods previously described [38, 103, 104, 107, 111, 112]. In brief, Ub$_6$ and USP5 were produced in bacteria, and eluted from glutathione sepharose beads by PreScission Protease (GE Healthcare). 1 µM (final) Ub$_6$ or 1 µM (final) commercial Ub chain (Boston Biochem) and 50 nM (final) USP5 were combined together in kinase reaction buffer (0.5 M Tris pH 7.5, 0.5 M KCl, 0.2% DTT) and incubated at 37 °C for the indicated amounts of time. Fractions were collected at the indicated time points and reactions were stopped by adding SDS loading buffer and boiling for 1 minute. For deubiquitination of ubiquitinated Ub$_6$ species from Drosophila lysates, 40 whole flies expressing HA-Ub$_6$-STOP in all tissues (sqh-Gal4 was the driver) were homogenized in RIPA lysis buffer + PI, sonicated, centrifuged (15 min, 15000 × g at 4 °C) and supernatant was incubated with anti-HA bead-bound antibody (Sigma-Aldrich) for 1.5 hr. Beads were rinsed 10X with
RIPA + PI and 3X with kinase buffer, split equally and one side was supplemented with additional PI, whereas the other was supplemented with 100 nM (final) USP2 catalytic domain (Boston Biochem). Reactions were incubated at 37 °C for the times indicated in figures and reactions were stopped by the addition of 2X SDS sample buffer and by boiling for 1 minute.

2.2.7. Mammalian cells and procedures

HEK-293 and HeLa cells were from ATCC and were grown in DMEM with 10% FBS and 5% Penicillin-Streptomycin under conventional conditions. Cells were transfected using Lipofectamine LTX (Invitrogen) as directed by the manufacturer. Twenty-four hours after transfection, cells were harvested in boiling SDS lysis buffer.

2.3. Results

2.3.1. Expression of unanchored poly-Ub in Drosophila

We devised a strategy to express in Drosophila unanchored poly-Ub that cannot be dismantled by DUBs. While this approach introduces exogenous Ub, we reasoned that our plan would directly examine unanchored chains that cannot be removed through deubiquitination. We designed two chains that consist of six Ub in tandem and lack internal “GG” motifs that are necessary for isopeptide bond formation and their dismantling by DUBs (figure 2.1A). The first (Ub⁶-Stop) cannot be cleaved by DUBs and lacks a terminal “GG”, meaning that it cannot itself be conjugated onto other proteins. The second version is conjugatable; it does not have internal “GG” motifs, but contains a “GG” at the end (Ub⁶-GG). We tested two DUBs for their ability to cleave these chains. As shown in figure 2.1B, the chains we generated are not cleaved in vitro by USP5. USP5 rapidly cleaves all types of chains in vitro, including linear chains [38] and can also cleave chains with a free C-terminal “GG” or with an occupied “GG” (i.e. anchored chain) [1, 22, 38, 106, 113]. Figure 2.1F shows that another DUB, USP2, is also unable to cleave Ub⁶-Stop into single Ub. We began our studies with the non-cleavable, non-conjugatable Ub⁶-Stop. We should
note that utilizing a linear chain is currently the only way to ensure efficient and robust production of non-cleavable poly-Ub in an intact organism. Importantly, as we describe below, these linear
chains are quickly modified with different linkages to form various topologies.

We generated *Drosophila* lines that utilize the Gal4-UAS system [71, 72] to express Ub\(^6\)-Stop. We began by expressing Ub\(^6\)-Stop throughout the fly, using the driver sqh-Gal4, which expresses in all tissues, during development and in adults [114-119]. As shown in figure 2.1C, Ub\(^6\)-Stop migrates as a number of species on western blots. The lowermost band is the expected, unmodified version of the protein, whereas the higher species are most likely posttranslationally modified. Based on results from stringent immunopurifications (IPs) with a denature/renature step, higher species consist of ubiquitinated versions of Ub\(^6\)-Stop. We first co-expressed a mono-Ub transgene that is HA-tagged alongside Ub\(^6\)-Stop that is HIS\(^6\) tagged, then precipitated HIS\(^6\)-Ub\(^6\)-Stop and examined its labeling by the mono-Ub. As shown in figure 2.1D, Ub\(^6\)-Stop heavy molecular weight species, but not the unmodified form, are labelled by the antibody that detects the mono-Ub we introduced. This finding indicates that the Ub\(^6\)-Stop linear chain is itself posttranslationally modified by Ub. Based on the quantification of Ub\(^6\)-Stop conjugated and non-conjugated bands and smears, ~75% of the total Ub\(^6\)-Stop is modified (figure 2.1E).

To additionally confirm that higher molecular weight Ub\(^6\)-Stop species are ubiquitinated, we subjected Ub\(^6\)-Stop from flies to the catalytic domain of USP2 (USP2\(_{\text{CD}}\)). Within 10 minutes, the higher molecular weight species collapse to the expected, unmodified Ub\(^6\)-Stop in the presence of USP2\(_{\text{CD}}\) (figure 2.1F). A band immediately above Ub\(^6\)-Stop remains stable. Based on our stringent IPs (figure 2.1D,G), this is most likely mono-ubiquitinated Ub\(^6\)-Stop. USP2\(_{\text{CD}}\) might be unable to remove this modification. Also, we cannot formally discount the possibility of another type of posttranslational modification of this Ub\(^6\)-Stop band. This species notwithstanding, our results demonstrate that Ub\(^6\)-Stop is itself ubiquitinated in the fly.

To get a glimpse at the type of Ub linkages attached onto Ub\(^6\)-Stop, we conducted additional, stringent IPs and probed Ub\(^6\)-Stop with antibodies against K27, K48 and K63 linkages. We found that Ub\(^6\)-Stop is modified with K27, K48 and K63 linkages (figure 2.1G). Ub\(^6\)-Stop itself is essentially a linear chain; the fact that there are K27, K48 and K63 Ub-Ub conjugates on it
means that the higher molecular weight species of Ub\(^6\)-Stop constitute branched chains with various Ub-Ub linkages. These types of poly-Ub species exist in cells. Our analysis does not distinguish whether a specific branch of Ub moieties added onto Ub\(^6\)-Stop comprises the same type or different types of linkages. Based on these biochemical data, we conclude that higher molecular weight species of Ub\(^6\)-Stop contain branched chains and various Ub-Ub linkages.

When compared to the rest of Ub in the fly by western blotting, Ub\(^6\)-Stop species are abundant in *Drosophila*. We observe the unmodified band of Ub\(^6\)-Stop, as well as an overall darkening of the Ub smear above it (Figure 2.1H), which most likely consists of Ub\(^6\)-Stop conjugated with endogenous Ub. Quantification of these data shows a statistically significant increase in Ub signal in the presence of Ub\(^6\)-Stop, when expressed in all tissues (figure 2.1H, quantification box). Thus, Ub\(^6\)-Stop species appear to be prominently expressed.

To assess how quickly Ub\(^6\)-Stop is ubiquitinated, we used the inducible, RU486-dependent ubiquitous driver, tubulin-Gal4-GS to drive the Ub\(^6\) construct. We raised flies in media without RU486. On day 1 as adults, flies were switched to RU486-food for 0–7 days. Ub\(^6\)-Stop is rapidly produced (figure 2.1I). Within 24 hours of being placed into RU486-containing media, we observe prominent Ub\(^6\)-Stop species, which reach an equilibrium over 3–4 days. This is most likely due to initial Ub\(^6\)-Stop production followed by degradation as the process stabilizes.

Next, we assessed the distribution of Ub\(^6\)-Stop into major cellular sub-compartments. We found that these species are cytoplasmic (figure 2.2A). We also found that their expression does not impair proteasome-dependent degradation (figure 2.2B,C). We examined endogenous cyclin A, a proteasome substrate, and CL1-GFP, a reporter of proteasome activity in mammalian and fly systems; the CL1 degron targets GFP for ubiquitination and subsequent proteasome-dependent degradation [38, 120-122]. Panels 2B and 2C show that the levels of neither proteasome substrate increase in the presence of Ub\(^6\)-Stop. In fact, we observed diminished levels of CL1-GFP with Ub\(^6\)-Stop, indicating increased—not decreased—proteasome activity. The
levels of cyclin A did not change in the presence of Ub\textsuperscript{6}-Stop. We also blotted for VCP, whose expression is increased when proteasome function is reduced in the fly [123] and did not find significant differences in the presence of Ub\textsuperscript{6}-Stop (figure 2.2C). Lastly, endogenous proteasome subunits were not significantly impacted by the presence of Ub\textsuperscript{6}-Stop (figure 2.2D). Results in
figure 2.2B–D indicate that Ub⁶-Stop does not inhibit the activity of the proteasome in vivo and that its presence does not perturb levels of endogenous proteasome components.

2.3.2. Expression of unanchored poly-Ub is not devastating in Drosophila

We examined lethality from unanchored chains in Drosophila by driving the expression of Ub⁶-Stop through sqh-Gal4. This is a highly robust driver that others and we have used with strong outcomes, such as early developmental lethality from knockdown of various genes and high toxicity from expression of various, mutant proteins [36, 38, 100-102, 114-118, 124, 125]. Drosophila undergo several developmental stages, none of which appears impacted by Ub⁶-Stop (figure 2.3A). When we tracked adult fly longevity in the absence or presence of Ub⁶-Stop, we again did not notice marked deviation between the two groups (figure 2.3B). This lack of a statistically significant difference in longevity in adult flies expressing Ub⁶-Stop was also noticeable when adults were stressed with heat. As shown in figure 2.3C, flies expressing Ub⁶-Stop and placed at 30 °C live similarly to their non-Ub⁶-Stop counterparts.

We subsequently expressed Ub⁶-Stop in select fly tissues—neurons, glia and muscle cells—using drivers common to the fly community. We selected this approach because tissue-specific drivers can express UAS transgenes more strongly in that particular tissue than ubiquitous drivers, as exemplified in figure 2.3D. Figure 2.3D shows expression of Ub⁶-Stop in different tissues; the muscle driver expresses this construct very highly. Developmental observations did not show marked differences in lethality from Ub⁶-Stop expressed in the tissues tested (figure 2.3E). When we examined adult fly longevity, 50% were dead a few days earlier than controls in the presence of Ub⁶-Stop (figure 2.3F). Comparison of the day when all flies were dead showed that with muscle expression, adults expressing Ub⁶-Stop persisted longer than controls. Statistical analyses of these results revealed a significant difference in the overall longevity of flies expressing Ub⁶-Stop in glial or neuronal cells compared to control flies: presence of Ub⁶-Stop in these tissues led to overall shorter lifespan. Still, this is a mild deviation. Muscle-
specific expression did not lead to a statistically different lifespan compared to controls.

Collectively, these data lead us to the conclusion that Ub₆-Stop is not devastating overall.

Figure 2.3. Expression of Ub₆-Stop everywhere or in specific tissues is not lethal to Drosophila. (A) Summary of the effects of expression of Ub₆-Stop throughout fly development. Driver was sqh-Gal4. Control: sqh-Gal4 on the genetic background used to generate the Ub⁶ flies. We monitored daily lethality at larval, pupal and pharate adult stages among groups and did not notice differences in their development. Little to no developmental lethality occurred in all crosses. We monitored at least 10 independent crosses, all at 25 °C. (B,C) Percent longevity of adult flies not expressing or expressing Ub₆-Stop at the indicated temperatures. P values were calculated using log-rank (Mantel-Cox) tests. (D) Western blots showing the expression levels of Ub₆-Stop in the indicated tissues. We used one day old, whole adult flies for all lysates. Results are representative of experiments conducted independently four or more times, with similar results. Asterisk: nonspecific band. (E) Summary of lethality during development when Ub₆-Stop was expressed in specific tissues, as in (A). We monitored lethality at larval, pupal and pharate adult stages. We did not notice differences in their development and little to no developmental lethality occurred in all crosses. We monitored at least 10 independent crosses, all at 25 °C. (F) Percent longevity of adult flies not expressing or expressing Ub₆-Stop in the indicated fly tissues. Controls consisted of the respective drivers on the genetic background utilized to generate Ub₆ flies, but without the transgene. P values were calculated using log-rank (Mantel-Cox) tests. In all panels, flies were heterozygous for driver and Ub⁶.
Longevity studies with the ubiquitous driver (figure 2.3A–C) and tissue-specific drivers (figure 2.3D–F) were conducted at different times, maintained in different rooms and reared and aged in fly media prepared with different batches of primary ingredients. These factors most likely account for the overall shorter longevity of flies in figure 2.3F compared to 3B. However, control flies for each experiment (figure 2.3A,B,C,E,F) were collected, aged and observed side by side with the Ub\textsuperscript{6}-Stop flies.

### 2.3.3. Protein levels of Ub\textsuperscript{6}-Stop depend on the proteasome

Since Ub\textsuperscript{6}-Stop cannot be disassembled by DUBs, we wondered whether it can be regulated by the proteasome. First, we assessed its persistence in vivo. We again utilized the RU486-dependent, ubiquitous driver, tubulin-Gal4-GS. Flies that contained one copy each of the driver and Ub\textsuperscript{6}-Stop transgenes were reared in food without RU486 until day 1 as adults, then placed on media with RU486 for 7 days to induce transgene production. On day 7, they were switched to media without RU486 to halt Ub\textsuperscript{6}-Stop expression and collected at different points. As shown in figure 2.4A, Ub\textsuperscript{6}-Stop protein is mostly degraded within seven days, although we still observe it at 14 days. Thus, this protein is turned over in the intact fly. Next, we examined whether the proteasome regulates its levels.

We used RNA-interference (RNAi) to specifically knock down genes that encode proteasome components. Where available, we employed more than one RNAi line for each gene. We conducted RNAi-based studies in fly eyes, because knockdown of most of the genes we targeted is lethal when performed everywhere. We targeted \(\alpha\) and \(\beta\) subunits of the 20S proteolytic core and the following components of the 19S proteasome: Rpn1 (helps with Ub binding and processing at the proteasome) [126], Rpn9 (anchors other components to the 19S) [127], Rpn11 (removes Ub chains) [20, 96], Rpt5 (facilitates interaction of 19S with 20S) [128] and
Targeting each of these subunits through RNAi led to higher levels of Ub\(^6\)-Stop protein compared to controls; controls consisted of the background genetic line of RNAi constructs in the absence of any knockdown (figure 2.4B–E). Similarly, knockdown of Sem1 led to prominently higher levels of Ub\(^6\)-Stop (figure 2.4D,E). Sem1 is necessary for 19S assembly [130] and is a stoichiometric component of the 19S, where it functions as a Ub receptor [130-132]. Collectively, these results strongly implicate the proteasome in Ub\(^6\)-Stop degradation.

Ubiquitinated proteins can come into direct contact with the proteasome, or can be assisted by Ub-binding proteins, referred to as proteasome shuttles. To examine the involvement
of proteasome shuttles in Ub$^6$-Stop turnover, we investigated the segregase, VCP, which functions in part to help deliver ubiquitinated proteins to the proteasome for degradation, as well as its cofactors, p47 and Ufd1-like [133-138]. We also targeted the proteasome shuttle protein Rad23 [139-142]. Knockdown of VCP (figure 2.4C,E), p47 and Ufd1-like (figure 2.4D,E) each led to consistently higher protein levels of Ub$^6$-Stop. Knockdown of Rad23 did not have as prominent of an effect on Ub$^6$-Stop protein levels compared to p47, Ufd1-like and VCP (figure 2.4D,E).

Subsequently, we examined the interaction of Ub$^6$-Stop with VCP and the proteasome. We expressed Ub$^6$-Stop in fly eyes, precipitated it under mild conditions and examined its interaction with endogenous Rpn9 (anchors Rpn10 to the 19S), Rpn10 (Ub receptor), 20Sα (proteolytic portion) and VCP [127, 143, 144]. Ub$^6$-Stop co-precipitated VCP, Rpn10 and Rpn9. Unlike with the 19S components, we were unable to specifically co-precipitate 20Sα with Ub$^6$-Stop (figure 2.5A,B). This could be due to dissociation of the 19S and 20S components during the IP. The rest of the results from panels 5A and B, however, indicate that Ub$^6$-Stop comes into physical contact with the proteasome and VCP.

Lastly, we examined the effect of p47 in the interaction of Ub$^6$-Stop with VCP and, downstream from this AAA ATPase, the proteasome [145]. We targeted this VCP cofactor based on data that it binds branched chains [12] and because its knockdown has a clear impact on Ub$^6$-Stop levels (figure 2.4). As shown in figure 2.5C, knocking down p47 leads to decreased levels of VCP and Rpn10 that co-precipitate with Ub$^6$-Stop. Knockdown of p47 did not reduce the amount of Rpn10 that co-precipitates with Ub$^6$-Stop as much as the levels of VCP that co-precipitates with Ub$^6$-Stop. This may be due to VCP-independent routes through which Ub$^6$-Stop reaches the proteasome. Collectively, data in figure 2.5 suggest that Ub$^6$-Stop comes into contact with the proteasome at least in part through p47 and VCP.

2.3.4. Ub$^6$-GG can be conjugated en bloc in mammalian cells and in Drosophila

Our work thus far focused on Ub$^6$-Stop, which cannot be conjugated onto other proteins. We studied these chains to examine the regulation of unanchored species when they cannot be
cleaved into mono-Ub. Still, the prospect exists that unanchored chains, which normally contain a terminal “GG”, might become conjugated onto other proteins, effectively eliminating them from the unanchored pool. The possibility of *en bloc* conjugation of an entire Ub chain has been considered for some time in the Ub community [75-78, 146]. We reasoned that our non-cleavable Ub species could be used to assess *en bloc* transfer within the frame of unanchored poly-Ub regulation.

We transiently expressed Ub<sup>6</sup>-Stop and Ub<sup>6</sup>-GG in mammalian cells. As shown in figure 2.6A, expression of Ub<sup>6</sup>-GG leads to a smear above the unmodified band. Unlike in flies, we do not observe a smear above Ub<sup>6</sup>-Stop when it is transiently transfected in cells, even though it is degraded via the proteasome. Using stringent, denature/renature IPs of Ub<sup>6</sup>-GG from mammalian cells, we found an endogenous protein chemically modified by this chain, ataxin-3 (figure 2.6B), whose ubiquitination we have documented before [103, 107, 111]. Ataxin-3 is a DUB whose catalytic activity does not eliminate its own ubiquitination [108]. Mutations in the

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**Figure 2.5. Ub<sup>6</sup>-Stop interacts with the proteasome, VCP and p47 in Drosophila.** (A–C) Western blots of co-IPs from whole fly heads expressing Ub<sup>6</sup>-Stop in fly eyes. Flies were heterozygous for GMR-Gal4 and HA-tagged Ub<sup>6</sup>-Stop. Asterisks: non-specific bands in lanes not expressing HA-Ub<sup>6</sup>-Stop. Results are representative of experiments conducted independently at least three times, with similar results. In each panel, “Ctrl” signifies anti-HA-bead-bound antibody that was incubated with fly lysates that do not express any HA-tagged Ub<sup>6</sup>-Stop. For quantified blots in panel (C), we loaded gels to achieve comparable levels of Ub<sup>6</sup>-Stop in the IP lanes. The entire HA-Ub<sup>6</sup> smear was quantified. Shown in histograms are means −/+ SD. P values are from two-tailed Student’s t-tests.
polyglutamine region of this DUB cause the neurodegenerative disease, Spinocerebellar Ataxia Type 3 [147]. Unmodified ataxin-3 migrates at ~42 kDa. Ub$^6$-GG migrates immediately below
50 kDa. We would expect ubiquitinated forms of ataxin-3 by Ub\textsuperscript{6}-GG to appear ≥90 kDa. In figure 6B, the ataxin-3-positive smear from Ub\textsuperscript{6}-GG denature/renature IPs begins below 100 kDa and extends all the way to the top, consistent with Ub\textsuperscript{6}-GG-ataxin-3.

We then turned to flies. Expression of the non-cleavable, conjugatable Ub\textsuperscript{6}-GG in all tissues does not cause marked lethality (figure 2.6C). To examine whether Ub\textsuperscript{6}-GG can be utilized en bloc in flies, we again investigated ataxin-3, which is ubiquitinated in Drosophila [111, 148]. Here, we used ataxin-3 with an expanded polyglutamine tract (from wild-type with ~20 repeats to pathogenic-range 77 glutamine residues), because this version of the protein is well ubiquitinated in the fly (our unpublished observations). We expressed ataxin-3 in the absence or presence of Ub\textsuperscript{6}-GG pan-neuronally and utilized whole, intact flies to isolate HIS\textsuperscript{6}-Ub\textsuperscript{6}-GG under stringent, denaturing conditions. As shown in figure 2.6D, in the presence of Ub\textsuperscript{6}-GG we observe ataxin-3 species in the high molecular weight portion of the pulldown lane, but not in the negative control lane, which has ataxin-3 but lacks Ub\textsuperscript{6}-GG. We do not notice unmodified ataxin-3 species, intact or proteolytically cleaved, in either of the IP lanes (figure 2.6D). Polyglutamine-expanded, unmodified ataxin-3 migrates at ~60 kDa. Accounting for Ub\textsuperscript{6}-GG (~50 kDa), we would expect the ubiquitinated species of ataxin-3 above the 100 kDa band, which is what we see (figure 2.6D). There isn't a marked change in the higher molecular weight species of ataxin-3 in input lanes when Ub\textsuperscript{6}-GG is expressed alongside this protein, but we clearly observe ataxin-3 signal in the Ub\textsuperscript{6}-GG pulldown lane. Our interpretation of these results is that a portion of ataxin-3, in the high molecular weight part of the gel, is modified with Ub\textsuperscript{6}-GG. Thus, some of ataxin-3 in the fly can be modified with the non-cleavable, conjugatable Ub\textsuperscript{6} species, without the need for this type of chain to be first cleaved into mono-Ub. We conclude that unanchored poly-Ub can be utilized en bloc for conjugation onto other proteins in an intact organism, without first being disassembled into mono-Ub.
2.4. Discussion

We set out to examine what happens to unanchored chains when they cannot be disassembled by DUBs. We generated new *Drosophila* models of poly-Ub, which we believe will find future use in the fly and ubiquitin communities. The unanchored chains that we constructed exist in different lengths and topologies *in vivo*, from unmodified Ub$^6$ to markedly higher molecular weight species, and contain various linkages. Nearly 75% of Ub$^6$ appears as modified bands/smears conjugated with endogenous Ub. These free chains were degraded by the proteasome and could also be attached onto other proteins, without the need to be first deconstructed into mono-Ub. Based on these findings, we propose a model of unanchored chain management that comprises four potential routes (figure 2.7): the first is canonical, where unanchored poly-Ub is dismantled and then reutilized [1, 21, 22, 92, 96]. Besides it, we posit that there may be additional options for unanchored chains: they can be degraded by the proteasome, they can be eliminated by being used *en bloc* to be conjugated onto other proteins in the cell, or they can be bound by Ub-binding proteins and maintained in a separate or “reserve” pool until they re-enter utilization, if or when needed. The last possibility is not necessarily mutually exclusive with the other potential avenues and may serve as a feeding route for the other options.

How is the decision made to degrade or reutilize an unanchored chain rather than dismantle it? Under some conditions, it might be more advantageous to use a ready-made chain than to make a new one to attach to a specific substrate, perhaps during low energy states or when increased rates of protein turnover are required—in other words, chain shuffling among different proteasome substrates could enhance their targeting and degradation.

The unanchored chains that we designed and expressed in flies do not represent all of the various types of Ub linkages and species that can be found in the cell. In fact, linear chains are a minor constituent of the total Ub pool [149] and the functions of these types of chains are not entirely known [150], hindering to some extent the utility of the lines that we generated toward understanding more broadly Ub-dependent pathways. Still, the linear Ub$^6$ that we constructed
exist as modified and unmodified species of various lengths and topologies in *Drosophila*. We contend that these species represent different types of free poly-Ub *in vivo* and, at the very least, can be used to provide clues into regulatory mechanisms that dictate what happens to branched chains containing multiple Ub-Ub linkages. It was recently reported that branched chains consisting of K48 and K11 linkages are generated by E2/E3 complexes and play critical roles in targeting misfolded proteins for proteasomal degradation [12].

The VCP cofactors Ufd1-like and p47 as well as VCP itself are critical for the levels of unanchored poly-Ub. The proteasome-associated protein, Rad23 also appeared important in this process. We did notice variation in the extent of effect with different RNAi lines targeting a specific gene; this is most likely due to differences in the efficacy of each line in reducing the mRNA levels of the targeted fly gene. Based on our results, VCP and its co-factors are key players for the protein levels of unanchored poly-Ub in the fly. This is not surprising for chains comprising various

**Figure 2.7. Model of unanchored poly-Ub regulation in vivo.** Unanchored poly-Ub can be dismantled into mono-Ub and reutilized in future Ub reactions by the coordinated action of E1/E2/E3. This is the canonical model of Ub recycling. Our results in Drosophila suggest the possibility of other, non-mutually exclusive mechanisms of unanchored Ub chain control: their degradation as whole units by the proteasome; their reutilization en bloc, enabling the conjugation of the entire chain onto a substrate without the prior need for dismantling into mono-Ub; or their maintenance in a separate, "reserve" pool through interaction with Ub-binding proteins. The "reserve" pool could feed into the other routes.
linkages (K48, K63 and K27, at least), since p47 and VCP are adept at recognizing branched chains in mammalian cells [12].

Unanchored chains have been argued to compete with ubiquitinated proteasome substrates for access to the 26S proteasome [43, 44, 47, 96]. However, unanchored poly-Ub species that we induced in the fly do not negatively impact degradation of proteasome substrates, even though these chains interact with the proteasome. We examined two substrates, endogenous cyclin A and the reporter CL1-GFP. The protein levels of neither substrate were increased. In fact, we observed lower protein levels of CL1-GFP, indicative of enhanced degradation of this fusion protein. We also did not observe changes in the levels of VCP, which is upregulated when the fly proteasome is inhibited [123]. Our results suggest that unanchored chains need not play an exclusively inhibitory role for the proteasome. Binding of free poly-Ub to various Ub-binding proteins could sequester the chains away from the proteasome, keeping the degradative machinery unperturbed. But, what might account for lower levels of CL1-GFP? For a subset of proteins degraded by the proteasome, unanchored poly-Ub might enhance substrate delivery to this machinery as a result of recruitment of Ub-binding proteins that normally delay their degradation, but which are now occupied with free poly-Ub. In the case of CL1-GFP, it might be that a protein that would normally bind to CL1-GFP and restrain its degradation is now occupied with Ub6, leading to more prompt degradation of CL1-GFP. Other possibilities exist. Future work is required to untangle these and other details.

The discrepancy between our work and prior reports that unanchored chains impede proteasome activity [43, 44, 47, 96] could be due to various reasons and highlights a need for additional studies of unanchored poly-Ub regulation. Some earlier work was conducted in cultured mammalian cells using transient expression. Perhaps, unanchored poly-Ub transiently inhibits the proteasome and becomes toxic in isolated cells, whereas *in vivo* free chains are easily managed. Other work focused on the function of specific DUBs, such as USP5, whereas we assessed unanchored poly-Ub more directly. Proteasome inhibition when certain DUBs are absent might
result from perturbation of specific substrates rather than general effects from unanchored poly-Ub. For example, similar to others [37], we observed impeded proteasome function when USP5 was knocked down in the fruit fly [38]. However, USP5 mutation or knockdown might lead to inhibited proteasome activity as a result of accumulation of its substrates, independently of unanchored chains. In fact, not all proteasome substrates are impacted by USP5 knockdown [47] and there is evidence that USP5 has specific substrates [113, 151, 152]. The tools that we generated here will be beneficial for future work to continue assessing the consequences of unanchored poly-Ub in intact organisms.

Lack of consistent and marked lethality from unanchored chains during development or in adults, including under heat stress, suggests that they are not necessarily toxic. Nonetheless, conditions or tissues where unanchored poly-Ub can be problematic may exist. For example, we did observe mild reduction in the lifespan of flies expressing Ub6-Stop in glial cells or neurons specifically. A study of ubiquitin homeostasis at the Drosophila neuromuscular junction (NMJ) during larval development [97] utilized mono-Ub transgenes that can be modified with endogenous Ub, but which cannot themselves be added onto other substrates because they lack a terminal “GG”. Post-synaptic presence of these transgenes caused morphological anomalies under some circumstances, but not others. If K48 linkages could be attached onto the transgene, there were mild, but statistically significant, anomalies. If K48 linkages could not be made onto the mono-Ub transgene (other chains could be constructed) there was no toxicity [97]. Thus, some types of unanchored linkages appeared mildly problematic in this assay, while others did not. Whether NMJ anomalies disappeared with continued development, or if these constructs were lethal throughout the fly was not clear. Together with our results, this earlier study [97] substantiates the conclusion that unanchored chains need not be toxic. Although we used robust drivers without devastating lethality effects, we will not discount the possibility of anomalies caused by free chains at very high levels, under certain stressors, or in specific types of assays.
The point we want to highlight is that unanchored chains can be handled well \textit{in vivo} and that the extent of their toxicity should be reevaluated.

In summary, unanchored chains can be managed \textit{in vivo} in ways that do not require their disassembly by DUBs: they can be degraded and they may even be conjugated \textit{en bloc} to other proteins. Our work presents new possibilities into Ub recycling and reutilization.
CHAPTER 3: UNANCHORED UBIQUITIN CHAINS DO NOT LEAD TO MARKED ALTERATIONS IN GENE EXPRESSION IN DROSOPHILA MELANOGASTER

3.1. Introduction

Cellular and organismal physiology and homeostasis are regulated at multiple, interdependent levels that extend from DNA-based regulation of gene expression to the epigenetic control of genes themselves and of their products. Among the more flexible systems of epigenetic control is the post-translational modification of cellular proteins by various adducts, including ubiquitination, phosphorylation, methylation and acetylation. Ubiquitination represent a highly malleable system of post-translational regulation of proteins and the complexes in which they participate[1, 2]. Ubiquitin (Ub), itself a small protein of approximately 8.5 kDa, is highly conserved among all eukaryotic species and regulates proteins in various ways, from tagging them for proteasomal degradation to directing their participation in cellular signaling pathways[1, 87, 153].

Ubiquitin conjugation onto another protein, what is termed “ubiquitination”, requires the concerted effort of three types of enzymes: an E1 activating enzyme, an E2 conjugating enzyme and an E3 ligase (Figure 1A). This ATP-dependent process results in an iso-peptide bond between the C-terminal “GG” motif of a Ub molecule and the substrate protein, typically at a lysine residue. Ub itself can also become ubiquitinated, resulting in a poly-Ub chain defined by the specific modified lysine residue or, in the case of M1/linear chains, the methionine residue[1, 6, 12]. The type of chain created has a distinct effect on the fate of its substrate protein; for instance, K48 chains are known for their involvement in targeting proteins for proteasomal degradation[8]. Ubiquitination is reversible; deubiquitinases (DUBs) remove Ub from a protein or edit the length of a chain[2].

Unanchored Ub chains--poly-Ub that is not tethered onto a substrate protein--also exist in the cell and can arise when a DUB removes an intact chain from a protein, or can be generated
Figure 3.1. Unanchored poly-Ub. (A) Unanchored poly-Ub can arise after E1/E2/E3 cycles build a Ub chain onto a substrate, after which a DUB removes the chain as a single species. It is believed that these untethered chains are then dismantled by additional DUBs to yield mono-Ub that can be recycled in new ubiquitination events. (B) Schematic of the two types of Ub6 chains we designed for expression in Drosophila. Both Ub6-Stop and Ub6-GG are head-to-tail hexa-Ub that cannot be dismantled by DUBs. Ub6-GG, but not Ub6-Stop, can be conjugated onto other proteins. (C) Ubiquitous Ub6 expression does not affect the development or the lifespan of the fly (Blount et al., 2018). One-day-old adults were collected and processed for RNA-Seq and qRT-PCR analyses.

Although unanchored poly-Ub is not well understood, it has been implicated as a participant in several cellular processes, including NF-κB signaling and autophagy[2, 21, 26, 33, 57, 68, 88, 92-94, 154]. The currently prevailing view is that unanchored Ub chains are quickly disassembled by DUBs and recycled as mono-Ub (Figure 1A)[1, 21, 22, 87]. Studies in yeast and in cultured mammalian cells have suggested that the buildup of free poly-Ub might become toxic by perturbing Ub-dependent proteasomal degradation[37, 43, 44, 47, 95, 96].
Intriguingly, when we examined the toxicity of untethered chains in an *in vivo* context, we observed that the extended presence of untethered Ub chains is not necessarily deleterious to an intact organism, *Drosophila melanogaster* [70]. For these studies in *Drosophila*, we designed head-to-tail hexa-Ub chains that lack all “GG” motifs (Ub^6; Figure 1B), making them both resistant to cleavage by DUBs and unable to be conjugated onto other proteins; these resemble linear, unanchored Ub chains that are endogenously present [1, 21, 22, 87]. When expressed in all tissues, unanchored poly-Ub does not negatively impact the lifespan of the fly (Figure 1C) [70]. It has been suggested that free poly-Ub could interfere with the proteasome. However, we observed no deficiencies in proteasome subunit expression or function; in fact, untethered poly-Ub were degraded by the proteasome [70]. We also observed that a pre-existing Ub chain can be used *en bloc* to ubiquitinate a protein *in vivo* [70].

Throughout our studies [70], we became confident that unanchored poly-Ub is not inherently or especially toxic, but it was still unclear whether the presence of either form induces a concerted cellular response against them. While both Ub^6_ constructs resemble linear, unanchored poly-Ub, their inability to be cleaved is unnatural. Does the introduction of these exogenous chains bring about an organismal response, or are they as readily tolerated as they seem to be? Is there an upregulation of dismantling DUBs, like USP5, which is widely reported to disassemble free poly-Ub [1, 22, 38, 106, 113]? Is there a change in the expression of E2/E3 pairs that might be able to take advantage of premade chains? Using RNA-Seq to compare Ub^6-GG and Ub^6-Stop to non-Ub^6-expressing controls we observed that ubiquitous expression of each Ub^6 construct induces significant changes in the expression of approximately 90 genes, with no clear indication of a specific cellular response mounted. Surprisingly, only two Ub-related genes were affected at the transcript level, and our analyses did not reveal a coordinated effect on pathways that are known to involve unanchored poly-Ub. According to these analyses, unanchored poly-Ub does not elicit a marked organismal response in *Drosophila*, suggesting that these species are not inherently problematic.
3.2. Materials and methods

3.2.1. Fly lines

Generation of transgenic *Drosophila* lines was described previously [70]. Ubiquitous gene expression was driven by sqh-Gal4 [71, 72, 114-116, 118, 119], with all flies heterozygous for the transgene and the driver. In the case of controls, all flies were heterozygous for sqh-Gal4 on the genetic background of Ub6 flies. Crosses were maintained in diurnal incubators at 25°C and ~60% humidity, on conventional cornmeal media. One-day-old adult offspring were collected for RNA isolation.

3.2.2. RNA isolation

Total RNA was extracted from ten whole flies per group using TRIzol reagent (Invitrogen), following the manufacturer’s protocol. RNA was then treated with TURBO DNase (Ambion) to eliminate contamination by DNA.

3.2.3. RNA-Seq

RNA expression analysis was conducted at the Wayne State University Applied Genomics Technology Center. Four biological replicates were used for each genotype. An aliquot of the RNA was assessed by microfluidics using the ScreenTape for the Agilent 2200 TapeStation. The electrophoretogram, RNA integrity number (RIN), and the ratio of the 28S:18S RNA bands were collectively examined to determine overall quality of the RNA (Table S3). RNA-Seq, primed from the poly(A) tail, was used to determine expression profiles. Lexogen’s QuantSeq 3′mRNA-Seq Library Prep Kit (FWD for Illumina) was utilized for building RNA-Seq libraries from 250 ng of total RNA in 5 µl of nuclease-free ultrapure water. Libraries were quantified on the Qubit and Agilent 2200 TapeStation using the DNA High Sensitivity Screen tape. The barcoded libraries were multiplexed at equimolar concentrations and sequenced with 50 bp reads in rapid mode on an Illumina HiSeq 2500. Data were de-multiplexed using Illumina's CASAVA 1.8.2 software. After quality was assessed [155] reads were aligned to the *Drosophila* genome (Build dm3) with STAR_2.4 [156] and tabulated for each gene region [157]. All raw data was uploaded into NCBI
GEO database (awaiting GEO ID number). Differential gene expression analysis was used to compare transcriptome changes between conditions using edgeR v.3.22.3 [158]. All conditions were individually compared (i.e. Ub⁶-GG versus control, Ub⁶-Stop versus control, and Ub⁶-Stop versus Ub⁶-GG) and transcripts were defined as significantly differentially expressed at absolute Log2 fold change >1, FDR <0.05. Dataset S1 contains all differentially expressed transcripts for each comparison.

3.2.4. DAVID/Pathway analysis

Differentially expressed (absolute Log2 fold change >1, FDR<0.05) RNA-Seq transcripts at each level of comparison (Ub⁶-GG versus control, Ub⁶-Stop versus control, Ub⁶-Stop versus Ub⁶-GG) were used to identify affected pathways. Transcripts were separated into lists of upregulated and downregulated genes for each condition. Each list was uploaded into the Functional Annotation tool provided by DAVID (https://david.ncifcrf.gov, v. 6.8) as a gene list and submitted using the official gene symbol as identifier and D. melanogaster as background (or Homo sapiens where indicated). Charts were created from several terms of interest, including enriched Biological Process (BP_DIRECT), Molecular Function (MF_DIRECT), and Cellular Component (CC_DIRECT) gene ontology as well as KEGG Pathway (KEGG_PATHWAY) terms. Terms were included as enriched if P-value<0.05.

3.2.5. Quantitative real time PCR

qRT-PCR was performed on select genes to validate RNA-Seq results. High-Capacity cDNA Reverse Transcription Kit (ABI) was used to obtain a cDNA library, following the manufacturer's protocol. Pre-amplification of the genes of interest was performed using TaqMan PreAmp Master Mix (Thermo Fisher Scientific). A Gilson 268 PIPETMAX liquid handling platform was used to plate Fast SYBR Green (ABI) qRT-PCR reactions in triplicate in 384-well plates. Messenger RNA levels were quantified with QuantStudio 5, using 2⁻ΔΔCt (cycle threshold) methods and normalizing all transcripts to the reference gene, Rp49. All primer sequences are listed in Table S3.9.
3.3. Results

3.3.1. Unanchored Ub chain design and expression in Drosophila for RNA-Seq analyses

As a strategy to study unanchored poly-Ub in Drosophila, we designed two types of Ub chain transgenes, each consisting of six Ub in tandem, without internal di-glycine, ‘GG’ motifs that are required for dismantling into mono-Ub by DUBs (figure 3.1B; [70]). The first chain type, Ub<sup>6</sup>-Stop, also lacks the C-terminal ‘GG’ motif required for conjugation onto substrate proteins. The second type, Ub<sup>6</sup>-GG, contains a C-terminal ‘GG’ motif, allowing the full chain to form iso-peptide bonds onto other proteins in mammalian cells and in vivo in the fly, as we demonstrated before [70]. Although the use of these chains introduces exogenous poly-Ub, this strategy permits us to directly examine the effects of intact, free poly-Ub on the Drosophila transcriptome. Presently, there is a lack of tools to more directly investigate unanchored poly-Ub in the fly; for example, targeting of DUBs implicated in free Ub chain disassembly would also impact other protein substrates that these DUBs have.

We utilized the binary Gal4-UAS expression system to drive our Ub<sup>6</sup> transgenes in the fly. In this system, transgenes with upstream activating sequence (UAS) sites are activated under the control of the transcription factor Gal4, itself expressed in the pattern of a specific gene [71, 72]. For our work in this study, we selected the Gal4 driver sqh-Gal4 [114-116, 118, 119] to express either form of Ub<sup>6</sup> in all fly tissues, throughout development and in adults. This driver employs the promoter and expression pattern of the gene spaghetti squash (sqh), which encodes the regulatory light chain of non-muscle type 2 myosin. sqh-Gal4 is a strong driver that leads to high levels of UAS-based transgene expression (such as our Ub<sup>6</sup>), during all developmental stages and throughout adulthood. We and others have used this driver in the past with robust outcomes, including lethality during various developmental stages and in adults as a result of the knockdown of various genes, and high toxicity from the expression of mutated or toxic proteins [36, 38, 100-102, 115-118, 125].
Our previously published work showed that the ubiquitous expression of Ub⁶ (via sqh-Gal4) had no significant effect on the development or lifespan of adult flies under normal conditions or during heat stress (30°C), indicating that robust levels of Ub⁶ are not especially detrimental (figure 3.1C; Table S3.1; [70]). Still, the possibility remains that cells could mount a response against them. One may surmise that in response to the presence of Ub⁶, DUBs or proteasomal proteins might be upregulated to attempt to clear the chains from the cell. Conceivably, Ub⁶ might also influence normal cellular processes, for instance by its recruitment into pathways that involve unanchored, linear poly-Ub, such as NF-κB signaling [57, 90, 94, 153]. Thus, we set out to examine if there are changes at the transcriptome level in response to Ub⁶.

We reasoned that we could detect changes in the fly transcriptome as a result of the expression and presence of untethered poly-Ub through RNA-Seq analysis. We selected to examine adult flies that were one day old as a middle point between developmental stages and adulthood, neither of which was impacted by the expression of Ub⁶ (Table S3.1; [70]. We extracted total RNA from one-day-old whole flies using TRIzol. The isolated RNA was then quality tested by electrophoretogram, RNA Integrity Number and the ratio of the 28S:18S RNA bands, and RNA-Seq was performed by the Applied Genomics Technology Center at Wayne State University (please see the Materials and Methods). The differentially expressed transcripts were analyzed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) [159].

While Ingenuity Pathway Analysis (IPA) is often the tool of choice to analyze RNA-Seq results, human orthologues exist for only about two-thirds of the transcripts affected by either form of Ub⁶ (Dataset S1) and the IPA databases available to us do not emphasize Drosophila genes. Because the success of IPA is heavily dependent on having access to the most applicable database [160], we opted to perform our analyses using DAVID. DAVID avoids stretching our observations to fit within the context of organisms other than Drosophila, or excluding Drosophila-specific genes involved in pathways of interest. Other fly laboratories have shown that DAVID
analysis recognizes and compares genes from the fly genome, while also providing the tools to
group functionally related genes and terms into a manageable number of biological modules
(Table S3.2; [159, 161-164]. figure 3.1C outlines our experimental workflow. We note at this point
that all of the genes discussed here are identified by their *Drosophila* symbol/name. The names
of their potential human orthologues, where applicable, are also provided in the text and tables.

3.3.2. DAVID analyses

We found a limited number of altered transcripts in flies expressing non-conjugatable and
conjugatable untethered Ub\(^6\) chains: 94 transcripts were altered in Ub\(^6\)-Stop and 86 were altered
in Ub\(^6\)-GG flies compared to controls, including 26 transcripts affected in both lines
(figure 3.2; Dataset S1). Controls were flies with the same genetic background used to generate
Ub\(^6\) flies, crossed to the sqh-Gal4 driver, ensuring that flies were as comparable as possible at
the genetic level. The majority of altered transcripts was upregulated (65 from each condition),
with 22 transcripts overlapping between groups (figure .2). Only 30% of all
identified *Drosophila* transcripts have assigned gene names, indicating that most of the affected
genes have not drawn sufficient genetic or functional attention in the fly, and 27% of the genes
have no predicted function.

To place these altered transcripts into physiological context, we submitted the differentially
expressed transcripts to DAVID [159]. This allowed us to determine enriched gene ontology terms
and pathways, assessing biological processes, molecular functions and KEGG (Kyoto
Encyclopedia of Genes and Genomes) pathways (Tables 3.1–3.3). Five biological process terms
(proteolysis, peptide catabolic process, mannose metabolic process, protein deglycosylation and
melanin biosynthetic process from tyrosine) were enriched in upregulated Ub\(^6\)-GG transcripts,
whereas for Ub\(^6\)-Stop, three terms (proteolysis, peptide catabolic process and transmembrane
transport) were enriched in upregulated and six terms (carbohydrate metabolic process, folic acid-
containing compound biosynthesis process, tetrahydrofolate interconversion, *de novo* IMP
biosynthetic process, one-carbon metabolic process and oxidation-reduction process) were
enriched in downregulated transcripts. The biological process terms ‘proteolysis’ and ‘peptide catabolic process’ were enriched in upregulated transcripts from both groups, the former associated with ~22% of all upregulated Ub\(^6\)-GG transcripts and ~18% of upregulated Ub\(^6\)-Stop
transcripts (Table 3.1; Table S3.3 lists all genes for each term). Five molecular function terms, the most prominent of which were ‘serine-type endopeptidase activity’ and ‘peptide binding’ were enriched in upregulated transcripts from both groups. The molecular function category overall reported nine enriched terms in upregulated Ub⁶-GG transcripts, as well as five upregulated and six downregulated transcripts for Ub⁶-Stop (Table 3.2; Table S3.4 lists all genes associated with each term).

As in other transcriptomic studies [165, 166], we assessed differentially expressed genes for both enriched Gene Ontology terms and KEGG pathways, in order to provide both a gene-specific and broader pathway context. For KEGG analysis, two pathways (lysosome and other glycan degradation) were enriched in upregulated Ub⁶-GG transcripts, with three (one carbon pool by folate, metabolic pathways and galactose metabolism) enriched in downregulated Ub⁶-Stop transcripts. Although few KEGG pathways were enriched, the term ‘metabolic pathways’ was associated with 34% of downregulated transcripts in Ub⁶-Stop flies.

<table>
<thead>
<tr>
<th>Table 3.1. Biological processes</th>
<th>Gene Ontology: biological process</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Term</td>
</tr>
<tr>
<td>Ub⁶-GG versus control</td>
<td></td>
</tr>
<tr>
<td>Upregulated</td>
<td>proteolysis* #</td>
</tr>
<tr>
<td></td>
<td>peptide catabolic process*</td>
</tr>
<tr>
<td></td>
<td>mannose metabolic process</td>
</tr>
<tr>
<td></td>
<td>protein deglycosylation</td>
</tr>
<tr>
<td></td>
<td>melanin biosynthetic process from tyrosine</td>
</tr>
<tr>
<td>Downregulated</td>
<td>None detected</td>
</tr>
<tr>
<td>Ub⁶-Stop versus control</td>
<td></td>
</tr>
<tr>
<td>Upregulated</td>
<td>proteolysis* #</td>
</tr>
<tr>
<td></td>
<td>peptide catabolic process*</td>
</tr>
<tr>
<td></td>
<td>transmembrane transport</td>
</tr>
<tr>
<td>Downregulated</td>
<td>carbohydrate metabolic process +</td>
</tr>
<tr>
<td></td>
<td>folate acid-containing compound biosynthesis process #</td>
</tr>
<tr>
<td></td>
<td>tetrahydrofolate interconversion #</td>
</tr>
<tr>
<td></td>
<td>de novo IMP biosynthetic process #</td>
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<tr>
<td></td>
<td>one-carbon metabolic process #</td>
</tr>
<tr>
<td></td>
<td>oxidation-reduction process + #</td>
</tr>
<tr>
<td>Ub⁶-Stop versus Ub⁶-GG</td>
<td></td>
</tr>
<tr>
<td>Upregulated</td>
<td>None detected</td>
</tr>
<tr>
<td>Downregulated</td>
<td>carbohydrate metabolic process +</td>
</tr>
<tr>
<td></td>
<td>oxidation-reduction process +</td>
</tr>
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</table>

Differentially expressed (absolute Log2 fold change>1, FDR<0.05) RNA-Seq transcripts at each level of comparison (Ub⁶-GG versus control, Ub⁶-Stop versus control, Ub⁶-Stop versus Ub⁶-GG) were separated into lists of upregulated and downregulated genes for each condition. Each list was uploaded into DAVID’s functional annotation tool (https://david.ncifcrf.gov, v. 6.8) as a gene list and submitted using the official gene symbol as identifier and D. melanogaster as background. The term BP_DIRECT was selected for chart creation within the Gene Ontology category, and terms were included as enriched if P-value<0.05. * indicates overlap in terms between Ub⁶-GG versus control and Ub⁶-Stop versus control comparisons; + indicates overlap between Ub⁶-Stop versus control and Ub⁶-Stop versus Ub⁶-GG comparisons; # indicates overlap in terms between analyses using fly genes and human orthologues, shown in Table S6.
Table 3.2. Molecular functions

<table>
<thead>
<tr>
<th>Term</th>
<th>Genes in functional categories</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ub&lt;sup&gt;6&lt;/sup&gt;-GG versus control</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Upregulated</strong></td>
<td>peptide binding&lt;sup&gt;*&lt;/sup&gt;</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>metallo-aminopeptidase activity&lt;sup&gt;*&lt;/sup&gt;</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>alpha-mannosidase activity</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>metalloprotease activity*</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>serine-type endopeptidase activity&lt;sup&gt;*&lt;/sup&gt;</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>carbohydrate binding</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>zinc ion binding</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>hydrolase activity</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>hydrolase activity, acting on C-N (not peptide) bonds, in linear amides&lt;sup&gt;*&lt;/sup&gt;</td>
<td>2</td>
</tr>
<tr>
<td><strong>Downregulated</strong></td>
<td>None detected</td>
<td></td>
</tr>
</tbody>
</table>

| **Ub<sup>6</sup>-Stop versus Ub<sup>5</sup>-GG**            |                                 |         |
| **Upregulated**                                            | peptide binding<sup>*</sup>      | 3       |
|                                                             | metallo-aminopeptidase activity<sup>*</sup> | 3       |
|                                                             | serine-type endopeptidase activity<sup>*</sup> | 6       |
|                                                             | metalloprotease activity*        | 3       |
|                                                             | hydrolase activity               | 2       |
|                                                             | hydrolase activity, acting on C-N (not peptide) bonds, in linear amides<sup>*</sup> | 2       |
|                                                             | methylene-tetrahydrofolate cyclohydrolase activity<sup>#</sup> | 2       |
|                                                             | methylene-tetrahydrofolate dehydrogenase (NAD<sup>+</sup>) activity<sup>#</sup> | 2       |
|                                                             | formate-tetrahydrofolate ligase activity<sup>#</sup> | 2       |
|                                                             | maltose alpha-glucosidase activity<sup>+</sup> | 2       |
|                                                             | alpha-1,4-glucosidase activity<sup>+</sup> | 2       | 3.10E-02 |

| **Ub<sup>6</sup>-Stop versus Ub<sup>5</sup>-GG**            |                                 |         |
| **Downregulated**                                          | maltose alpha-glucosidase activity<sup>+</sup> | 5       | 1.60E-09 |
|                                                             | alpha-1,4-glucosidase activity<sup>+</sup> | 5       | 2.00E-09 |
|                                                             | catalytic activity<sup>+</sup>     | 6       | 4.00E-07 |

Differentially expressed (absolute Log2 fold change > 1, FDR<0.05) RNA-Seq transcripts at each level of comparison (Ub<sup>6</sup>-GG versus control, Ub<sup>6</sup>-Stop versus control, Ub<sup>6</sup>-Stop versus Ub<sup>5</sup>-GG) were separated into lists of upregulated and downregulated genes for each condition. Each list was uploaded into DAVID’s functional annotation tool (https://david.ncifcrf.gov, v. 6.8) as a gene list and submitted using the official gene symbol as identifier and D. melanogaster as background. The term MF_DIRECT was selected for chart creation within the Gene Ontology category, and terms were included as enriched if P-value<0.05. * indicates overlap in terms between Ub<sup>6</sup>-GG versus control and Ub<sup>6</sup>-Stop versus control comparisons; # indicates overlap between Ub<sup>6</sup>-Stop versus control and Ub<sup>6</sup>-Stop versus Ub<sup>5</sup>-GG comparisons; Indicates overlap in terms between analyses using fly genes and human orthologues, shown in Table S7.

(Table 3.3; Table S3.5 lists all genes associated with each term). The relatively modest number of enriched gene ontology terms and pathways is not unexpected, considering the limited number of differentially expressed transcripts that reached statistical significance.

Next, we examined the possibility that unanchored Ub chains that cannot be conjugated elicit a response different from chains that can be conjugated. Thus, we directly compared flies expressing Ub<sup>6</sup>-Stop to those expressing Ub<sup>5</sup>-GG. Only 21 transcripts were altered in Ub<sup>6</sup>-Stop compared to Ub<sup>5</sup>-GG, 17 of which were downregulated (figure 3.2 and Dataset S1; no transcripts were altered across all comparisons). We again relied on DAVID to analyze differentially expressed transcripts for gene ontology terms and pathways (Tables 3.1–3.3; Tables S3.3–S3.5 list all genes associated with each term). Two biological processes (carbohydrate metabolic process and oxidation-reduction process) and three molecular functions (maltose alpha-
glucosidase activity, alpha-1,4-glucosidase activity and catalytic activity) were enriched in downregulated Ub^6^-Stop transcripts in comparison to both Ub^6^-GG and controls (Tables 3.1-3.2; Tables S3.3,S3.4). For KEGG analysis, four pathways were enriched in downregulated Ub^6^-Stop transcripts, with two of the four (galactose metabolism and metabolic pathways) enriched in comparison with both other conditions (Table 3.3; Table S3.5). While these transcriptomic and pathway analysis outcomes suggest a response specific to flies expressing non-conjugatable free Ub chains, the small number of differentially expressed transcripts limits this interpretation.

Lastly, we used DAVID to analyze only the set of human genes that have fly orthologues, in case additional or different pathways arise that might not have been captured by analyzing the fly genes exclusively. As shown in Tables S3.6–S3.8, there was general agreement with the fly-based DAVID analyses. The biological processes, molecular functions and pathways that were represented by the largest numbers of fly genes were well conserved between the two sets of analyses, including proteolysis and carbohydrate metabolic process, zinc-ion binding and serine-type endopeptidase activity, and lysosome and metabolic pathways. Still, biological processes and molecular functions arose that were not observed from fly-based gene analysis. These
differences were most often represented by small numbers of genes, generally two to four. Among biological processes, these include upregulation of regulation of cell shape, response to pH and termination of signal transduction (comparing Ub⁶-GG versus control), upregulation of biotin metabolism (comparing Ub⁶-Stop versus control), and downregulation of amino acid transport and protein tetramerization (comparing Ub⁶-Stop versus control). Among molecular functions that emerged from the human gene-based analyses, apolipoprotein binding and mannose binding were upregulated (comparing Ub⁶-GG versus control), zinc-ion binding was upregulated (comparing Ub⁶-Stop versus control), and electron carrier activity and oxidoreductase activity were downregulated (comparing Ub⁶-Stop versus Ub⁶-GG). Based on KEGG pathway analyses, the following differences were observed in the human-based analyses when compared to the fly-based analyses: upregulation of metabolic pathways (comparing Ub⁶-GG versus control), downregulation of the biosynthesis of antibiotics and glycerolipid metabolism (comparing Ub⁶-Stop versus control), and downregulation of the biosynthesis of antibiotics (comparing Ub⁶-Stop versus Ub⁶-GG). Collectively, while there are some variations between the two sets of analyses, the overall outcomes are not markedly different. Importantly, considering the small numbers of genes representing the divergences between the two sets of analyses, it is warranted that differences in outcomes be interpreted with caution.

Overall, expression of unanchored Ub chains in Drosophila has a seemingly minimal impact on transcriptomic response, as the number of altered transcripts (<100 for each group) is markedly low in comparison to the majority of reports by other, whole-fly RNA-Seq studies [167-170]. Although one study reported a comparable 57 genes affected by formaldehyde exposure [167], most were within the range of hundreds to several thousand genes, in studies ranging from cold acclimation to infection [167-170].

3.3.3. Validation by qRT-PCR

To validate RNA-Seq observations, we selected twelve genes from several pathways for confirmation by qRT-PCR. cDNA libraries were obtained from the same RNA used for RNA-Seq,
as well as from RNA extracted from new genetic crosses, for at least three biological replicates per genotype. All primer sequences are listed in Table S3.9. In most cases, results from RNA-Seq were confirmed by statistically significant changes, in the same direction, by qRT-PCR (figure 3.3). Our overall confirmation success is well within the range of confirmation reported widely in the literature [171-174]. qRT-PCR-validated transcripts include several genes with marked expression changes observed by RNA-Seq: CG32751 (~78-fold increase); Drsl3 and LysE (~tenfold increase); Mal-A7 and CG2650 (~tenfold decrease) (Dataset S1 lists all log2 fold changes determined by RNA-Seq).

The direction of change for the transcripts that were confirmed to reach statistical significance by qRT-PCR was the same between RNA-Seq and qRT-PCR results, with one exception: Takl1 in response to Ub^6-Stop (figure 3.3; please see the Discussion for additional information on Takl1 protein). By RNA-Seq, Takl1 on the Ub^6-Stop background nearly missed the

<table>
<thead>
<tr>
<th>Ub^6-GG</th>
<th>RNA-Seq Results</th>
<th>qRT-PCR Results</th>
<th>pValue (qRT-PCR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CG1304</td>
<td>Increased</td>
<td>Increased</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>CG32751</td>
<td>Increased</td>
<td>Increased</td>
<td>0.202</td>
</tr>
<tr>
<td>CG4653</td>
<td>Increased</td>
<td>Increased</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>CG7631</td>
<td>Increased</td>
<td>Increased</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>qln</td>
<td>Decreased</td>
<td>Increased</td>
<td>0.133</td>
</tr>
<tr>
<td>Takl1</td>
<td>Increased</td>
<td>Increased</td>
<td>0.081</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Ub^6-Stop</th>
<th>RNA-Seq</th>
<th>qRT-PCR</th>
<th>pValue (qRT-PCR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CG11911</td>
<td>Increased</td>
<td>Increased</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>CG2650</td>
<td>Decreased</td>
<td>Decreased</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>CG7631</td>
<td>Increased</td>
<td>Increased</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Drsl3</td>
<td>Increased</td>
<td>Increased</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>LysE</td>
<td>Increased</td>
<td>Increased</td>
<td>0.05</td>
</tr>
<tr>
<td>Mal-A7</td>
<td>Decreased</td>
<td>Decreased</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Takl1</td>
<td>Increased</td>
<td>Decreased</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Ubi-p5E</td>
<td>Decreased</td>
<td>Decreased</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Figure 3.3. qRT-PCR validation of differential expression trends for select RNA-Seq hits. Asterisk indicates inconsistency between RNA-Seq and qRT-PCR results. All P-values were determined using a one-tailed Student's t-test comparing gene expression fold change.
FDR cutoff (FDR=0.047; GEO data available online). We interpret this divergence in outcomes for *Takl1* as an indicator of lack of overall change in its expression in the presence of Ub$^6$-Stop. It is not uncommon for the direction of change in RNA-Seq results to differ from the direction of change observed from qRT-PCR data [171-174]. These discrepancies can arise for a variety of reasons, including the housekeeping gene used [173] as well as the length of the identified genes [175]. The rest of the genes we tested by qRT-PCR, whose differential expression reached statistical significance, matched the direction of fold change observed with RNA-Seq. The expression pattern of the genes assessed by qRT-PCR in the fly is summarized in Table S3.9, although we note that this table only lists the tissues in which the genes are more highly expressed; they may also be present in other tissues.

3.4. Discussion

Here, we evaluated whether there is an organismal response at the gene expression level in the presence of unanchored poly-Ub in *Drosophila*. Unanchored Ub chains are thought to be transient, toxic residents of the cellular milieu [37, 43, 44, 47, 95, 96]. However, our previously published work showed that this might not need to be the case *in vivo*: we observed little to no toxicity from the presence of unanchored poly-Ub in intact flies at all ages and stages examined [70]. Thus, we wondered whether lack of clear toxicity is due to a mounted defense or response, which we elected to start examining by RNA-Seq.

To model unanchored poly-Ub in the fly, we utilized transgenes that express six Ub moieties in tandem, lacking internal motifs that enable their disassembly into mono-Ub; one transgene encodes Ub$^6$ that cannot be attached onto other proteins, whereas the other enables this option (figure 3.1B). Transgenes were expressed in all fly tissues and at all stages of development and in adults, and RNA-Seq analyses were conducted using one-day-old flies. While planning this study, we had several hypotheses on the types of genes that could be affected by untethered Ub chains that we generated and expressed in *Drosophila*. Primarily, we thought that genes involved in Ub-dependent processes and pathways, as well as proteasome-related genes,
might be altered. These hypotheses were based on the current notion that unanchored poly-Ub is rapidly disassembled [1, 15, 21, 22, 87] and on our previous work showing that Ub⁶ is degraded by the proteasome [70]. For Ub⁶-GG, we further hypothesized a potential upregulation of Ub conjugation systems that could transfer these chains onto other proteins as single units. These hypotheses went unsupported by our RNA-Seq data; our analyses did not reveal a detectable difference in the transcription of those genes, with the exception of one E3 ligase, qin (figure 3.4), whose change at the transcript level was not confirmed through qRT-PCR. qin is a Tudor domain protein involved in the production of piRNAs that repress transposons in germline cells. It contains a RING domain and two B-Box domains, indicating E3 ligase activity, but its E3 function has not been characterized [176-178]. The lack of congruency between RNA-Seq and qRT-PCR results for qin argues against a marked effect from unanchored, conjugatable poly-Ub on the transcript levels of this E3 Ub ligase.

Insofar as Ub-related genes are concerned, we noted a reduction in the levels of Ubi-p5E in the presence of Ub⁶-Stop (Figs 3.,4;4; Dataset S1). Ubi-p5E is one of five Drosophila genes that encode mono-Ub either as a linear chain of multiple Ub moieties or in fusion to ribosomal components [179-184]. Ub genes expressed as linear chains, such as Ubi-p5E, are believed to be processed by DUBs down to mono-Ub [185, 186]. Reduced levels of this specific Ub-encoding gene, but not of the other four, suggest that the presence of unanchored poly-Ub that is not conjugatable to other proteins could be perceived as Ubi-p5E product. What senses this unanchored chain and the processes through which Ubi-p5E is regulated are unclear. One might surmise that lower levels of Ubi-p5E translate into reduced mono-Ub protein in the fly and impaired Ub-dependent processes; in turn, this would be expected to lead to upregulation of other Ub-encoding genes or of DUBs that process it, none of which was detected by our analyses. Whether there is a physiological significance attached to the reduced levels of Ubi-p5E in the presence of Ub⁶-Stop presently is unclear; perhaps Ubi-p5E regulation can be utilized in the future to understand unanchored poly-Ub sensing at the protein level and its downstream events.
We did not detect coordinated changes in pathways that involve unanchored poly-Ub, such as NF-κB. One gene altered in both the Ub⁶-Stop and Ub⁶-GG conditions, Takl1 (Tak1-like 1), is closely related to Tak1, a MAP3K required for the immune activation of NF-κB and JNK pathways [57, 187]; little is known about fly Takl1 itself. We caution here that the direction in the difference of transcript levels for Takl1 differed between RNA-Seq and qRT-PCR results, arguing against a clear effect from unanchored poly-Ub on this gene's expression. Ub⁶-Stop also led to differential expression of two transcripts linked to immunity: the upregulation of LysE (Lysozyme E) and the downregulation of DptA (Diptericin A). LysE protein is expressed in the midgut, where it is believed to aid in the digestion of food-borne bacteria [188]. DptA is an antimicrobial protein whose expression is induced by the immune deficiency pathway via NF-κB-related proteins [81, 189]. While these genes are likely participants in immunity or some NF-κB processes, their limited number is not indicative of a major alteration in those processes, and our DAVID analyses did not point to an effect on immunity or NF-κB signaling as a whole.

The small number of enriched gene ontology terms and pathways that we observed is reasonable considering the limited input of differentially expressed genes, suggesting that the alteration of these genes does not overtly perturb critical physiological processes. One interesting exception is the enrichment of proteolysis and peptide catabolic process in both Ub⁶ species.

Figure 3.4. Overview of the types of genes and pathways that we hypothesized might have been upregulated to dismantle, clear, or re-utilize Ub⁶, compared to the observed outcome by RNA-Seq.
While there is a consensus among the two types of untethered Ub chains in the upregulation of proteolysis-related genes, the number of impacted genes is not large. The GO category ‘proteolysis’ comprised about 15 genes, including two genes in the Trypsin family (zetaTry and Jon65Aii). Several proteolysis-related genes share homology with human PRSS genes (serine proteases; CG18493, CG31266, CG8299, CG9763, CG11911, CG11912, CG6048, zetaTry) and Aminopeptidase N (CG31198, CG31343, CG42335). Those genes might be involved with the disassembly of unanchored chains (figure 3.4), but it is unlikely that they act alone, as we observed previously that the proteasome is critically important for the degradation of unanchored chains in the fly [70]. The increase in proteolysis-related genes suggests an attempt by the organism to remove these species via specific peptidases, which might indeed play a role in the removal of endogenous, unanchored poly-Ub; this possibility requires future attention. Our previous work showed that the proteasome is important for degradation of free poly-Ub [70]. Perhaps the proteasome and these peptidases work in concert to dismantle free poly-Ub (figure 3.4).

A caveat to note is that our studies examined changes at the gene expression level. Perhaps control of untethered Ub$^6$ species is coordinated by changes at the protein level. Changes in fly proteomics as a result of untethered poly-Ub await investigation. Suffice it to say here that, based on western blotting, none of the proteasome subunits we examined before showed a difference at the protein level [70]. It is also important to note that the majority of the differentially expressed transcripts identified here are unannotated, leaving open the possibility that some of them have undiscovered roles in the handling of unanchored poly-Ub.

Because we utilized ubiquitous expression and whole flies for RNA-Seq analyses, it is possible that tissue- or system-specific responses are masked by conflicting changes in other tissues [190]. We elected to examine changes in the whole fly, as done in prior work that identified numerous genes altered due to specific types of conditions or insults [167-170]. As we continue
to investigate unanchored poly-Ub *in vivo*, should we observe a need to examine transcriptome changes in a tissue-specific manner we will be well positioned to do so. However, our current work with unanchored poly-Ub species overall indicates that these members of the Ub family are not inherently toxic and do not present cells with particularly egregious insults that necessitate large, coordinated responses.
CHAPTER 4: Isoleucine 44 Hydrophobic Patch Controls Toxicity of Unanchored, Linear Ubiquitin Chains Through NF-κB Signaling

4.1. Introduction

Post-translational modification adds a critical layer of complexity to the genome, allowing for regulation of many cellular functions. Methylation of histones in nucleosomes controls the expression of specific genes [191], phosphorylation of enzymes determines their activity [192], and glycosylation affects a protein’s conformation and ability to form complexes [193]. Ubiquitin (Ub) is a small protein adduct that is arguably known best for its role in protein quality control due to its participation in proteasome-dependent degradation [8]. Beyond that, Ub is an essential player in nearly all cellular processes, making it vital to eukaryotic cell health. A protein’s ubiquitination status can lead to various outcomes, from alteration of its protein-protein interactions, to changes in its localization, function, enzymatic activity and half-life [1, 87, 103, 107, 111].

Ubiquitination is a sequential, ATP-dependent process that requires a Ub activating enzyme (E1), a Ub conjugating enzyme (E2), and a Ub ligase (E3) to covalently attach a Ub molecule to a substrate protein, typically at one of the substrate’s lysine residues [1]. With seven lysine residues of its own, Ub itself becomes ubiquitinated, resulting in the formation of poly-Ub chains with unique topologies dependent on the specific lysine residue linkers. Head-to-tail chains also exist, linked at the N-terminal methionine, as well as chains that contain a mixture of linkage types [6, 12]. Ub chains that are not attached to a protein – known as unanchored, untethered, or free poly-Ub – can be synthesized by specialized E2s and E3s, and also arise from the removal of a preexisting chain from a ubiquitinated protein by deubiquitinating enzymes (DUBs) [2].

Ubiquitination is editable and reversible, ensuring that ubiquitinated substrates are targeted to the proper cellular complex while maintaining an available pool of Ub for reutilization. DUBs are a family of enzymes responsible for cleaving the linkages of Ub with its substrate or the linkages between Ub monomers in a poly-Ub chain. Depending on the DUB, it may influence the
outcome for a ubiquitinated protein by editing the length or topological makeup of the Ub chain, or by removing the chain entirely [2, 20, 22]. In the case of unanchored poly-Ub, specific DUBs break down multiple, linked Ub molecules into mono-Ub; such is the case with Ubiquitin Specific Protease 5, which has been evidenced to disassemble unanchored chains [33]. Processing of unanchored poly-Ub is important, as these chains participate in several processes, such as NF-kB signaling, and because disassembly of unanchored poly-Ub can replenish the pool of mono-Ub for reuse [22, 35, 57, 68, 88, 186].

Unanchored poly-Ub has not been as well studied as other Ub species. There are several concepts regarding free poly-Ub based on studies in yeast and cultured mammalian cells: that it exists only at very low concentrations, that it is quickly disassembled by DUBs to be reused as mono-Ub, and that its accumulation is toxic [2, 21, 22, 37, 87]. It has been proposed that unanchored poly-Ub interferes with the normal function of the proteasome, either by directly interacting with and occluding it – i.e., free poly-Ub could begin its own degradation process, but overwhelms the proteasomal machinery – or by outcompeting ubiquitinated proteasomal substrates [43, 44, 47].

Our previous work introduced transgenic, DUB-resistant, untethered Ub chains into a physiological system, *Drosophila melanogaster*, to address questions surrounding the potential toxicity and handling of free poly-Ub in an intact, multicellular organism [70, 79]. We synthesized DNA to express six Ub molecules in tandem, without any internal “GG” motifs (denoted as Ub⁶), which prevents them from being cleaved into mono-Ub by DUBs. Ub⁶ chains exhibited little-to-no toxicity, did not interfere with proteasomal function, and were readily subjected to proteasomal degradation [70]. These chains could also be transferred to a substrate protein *en bloc* in flies and in mammalian cells, without the need for prior disassembly into mono-Ub [70]. The DUB-resistant, free Ub⁶ chains become ubiquitinated themselves through various linkages, transforming them into mixed-linkage, free chains [70]. Additional work indicated that continuous presence of these Ub chains *in vivo* does not cause marked changes at the transcriptome level.
in the fly [79]. Collectively, we interpreted these results to indicate that free poly-Ub is not necessarily detrimental \textit{in vivo} and that Ub recycling may take place not only at the level of the monomer, but also through the reutilization of intact, untethered Ub chains.

Nonetheless, considering earlier studies that suggested toxicity from free chains [43, 44, 96], we sought to understand under what circumstances unanchored poly-Ub might become problematic to the cell. One key feature of the transgenic Ub chains expressed in flies is their conjugation with endogenous Ub [70]. This phenomenon made us wonder whether ubiquitination of these chains is important for the lack of toxicity we observe. Therefore, we generated new, isogenic fly lines that express Ub\textsuperscript{6} that are identical to the previously described species, with the exception that they lack all internal lysine residues, rendering them ubiquitination-resistant. The new chains essentially resemble free, linear poly-Ub. By manipulating the potential of Ub\textsuperscript{6} to be ubiquitinated, rather than the cell’s ability to ubiquitinate proteins, we avoid impacting ubiquitination processes more generally and can attribute changes in toxicity to the presence of Ub\textsuperscript{6}.

We found stark differences in the toxicity of and response to ubiquitination-resistant Ub\textsuperscript{6} chains \textit{in vivo}. Depending on the type of tissue where lysine-less Ub\textsuperscript{6} was expressed, it led to either developmental lethality or markedly shortened adult lifespan. Ubiquitination-resistant Ub\textsuperscript{6} has an extended turnover cycle when compared to Ub\textsuperscript{6} with all its lysine residues intact. Additionally, ubiquitination-resistant Ub\textsuperscript{6} leads to the upregulation of several components of NF-\textkappa B pathways in the fly, indicating a mechanism for toxicity from these free chains in \textit{Drosophila}. Importantly, toxicity from ubiquitination-resistant Ub\textsuperscript{6} is reversed by mutating isoleucine (ile) residues at position 44 of each constituent Ub into alanine. Ile44 is a key part of a hydrophobic patch on Ub that controls many interactions [1, 5, 39-41]. We conclude that while unanchored poly-Ub is not inherently problematic \textit{in vivo}, there can be circumstances in which these species may become detrimental, such as when cells’ abilities to manipulate and handle them are severely
hampered. The studies that we describe here also introduce to the field new genetic tools for continued investigation of untethered poly-Ub.

4.2. Materials and Methods

4.2.1. Antibodies

Anti-HA (1:500 or 1:1,000; rabbit monoclonal; Cell Signaling Technology, #3724); anti-Ub (1:1,000; rabbit polyclonal; Cell Signaling Technology, #3933); anti-Tubulin (1:1,000, mouse monoclonal, Sigma-Aldrich, #T5168); anti-Relish (1:500 from concentrate; mouse monoclonal; Developmental Studies Hybridoma Bank, #21F3); anti-Lamin (1:1,000; mouse monoclonal; Developmental Studies Hybridoma Bank, #ADL84.12); anti-phosphorylated-p65 (Ser36) (1:1,000; rabbit monoclonal; Cell Signaling Technology, #3033); anti-p65 (1:1,000; rabbit monoclonal; Cell Signaling Technology, #8242); goat anti-mouse, peroxidase conjugated secondary (1:10,000; Jackson Immunoresearch); goat anti-rabbit, peroxidase conjugated secondary (1:10,000; Jackson Immunoresearch).

4.2.2. Fly generation

GenScript (genscript.com) synthesized Ub⁶ transgenes and cloned them into pWalium10.moe by using restriction sites EcoRI and XbaI, as also described before [70]. The amino acid sequences of the new lines generated for this study are shown in figures 4.1 and 4.5. The purified plasmid was injected into yw; attP40 by Duke University Model Systems, using the PhiC31 targeted integration to achieve site-specific, unidirectional integration into the second chromosome [194, 195]. The transformed chromosome was migrated onto the w¹¹¹⁸ parental line and balanced lines were confirmed through genomic DNA-based PCR assays for transgene insertion and orientation, as described before [102, 125]. All fly lines were sequence-verified.

4.2.3. Drosophila stocks and procedures

All flies were maintained in diurnal incubators at 25 °C and ~60% humidity, in conventional cornmeal media. Where noted, RU486 was added to the conventional media, as previously described [99]. tubulin-Gal4-GS was a generous gift of Dr. R. J. Wessells, Wayne State University;
sqh-Gal4 was a gift of Dr. Daniel Kiehart, Duke University; repo-Gal4 was gifted by Dr. Daniel Eberl, University of Iowa. Mef2-Gal4 (#27390) and elav-Gal4 (#458) were from Bloomington Drosophila Stock Center. The following RNAi lines were also from the Bloomington Drosophila Stock Center: Relish (#3361), Dif (#30513), cactus (#31713), dorsal (#27650), IKKBeta (#35186), kenny (#35572, 57759). dorsal overexpression line was from FlyORF (#000638). For longevity assays, male and female flies were collected as they eclosed from their pupal cases and aged on conventional cornmeal fly media at 25 °C, with ~20 flies per vial. Flies were transferred to fresh vials every 2–3 days until all were dead. Throughout this study, flies were heterozygous for driver and transgenes.

4.2.4. Western Blotting

Five whole adults flies or pharate adults, or ten dissected heads per group were lysed as described previously [70]. Blots were developed and quantified using a CCD-equipped VersaDoc system and Quantity One software (Bio-Rad), Syngene PXi4 and GeneSys software (Syngene), or ChemiDoc and ImageLab (Bio-Rad).

4.2.5. Immunoprecipitation and \textit{in vitro} deubiquitination

For precipitation of HA-Ub\textsuperscript{6} from flies, 40 flies per group were homogenized in RIPA lysis buffer (50 mM Tris, 150 mM NaCl, 0.1% SDS, 0.5% deoxycholic acid, 1% NP40, pH 7.4) supplemented with protease inhibitor cocktail (PI; Sigma-Aldrich), sonicated, centrifuged at 15,000 x g for 20 minutes at 4 °C, and then incubated with anti-HA antibody-bound beads for four hours, tumbling at 4 °C. Beads were rinsed 10 X (including 2 x 5 minutes tumbling at 4°C), then split into two microfuge tubes and incubated at 37 °C with either NEM (0.5 mM) or the catalytic domain of USP2 (0.1 uM; Boston Biochem). After incubation for the selected times, reactions were stopped by the addition of 6X SDS sample buffer and by boiling for 1 minute.

4.2.6. Soluble/insoluble fractionation

Five flies per group were mechanically homogenized in 200 μL of NETN lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, and 0.5% IPEGAL ca-630) supplemented with PI, sonicated
briefly, and were then centrifuged at 20,000 x g for 30 min at 4 °C. The resulting supernatant was removed, transferred into a new microfuge tube, and subsequently quantified by using the BCA assay (ThermoFisher). The remaining pellet was fully resuspended in 200 μL of PBS + 1% SDS by vortexing and boiling. Ten μg of the supernatant fraction and 7 μL of the pellet fraction were supplemented with loading buffer, boiled briefly and were then electrophoresed on SDS-PAGE gels for analyses by Western blots.

4.2.7. Quantitative real-time PCR

From 10 dissected pharate heads per group, total RNA was extracted using TRIzol reagent (Invitrogen) and treated with TURBO DNase (Ambion) to eliminate contaminating DNA. High-Capacity cDNA Reverse Transcription Kit (ABI) was used to obtain a cDNA library, and preamplification of the genes of interest was performed using SsoAdvanced Preamplification Supermix (Bio-Rad). Fast SYBR Green Master Mix (Thermo Fisher Scientific) was used in conjunction with a StepOne real-time PCR system. mRNA levels were quantified using \(2^{-\Delta\Delta C\text{t}}\) (cycle threshold) methods and normalizing all transcripts to the reference gene, rp49. The following primer sequences were used:

\[
\begin{align*}
\text{rp49} & \quad (F: \text{AGATCGTGAAGAAGCGCACCAAG}; \ R: \text{CACCAGGAACTTCTTGAATCCGG}) \\
\text{Relish} & \quad (F: \text{ATGAACTTGAACCAGGTGC}; \ R: \text{TGCCGACTTGCGGTTATTGA}) \\
\text{Dif} & \quad (F: \text{CAGAGTTCCAACCCACGGAC}; \ R: \text{AGGAGTTCTGGATTCCGGGTAGT}) \\
\text{dorsal} & \quad (F: \text{AGAGCCCGCAGGTTTTT}; \ R: \text{TGCCATCCTGGTGTCATTTC}) \\
\text{cactus} & \quad (F: \text{ATCCAACGACAAAGCGGTCA}; \ R: \text{GATTTTCCCTCCCTGCGT}) \\
\text{kenny} & \quad (F: \text{TACCTCGCGCTAAAGAGCAC}; \ R: \text{CAGCTCTTGGTGGTCCACGC})
\end{align*}
\]

4.2.8. Subcellular fractionation

ReadyPrep Cytoplasmic/Nuclear Protein Extraction Kit (Bio-Rad) was used to separate nuclear and cytoplasmic proteins from 10 pharate adult dissected heads (figure 4.5E), or individual wells in a 24-well culture plate for experiments with HEK-293T cells. The manufacturer's protocol was used, with two variations: 1) nuclear fractions were rinsed once with cytoplasmic
protein exclusion buffer (CPEB) for 10 minutes, tumbling at 4°C before subsequent incubations in CPEB on ice; 2) cytoplasmic fractions were centrifuged 3 times, with the supernatant transferred to a fresh tube between spins.

4.2.9. Mammalian cell maintenance and transfection

HEK-293T cells were maintained under conventional conditions in DMEM with 10% FBS and 5% Penicillin-Streptomycin. Cells were transfected using Lipofectamine LTX (Invitrogen), using the manufacturer’s protocol. Twenty-four hours after transfection, cells were collected and rinsed in cold PBS, then used for whole cell lysis (using the same buffer and protocol as for blots with flies), or for subcellular fractionation. DNA constructs used for transfections were the same as those synthesized for fly generation, but subcloned into the mammalian vector pcDNA3.1.

4.3. Results

4.3.1. New Drosophila lines expressing ubiquitination-resistant, unanchored chains

As introduced above, we previously examined whether expression of unanchored poly-Ub is toxic in Drosophila. Using the Gal4-UAS system [71, 72], we expressed Ub⁶ chains that cannot be dismantled by DUBs (e.g. Ub⁶-Stop in figure 4.1A). These poly-Ub resemble linear, untethered chains that exists in nature [1, 21, 22]. The chains were constructed to either remain unanchored, due to the lack of the terminal “GG” motif that would enable their isopeptide-bond fusion to another protein (Ub⁶-Stop; figure 4.1A), or could be tethered wholesale onto another protein as a result of a single “GG” motif at the very end [70]. We found that these Ub⁶ species are innocuous [70, 79]. Nonetheless, as some studies concluded that untethered poly-Ub is toxic [43, 44, 96], we sought to explore when free chains might become detrimental to the cell. We focused on a potential role from the ubiquitination of untethered poly-Ub by endogenous Ub, since this is a major feature of transgenically-expressed chains [70].

We designed head-to-tail Ub⁶ that, similar to the prior constructs [70], does not contain “GG” motifs internally or at the very end, and is also devoid of lysine residues (lys to arg mutation), making it resistant to ubiquitination (Ub⁶-Stop-K0; figure 4.1A). Removing the potential for
ubiquitination of Ub\textsuperscript{6} allows us to examine the behavior of an untethered Ub chain that cannot be decorated by ubiquitination within the cell, in contrast to the mixed-linkage chains that arose and

Figure 4.1. New transgenic Drosophila lines expressing non-cleavable, unanchored ubiquitin chains. (A) Amino acid sequence, abbreviation and purpose of new HA-tagged ubiquitin chain transgenes. Neither chain can be conjugated onto another protein as it lacks a terminal “GG” motif that is required for isopeptide bond formation. Absence of “GG” motifs internally also ensures that they are not dismantled by DUBs [26]. All lysine (K) residues within Ub\textsuperscript{6}-Stop were mutated into the similar, but non-ubiquitatable amino acid, arginine (R) to prevent ubiquitination. (B) Western blots from flies expressing the noted transgenes in all neurons (elav-Gal4, adult lysates), muscle cells (Mef-Gal4, adult lysates), or all glial cells (repo-Gal4, pharate adult lysates). Delta symbol (Δ): signal underneath the main band of lysine-less Ub\textsuperscript{6} that we observe sometimes and could be a proteolytic fragment of the chain. (C) Western blots from stringently immunopurified, HA-tagged ubiquitin chains treated, or not, with the catalytic domain of USP2 for the indicated amounts of time. Mef2-Gal4 flies were one day old. Flies with tub-Gal4-GS driver were induced to express Ub\textsuperscript{6} for 7 days before being collected for protein extraction. (D) Western blots from soluble/pellet fractionation of flies expressing the noted ubiquitin chains in all muscle cells. Flies were one day old. The smear present in the Ub\textsuperscript{6}-Stop-K0 samples comprises SDS-resistant species as a result of the buffer used in this protocol. As shown in figure 1C, second lane from the left and USP2\textsubscript{200} treated lanes, similar smears from a different buffer and lysis protocol (Materials and Methods) are not collapsed by the addition of the DUB. Asterisks in panels: non-specific band detected by the anti-HA antibody.
were highly abundant in our previous studies [70].

We generated new transgenic flies that incorporate Ub\(^6\)-Stop-K0 at the same chromosomal site that contains Ub\(^6\)-Stop, attP40 on the second chromosome of *Drosophila*. The PhiC31 system of transgene integration ensures single copy, same orientation insertion of the intended construct into the same chromosomal site [70, 101, 194-197]. Following established protocols from our published work [102, 125], we utilized PCR-based assays and sequencing to ensure integration of the transgene into attP40 at the right locus and in the same orientation, and to ensure that the sequence inserted was the one intended (data not shown). With these isogenic lines on hand, we began our investigations. To characterize the biochemical properties of Ub\(^6\)-Stop-K0 in comparison to Ub\(^6\)-Stop, we first performed Western blotting using lysates collected from flies expressing either version of Ub\(^6\). We observed Ub\(^6\)-Stop-K0 at the expected molecular weight of \(~50\) kDa, without the distinct and clearly defined ubiquitination laddering that we see above Ub\(^6\)-Stop (figure 4.1B and other supportive information in figures 4.3 and 4.5). At this point, it is interesting to note that the precise type of ubiquitination laddering above the main band of Ub\(^6\)-Stop differs among different tissues. In muscle cells, there appears to be a higher preponderance of \(\text{>Ub}^1\) addition (Ub\(^3-5\), middle portion of the blots in figure 4.1B; see also figure 4.5E) compared to neuronal and glial cells, whose signal is stronger closer to the main, unmodified band. The reasons for these differences and their potential physiological consequences are unknown. Still, the key point from these comparisons is that Ub\(^6\)-Stop-K0 migrates similarly in different fly tissues.

We showed previously that the laddering above the main band of Ub\(^6\)-Stop comprises ubiquitinated species [70]. One of the methods we utilized was to incubate immunoprecipitated (IP-ed) Ub\(^6\)-Stop with the catalytic domain of USP2 (USP2\(_{\text{CD}}\)), a DUB that would remove Ub added onto Ub\(^6\) in any type of linkage, including met1/linear fusions [198], while leaving Ub\(^6\) itself intact due to absence of “GG” motifs. As shown in figure 4.1C, while USP2\(_{\text{CD}}\) readily removes conjugated Ub from Ub\(^6\)-Stop, as evidenced by the collapse of the higher molecular weight (HMW)
bands into the main Ub\textsuperscript{6} band, it has no detectable effect on Ub\textsuperscript{6}-Stop-K0. The faint HMW smear observed in the long exposure image in figure 4.1C is likely aggregated Ub\textsuperscript{6}-Stop-K0, as suggested by centrifugation protocols (figure 4.1D). Through centrifugation of fly lysates, we observe the presence of some Ub\textsuperscript{6}-Stop, and especially of Ub\textsuperscript{6}-Stop-K0, in the pellet fraction, suggesting that both chains may aggregate (figure 4.1D). Together, these data indicate that Ub\textsuperscript{6}-Stop-K0 is expressed in the fly and that it is resistant to ubiquitination.

4.3.2. Lysine-less, unanchored poly-Ub is highly toxic in \textit{Drosophila}

We found before that expression of Ub\textsuperscript{6}-Stop in all tissues or only in muscle cells did not impact longevity, while restricted expression in glial cells or neurons reduced adult fly longevity slightly, by a few days (normal flies can live around 90 days; [70]). Here, we used various Gal4-UAS drivers to test the impact of Ub\textsuperscript{6}-Stop and Ub\textsuperscript{6}-Stop-K0 in fly longevity. sqh-Gal4 is a commonly used driver that leads to expression of the transgene of interest in all tissues during development and as adults [70, 101, 115, 124, 196]. Flies heterozygous for sqh-Gal4 and Ub\textsuperscript{6}-Stop-K0 die as embryos or young larvae (figure 4.2A). elav-Gal4 (pan-neuronal expression) and repo-Gal4 (pan-glial expression) both lead to developmental death and drastically shortened adult lifespans when expressing Ub\textsuperscript{6}-Stop-K0 (figures 4.2A-C). In the case of glial expression, no Ub\textsuperscript{6}-Stop-K0-expressing adults emerged from most crosses that we set up; developing flies died as pharate adults. We were able to collect some live adults in later crosses for experiments described in figure 4.4 and their lives were significantly shorter than control adults. It is the longevity of these adults that is shown in figure 4.2C. Expression of Ub\textsuperscript{6}-Stop-K0 in muscle cells (Mef2-Gal4) results in a less dramatically, but still statistically significantly abbreviated lifespan (figures 4.2A, D). In essence, with every Gal4 driver used, Ub\textsuperscript{6}-Stop-K0 leads to markedly reduced lifespan compared to both Ub\textsuperscript{6}-Stop flies and control flies that are genetically similar but do not express either Ub\textsuperscript{6} (figure 4.2). Clearly, different drivers lead to variable toxicity from ubiquitination-resistant, linear poly-Ub. Among the tissues examined, muscle cells are least impacted. This is unlikely to be directly due to differences in relative amounts expressed by the different drivers, since we showed
before that muscle cells express Ub⁶ transgenes highly robustly [70]. More likely, different tissues handle and tolerate these species in different ways, leading to the observed variation in toxicity. These findings highlight the need to investigate Ub-dependent pathways in a tissue-specific manner to understand the full scope of their roles and importance in vivo. Collectively, these data point to marked toxicity from ubiquitination-resistant, untethered Ub⁶ species in Drosophila, unlike the ubiquitination-prone counterpart.

Figure 4.2. Lysine-less, non-cleavable ubiquitin chains are toxic in flies. (A) Summary of findings when the noted transgenes are expressed as indicated. “None” denotes flies that contained the indicated transgene without a Gal4 driver. Results are from crosses that were conducted simultaneously. A schematic of the fly life cycle, with the approximate number of days (“D”) spent in each stage, is provided. (B-D) Longevity assays of adult flies expressing Ub⁶ chains under the noted Gal4 drivers. “Ctrls” do not contain Ub⁶ transgenes but have the Gal4 driver on the genetic background used to generate the ubiquitin chain-containing flies. ‘p’ values are from log-rank tests.
4.3.3. Lysine-less, unanchored poly-Ub is more stable in the fly

Since endogenous ubiquitination may influence the toxicity of Ub\(^6\), we questioned whether fly cells clear Ub\(^6\)-Stop-K0 as readily as Ub\(^6\)-Stop, which can be ubiquitinated. Lys48- and lys11-linked ubiquitination are proteasomal degradation signals \([1, 8, 199, 200]\) that are present on Ub\(^6\)-Stop ([70] and unpublished observations from J. R. Blount and S. V. Todi). It is not unreasonable to hypothesize that their absence could hinder the degradation of Ub\(^6\)-Stop-K0. To determine whether Ub\(^6\)-Stop-K0 is turned over more slowly than Ub\(^6\)-Stop, we employed the RU486-dependent, ubiquitous driver, tub-Gal4-GS to perform an experiment akin to a pulse-chase [70]. Flies heterozygous for tub-Gal4-GS and either form of Ub\(^6\) were reared on regular fly food until they emerged from their pupal cases, at which point they were switched to RU486 food for 2 days to induce Ub\(^6\) transgene expression (the "pulse"). Adults were then switched to regular media to halt additional Ub\(^6\) production and were flash-frozen periodically to assess Ub\(^6\) disappearance by Western blotting (the "chase"). We selected an inducible, ubiquitous Gal4 driver and focused on adult flies to gain a general perspective on the turnover of these Ub species, aware of the fact that degradation rates may differ in a tissue- and developmental stage-dependent manner. Nonetheless, this approach provides us with critical insight into the handling of ubiquitination-prone and ubiquitination-resistant, linear poly-Ub at the level of the whole organism.

Figure 3 summarizes our findings. We observe that lack of ubiquitination stabilizes Ub\(^6\)-Stop-K0 compared to Ub\(^6\)-Stop. Fifty percent of Ub\(^6\)-Stop-K0 is degraded around 72h in comparison to less than 24h for Ub\(^6\)-Stop. By 7 days, both proteins reach comparable levels. These data suggest that internal lysine residues and their ubiquitination are critical, but not entirely essential for chain turnover in flies.

4.3.4. Toxicity of lysine-less, unanchored poly-Ub depends on NF-κB signaling

Recent studies focusing on innate immunity pathways have revealed roles for free poly-Ub in NF-κB signaling cascades \([50-52, 57, 60, 63]\). “NF-κB” refers to a family of inducible transcription factors which, in response to the activation of immune receptors by invading
microbes, induce the expression of antimicrobial peptides. In *Drosophila*, innate immunity is primarily regulated by two pathways that activate NF-κB: Imd and Toll (figure 4.4A). During the *Drosophila* immune response mediated by NF-κB pathways, a series of downstream regulators are involved (e.g. cactus, dorsal, kenny, and Relish; figure 4.4A). Lysine-less Ub⁶ resembles unanchored, linear chains that have been proposed to regulate components of NF-κB in the mammalian system [53, 58, 201]; therefore, we posited that Ub⁶-Stop-K0 might mimic endogenous poly-Ub involved in the Imd pathway and cause abnormal signaling, which could explain toxicity that we observe with the expression of Ub⁶-Stop-K0 chains (figure 4.2).

To address the possibility of aberrant NF-κB signaling in the toxicity of lysine-less Ub⁶, we used the repo-Gal4 driver to express in glia Ub⁶-Stop-K0 alongside RNAi transgenes targeting components of the *Drosophila* Imd and Toll pathways, and monitored effects on longevity. We
chose repo-Gal4 because Ub^{6}-Stop-K0 expression in glial cells causes toxicity in developing and adult flies (figures 4.2A, C); thus, any relief of this toxicity is observable along developmental and adult stages.

Independent knockdown of \textit{Relish} and \textit{Dif} significantly alleviates toxicity from Ub^{6}-Stop-K0, although neither restores adult fly lifespan to that of the controls that do not express Ub^{6}-Stop-K0.
K0 (figure 4.4B). A separate longevity study revealed that knockdown of *dorsal*, *cactus* and *IKKβ* also significantly reduces toxicity (figure 4.4C). In this second study, Ub^6^-Stop-K0 leads to widespread pharate adult lethality with no surviving adults (figure 4.2A summarizes fly stages of development). We have observed that transient factors, such as variation in ingredient lot numbers, can influence the development and survival of these flies; therefore, all comparisons in development and longevity are made only among flies reared simultaneously. Still, even though we notice some variation in the extent of toxicity, Ub^6^-Stop-K0 is consistently toxic and knockdown of NF-κB components consistently leads to increased survival (figures 4.4B, C). The one exception is kenny. Knockdown of *kenny* does not improve toxicity from Ub^6^-Stop-K0. No flies with pan-glial expression of both *kenny* RNAi and Ub^6^-Stop-K0 survive to adulthood, despite the eclosure of a small number of flies expressing Ub^6^-Stop-K0 alone (figure 4.4B). This observation indicates that toxicity-rescuing effects we see with NF-κB knockdown are specific to key genes.

In summary, targeting of several NF-κB players reduces the toxicity of lysine-less, free Ub^6^, implicating Ub^6^-Stop-K0 in immune signaling via both Imd and Toll pathways.

To further explore this implication, we performed qRT-PCR using dissected heads from pharate adults expressing Ub^6^-Stop-K0 and repo-Gal4 to drive expression in glial cells only, compared to those with the same genetic background but without the Ub^6^ transgene. We used pharate adult heads because most Ub^6^-Stop-K0 adult flies do not eclose and because repo is robustly expressed in the head (flyatlas.com). In the presence of Ub^6^-Stop-K0, we observe statistically significantly increased levels of *Relish*, *Cactus*, *Dif*, and *dorsal* mRNA. In agreement with our previous results, *kenny* mRNA levels trend upward, however without reaching significance (figure 4.4D). *dorsal* mRNA, in particular, is markedly upregulated. Importantly, overexpression of *dorsal* in glial cells recapitulates lethality in flies that do not express any Ub^6^, indicating that *dorsal* upregulation alone is sufficient for toxicity (figure 4.4E). These results illustrate a disturbance in normal NF-κB signaling in the presence of Ub^6^-Stop-K0 and present a mechanism for unanchored chain toxicity that relies on aberrant NF-κB upregulation.
4.3.5. Aberrant NF-κB signaling is mediated by the ile44-centered hydrophobic patch of lysine-less, linear poly-Ub

Proper folding of Ub molecules results in a hydrophobic patch centered around amino acids isoleucine (ile) 44, leucine 8, histidine 68, and valine 70 (figure 4.5A; [1]). This surface is the recognition site for many Ub-binding domains (UBDs) and is thus essential to the interaction of Ub with other proteins and for many of its actions (figure 4.5B; [4, 39-41]). NEMO, the NF-κB regulator, binds linear and lys63-linked chains [53, 56] and 3D analysis of this interaction shows a critical position for Ub’s ile44 (figure 4.5C). Likewise, TAB2 and TAB3, which activate TAK1 in mammalian NF-κB signaling, recognize the ile44 patch through their C-terminal Np14 zinc finger domains [202]. Reasoning that ile44 within Ub6-Stop-K0 is likely important for its incorporation into, or recognition by, NF-κB signaling, we generated an additional Ub6 chain identical to Ub6-Stop-K0, with the exception that every ile44 residue is mutated to alanine (Ub6-Stop-K0-ile44a; figure 4.5D), a mutation that is commonly used to disrupt the hydrophobic patch [39-41]. These

Figure 4.5. New transgenic, lysine-less ubiquitin chains with mutated isoleucine 44. (A) Surface representation of ubiquitin (PDB 1ubq) highlighting the structural location of the amino acids forming the hydrophobic patch involved in ubiquitin recognition (I44 in yellow, and L8, H68, and V70 in white). (B, C) Ribbon representation of ubiquitin (blue) displaying isoleucine 44 in the binding surface of different ubiquitin-interacting motifs in pink. (B, UIM; PDB 1yx5; C, the UBD domain of NEMO; PDB 2zvo). Inset highlights ubiquitin’s hydrophobic patch and the key position of isoleucine 44 (yellow). Images were generated using PyMol (Schrodinger, Inc). (D) Summary of the new ile44a transgenic line that we generated, its abbreviation and purpose. (E) Western blots from flies expressing the noted transgenes in all muscle cells (Mef2-Gal4, adult lysates) or all glial cells (repo-Gal4, pharate adult lysates).
new chains allow us to examine the influence of the ile44 hydrophobic patch on the toxicity of Ub6 chains. Expression of Ub6-Stop-K0-ile44a in Drosophila muscle cells and in glial cells leads to protein levels that are comparable to those of Ub6-Stop-K0. Similar to Ub6-Stop-K0, Ub6-Stop-K0-ile44a does not show the distinct HMW bands consistent with ubiquitination in Western blots. This is contrary to Ub6-Stop, which presents with clear and distinct laddering of ubiquitination above its main band (figure 4.5E).

To assess toxicity from Ub6-Stop-K0-ile44a, we used the tissue-specific drivers described earlier to express the new transgene and then performed longevity assays. In each case, Ub6-Stop-K0-ile44a flies survive longer than their Ub6-Stop-K0 counterparts (figures 4.6A, B). In fact, Ub6-Stop-K0-ile44a expression in muscle cells and in neurons does not have a statistically significant effect on adult fly lifespan compared to controls that express no Ub6. Driving expression in all fly cells or only in glia results in a modest reduction in longevity (figure 4.6B). It is particularly remarkable that disrupting the hydrophobic interaction interface of Ub eliminates developmental lethality from ubiquitous expression of Ub6-Stop-K0 and only mildly reduces adult fly longevity compared to controls without Ub6 (figure 4.6A, B).

Next, we examined whether reduced toxicity from Ub6-Stop-K0-ile44a coincides with normalized levels of genes involved in NF-κB signaling, compared to Ub6-Stop-K0. We recapitulated studies conducted for figure 4.4D by using new crosses and adding flies that express Ub6-Stop-K0-ile44a in all glia. As summarized in qRT-PCR results in figure 4.6C, the mRNA levels of dorsal and Relish are not statistically different in the presence of Ub6-Stop-K0-ile44a compared to flies not expressing any Ub6 transgenes, but continue to be upregulated in the presence of Ub6-Stop-K0.

We validated these findings by examining the protein levels of Relish (antibodies tested for other NF-κB components in flies were non-specific or inconsistent). We observed that in the presence of Ub6-Stop-K0, Relish protein levels are noticeably higher, compared to flies not
Figure 4.6. Isoleucine 44 mediates toxicity from free, linear, lysine-less ubiquitin chains. (A) Summary of findings when the noted transgenes are expressed as described. “None” denotes no Gal4 driver was present. Results are from crosses that were conducted simultaneously. (B) Longevity assays of adult flies expressing Ub6 chains under the noted Gal4 drivers. “Ctrls” do not contain Ub6 transgenes, but have the Gal4 driver on the genetic background used to generate the Ub6 chain-containing flies. ‘p’ values are from log-rank tests. (C) mRNA levels of the noted NF-kB-related genes by qRT-PCR in dissected heads of pharate adults expressing Ub6-Stop-K0 or Ub6-Stop-K0-i44a in glial cells, presented as fold change over controls without the Ub6 transgene. ‘p’ values are from Student’s t-tests comparing Ub6-expressing flies to controls. (D) Western blots from dissected pharate adult heads expressing the noted transgenes in all glial cells (left). Relish protein levels were normalized to direct blue and quantified (right). ‘p’ value is from Student’s t-tests comparing Ub6-expressing flies to controls that contain the driver on the same genetic background as ubiquitin chain-encoding flies. (E) Western blots from two independent cytoplasmic/nuclear fractionations of dissected pharate heads expressing, or not, Ub6 transgenes in glial cells. Due to the temporary closure of our institution as a result of the COVID-19 pandemic, we were unable to obtain additional, independent samples for this panel. We also encountered some difficulties with signal-to-noise ratio from anti-Relish antibody for this panel, but were unable to procure more reagents as a result of university closure. In (C) and (D), “N.S.” denotes no statistical significance comparing a specific Ub6 group to its respective control.

expressing any Ub6 transgene, or expressing the ubiquitinatable Ub6-Stop. In the presence of
Ub⁶-Stop-K0-ile44a, Relish levels return towards normality (figure 4.6D). Because NF-κB transcription factors translocate to the nucleus when activated, we performed subcellular fractionation to visualize nuclear Relish protein levels in heads dissected from pharate adults expressing Ub⁶ in glial cells. We observed that more Relish is present in the nuclei of Ub⁶-Stop-K0 pharate adults, compared to controls that are genetically similar, but lack Ub⁶ (figure 4.6E). Nuclear Relish levels in the presence of either Ub⁶-Stop or Ub⁶-Stop-K0-ile44a appear lower than those in the presence of Ub⁶-Stop-K0, indicating the importance of the chains’ susceptibility to ubiquitination and ile44. Collectively, these results lead us to conclude that ubiquitination-resistant, free, linear Ub chains can be highly toxic in flies at least in part due to NF-κB signaling that is dependent on the ile44-centered hydrophobic patch on Ub.

Due to a lack of quality antibodies for NF-κB components in flies, we performed subcellular fractionation experiments using cultured, HEK-293T human cells to validate our findings in figure 4.6E. In humans, p65 is an NF-κB transcription factor whose phosphorylation at ser536 controls its nuclear translocation and activity [203]. To examine p65 translocation, we transiently transfected HEK-293T cells with the Ub⁶ constructs and 24 hours later performed subcellular fractionation and Western blotting. Expression of the various Ub⁶ constructs in HEK-293T cells leads to ubiquitinated species for Ub⁶-Stop, but not so for lysine-less variants (figure 4.7A). As shown in figure 4.7B, Ub⁶-Stop-K0 expression leads to markedly more endogenous phosphorylated-p65 in the nucleus compared to cells transfected with empty vector; the ile44a mutation reverses this effect. There was no statistically significant difference in the total p65 levels in the cytoplasm or nucleus (figure 4.7B). Based on the increased nuclear translocation of activated p65, it is likely that Ub⁶-Stop-K0 induces the transcription of downstream NF-κB response genes; additional studies are needed to determine the precise outcomes. These results reinforce a role for ubiquitination-resistant, unanchored, linear poly-Ub in NF-κB signaling, notably in a human cell line.
4.4. Discussion

We have developed a model to study the control and function of untethered poly-Ub in an intact, multicellular organism, *Drosophila melanogaster*. By manipulating the properties of linear, unanchored poly-Ub, we propose that free chains are not intrinsically toxic, but that the ability of cells to control them, e.g. through endogenous ubiquitination, is crucial to their remaining innocuous. Ub⁶-Stop-K0, which is unreceptive to ubiquitination, causes aberrant NF-κB signaling and fly death that is largely dependent on amino acid ile44 on Ub. Our results, therefore, also demonstrate a critical role for ile44 on Ub in NF-κB-dependent pathways.

The Ub⁶ chains that we designed are head-to-tail by necessity; current technology limits the ability to stably, genetically encode other linkage types *in vivo*. Endogenous, unanchored poly-
Ub chains are diverse in their linkage types and lengths, and their composition controls their functions and interaction partners [1, 2, 6, 57, 60]. We previously characterized Ub6 species that are endogenously ubiquitinated, transforming them into branched chains with various lysine linkages [70]. Unlike them, Ub6-Stop-K0 and Ub6-Stop-K0-ile44a that we described here are homogenous, linear poly-Ub. We also established in prior work [70] and through the results of this study that each of the Ub6 species has distinct properties, from turnover rates to physiological outcomes. For these reasons, the transgenically-encoded Ub6 fly lines that we developed serve as valuable tools to study the function and regulation of unanchored poly-Ub in vivo.

It remains to be resolved which proteins recognize and interact with Ub6-Stop-K0. For example, upstream elements that lead to upregulated NF-κB component levels in the presence of lysine-less, linear poly-Ub need to be identified. Still, we infer that these elements likely depend on interacting with the ile44 hydrophobic patch, since mutating this amino acid residue markedly reverses Ub6-Stop-K0 toxicity in intact flies. Our results highlight NF-κB components as key players in toxicity from ubiquitination-resistant, linear chains. According to mammalian studies, canonical activation of NF-κB requires the linear Ub chain assembly complex (LUBAC), which attaches head-to-tail, met1-linked chains to NEMO, an NF-κB regulator, and participates in the synthesis of branched, unanchored poly-Ub chains composed of both met1- and lys63-linkages [57]. Free Ub chains are required for the activation of the MAP kinase kinase kinase, TAK1 and the subsequent phosphorylation and degradation of the inhibitory kinase, IKKβ [52]. Several other proteins within the pathway, including NEMO and the LUBAC proteins, contain UBDs that may interact with untethered chains and may be regulated by them [50]. Thus, it is likely that the presence of lysine-less, linear, unanchored poly-Ub places NF-κB in overdrive by interacting with NEMO and LUBAC. In the absence of a functional binding interface on Ub (the ile44a mutation),
NF-κB is not improperly triggered. Our results lead to the conclusion that toxicity induced by linear chains is due to overstimulation of the NF-κB pathway. In the case of the ile44a mutant, these linear chains are not recognizable by the cellular components so their expression per se does not induce toxicity.

As introduced earlier, flies have two NF-κB pathways, Imd and Toll. The Imd pathway is initiated when gram-negative bacteria are detected by peptidoglycan recognition protein-LC at the plasma membrane [204-206]. This event triggers the activation of Tab2/Tak1 that phosphorylates and activates the IKK complex (kenny and IKKβ) [207-209] which then, via Relish (the NF-κB transcription factor) activates antimicrobial genes including Diptericin and Cecropin ([81, 210, 211] and figure 4.4A). The involvement of unanchored poly-Ub in the Imd pathway is expected at multiple steps, based on mammalian NF-κB signaling orthologues. Ubc4, an E2 conjugase whose mammalian orthologue, E2-25K/Ube2K generates poly-Ub chains without a substrate protein [78], is required for Imd activation [212]. The concomitant knockdown of the E3
ligase Bendless and the E2 conjugase Uev1a abrogates Imd signaling in cultured insect cells [83], and Bendless/Uev1a together synthesize unanchored lys63-linked Ub chains in vitro [213]. To the best of our knowledge, there are no published reports showing a direct involvement of unanchored poly-Ub in the Imd pathway in *Drosophila*, but the importance of Ubcd4 and Bendless/Uev1a implicates these chains. Our results that linear poly-Ub activates Relish strengthen this connection and further indicate that free, linear Ub chains are involved in the Imd pathway in flies.

Unlike for the Imd pathway, where evidence from mammalian orthologues and functional assays of fly proteins indicates a role for free Ub chains, it is unclear whether unanchored poly-Ub is involved in the Toll pathway. Toll signaling begins when gram-positive bacteria or fungi are detected by extracellular peptidoglycan recognition proteins and glucan-binding proteins, triggering a cascade that results in the processing of the Toll ligand Spaetzle to facilitate its binding to the Toll receptor [214-216]. After activation, the Toll receptor binds the adaptor MyD88 and recruits Tube and Pelle to phosphorylate cactus [217], ultimately activating dorsal and Dif [218] and driving the transcription of antimicrobial peptides like Drosomycin and Defensin ([82] and figure 4.4A). Insofar as we know, our data that free, linear poly-Ub increases levels of cactus and dorsal are the first to link linear chains to the Toll pathway, thus merging gram-negative and gram-positive/fungi, NF-κB-centered processes under the regulation of linear Ub chains. Which proteins serve as “receptors” that recognize lysine-less, linear poly-Ub and lead to activation of the Toll pathway? Studies with unbiased approaches and targeted genetics will be necessary to uncover these details and also to identify downstream genes that are impacted by free, lysine-less Ub chains for both the Imd and Toll pathways. Based on increased nuclear levels of Relish and phosphorylated-p65 proteins in flies and mammalian cells expressing Ub5-Stop-K0, there is good reason to predict a genetic response to these transcription factors.

The extent of NF-κB component perturbation by linear Ub chains differed among experiments in this work. For example, in figure 4.6C, glial expression of Ub5-Stop-K0 did not
induce *dorsal* mRNA expression to the magnitude shown in figure 4.4D. As mentioned above, we have observed physiological effects from different fly media conditions, even using the same food recipe. Flies used in figure 4.4 were reared on a lot of food that was different from those in figure 4.6, which might have been more prone to mold or bacterial growth and could have itself induced background NF-κB signaling. We stress the importance of making comparisons only among flies and their respective controls that were maintained on the same batch of food and monitored at the same time. In our case, all flies from figure 4.4D were reared together and collected independently of those in figure 4.6C, which were reared and collected as a separate cohort. But, regardless of the observed experimental variation, the important point remains that the induction of both *dorsal* and *Relish* mRNA was consistent among flies reared simultaneously: Ub\(^6\)-Stop-K0 significantly increases their levels, whereas Ub\(^6\)-Stop-K0-ile44a does not have the same impact.

Our work provides new insight into Ub biology more generally. The current model of Ub utilization and recycling centers on the use of mono-Ub in a stepwise manner to generate poly-Ub and the disassembly of poly-Ub into mono-Ub before reuse (figure 4.8). We previously described [70] and further evidence here the possibility of alternative routes for Ub use and recycling, based on the notion that free Ub chains may not need to be disassembled into mono-Ub to be eliminated. They can be conjugated *en bloc* onto other proteins, or can be degraded without the need of disassembly into their building blocks [70]. These routes can effectively recycle untethered poly-Ub, which seems largely innocuous to an intact organism, except under highly specific conditions, such as the ones in the current study. We propose that cells might employ various types of Ub utilization, recycling and disposal routes, depending on homeostatic needs (figure 4.8). For example, conjugation of free poly-Ub onto specific proteins without prior disassembly might be beneficial under times of energy stress or proteotoxic pressure, where quick and efficient removal of certain proteins might benefit from Ub chain “hopping”. These and other possibilities deserve attention to understand Ub biology.
Drosophila is an excellent model organism due to its flexible genetics, easy and inexpensive maintenance, low number of chromosomes, and short life cycle. Importantly, most genes and pathways are conserved among Drosophila and vertebrates, allowing for reliable translation of findings from flies to higher-order organisms [219]. It will be important to expand our findings in a mammalian system to examine the role of endogenous, unanchored poly-Ub in various disease models and stress responses. Free Ub chains are upregulated under certain stress conditions, including inflammation [68] – what types of unanchored chains might we see in mammals with inflammatory diseases? Are there tissue-specific differences? Are there disease-specific variations? Diversity in unanchored Ub chain signaling – from chain length to composition to available binding interfaces – will be important to consider when studying the effects of these species going forward.

In summary, our results reinforce the notion that free Ub chains are not inherently detrimental. Our investigations contribute to the overall understanding of Ub biology, recognize the ile44-centered hydrophobic patch of Ub as an important site for toxicity, and identify ways to reduce and prevent deleterious effects from untethered Ub chains in vivo (figure 4.8). Continued work is necessary to probe into these possibilities in different organisms and under various physiological conditions.
CHAPTER 5: FUTURE DIRECTIONS

The utility of novel tools we have developed to study unanchored poly-Ub in Drosophila is far from exhausted, as they can be used to probe lingering mysteries surrounding free Ub chain toxicity, function, and regulation. For one, which E2s and E3s use preformed chains to ubiquitinate substrates? Could there be DUBs that attempt to process the Ub⁶ chains we designed, whether successfully or not? Our RNA-Seq studies identified transcriptional changes when conjugatable Ub⁶-GG is expressed all throughout the fly (Chapter 3), but this approach does not address the protein-protein interactions necessary for post-translational ubiquitination and deubiquitination. Mass spectrometry studies could shed light on these interactions by identifying any DUBs that associate with linear hexa-Ub, or the ubiquitination enzymes that use Ub⁶-GG to ubiquitinate en bloc (figure 2.6).

Mass spectrometry studies using intact organisms will be especially beneficial for identifying additional signaling capabilities of free poly-Ub. Our work in Drosophila has clarified the importance of context in the study of unanchored chains, as they behave differently in vivo than they do in in vitro proteasome assays and yeast (discussed in section 1.6). Exploratory studies will be necessary to identify new pathways that involve free chains. NF-κB pathways are well-studied, with many participating proteins already identified and a measurable transcriptional output with the induction of immune and inflammatory response gene expression. Thus, studying unanchored poly-Ub’s participation in these pathways is straightforward, compared to lesser known pathways. Our RNA-seq studies in flies detected changes in the expression of many orphan genes whose products have not yet been ascribed names or functions (Dataset S1) – could the resultant proteins be effectors or products of additional signaling pathways that involve free poly-Ub? Viewing this question through the lens of protein-protein interactions by performing mass spectrometry will not only clear up questions around post-translational effects of
unanchored chains – which may not be caught by RNA-Seq or *in vitro* experiments – but could also illuminate details of more enigmatic signaling pathways that involve uncharacterized proteins.

Tissue-specific context is also important when considering the effects and handling of unanchored chains, as neuronal and glial expression of Ub⁶-Stop-K0 or Ub⁶-Stop-K0-ile44a reduced fly lifespans compared to their respective expression in muscles (figures 4.2, 4.6). Even Ub⁶-Stop expression has a small but significant effect on lifespan when expressed in neurons, but not when expressed in any other tissue (figure 4.2). It is possible that unanchored chains are handled in distinct ways in different tissues, depending on the relative abundance of certain proteins or the unique cellular demands in those tissues. These variations in toxicity represent an ambitious and potentially lucrative research opportunity: delineating the intricacies of free poly-Ub function and toxicity in distinct cell types in the context of the whole organism. It is possible to isolate specific populations of cells from *Drosophila* using techniques including magnetic bead-based cell sorting [220]. Using Gal4-UAS to express Ub⁶ transgenes in specific tissues, then isolating Ub⁶-expressing cells to use in transcriptomics and proteomics studies would provide tissue-specific profiles on the effects of unanchored, linear poly-Ub, moving us towards a better understanding of how and when these chains are toxic, or how they affect the cellular landscape. Even beyond that, these studies could point toward intercellular signaling affected by untethered chains if they are also performed using non-Ub⁶-expressing cells from Ub⁶ flies (for example, are there changes in neuronal cells when Ub⁶ is expressed only in glia?). Lifestage-dependent effects are also evident from the developmental lethality with Ub⁶-Stop-K0 expression in various cell types (figure 4.2). The behavior of free poly-Ub is clearly complicated and changes depending on context, a fact that future studies will need to address in more detail.

Another question that unanchored ubiquitin research has not yet addressed is whether there might be some circumstances in which it is beneficial to have pre-made, unconjugated Ub chains ready for immediate use, such as when there is limited ATP to dedicate to chain elongation. This is particularly interesting in light of our finding that Ub⁶ transgenes are robustly expressed in
muscle cells compared to other tissues when using tissue-specific drivers (figures 2.3D, 4.1B). Importantly, this robust expression does not seem to increase toxicity, as longevity is unaffected by Mef2-driven (muscle cell-specific) Ub\(^6\)-Stop or Ub\(^6\)-Stop-K0-ile44a expression (figures 2.3F, 4.6A-B), and Ub\(^6\)-Stop-K0 expression in muscle cells has a far smaller effect on longevity than its expression in neurons, glia, or all cells (figure 4.2). It will be useful to decipher the handling of these chains in muscle cells under different conditions, including exercise and hypoxia, circumstances in which ATP stores are depleted. One interesting area to explore would be any effects from unanchored chains in cardiac muscles, since Ub\(^6\) expression was so strong in Drosophila muscle cells (figures 2.3D, 4.1B). There is evidence for the involvement of the ubiquitin-proteasome system in many cardiovascular diseases. For example, it has been shown that oxidative stress caused by heart disease, ischemia, and aging can lead to proteasomal dysfunction and the buildup of aggregated proteins [221]. A contributing factor in some cases is an increase in reactive oxygen species, which can inhibit certain DUBs including USP14 [221, 222], which notably creates untethered Ub chains at the proteasome by deubiquitinating poly-Ub-ed substrates en bloc [27, 223]. When USP14 cannot deubiquitinate substrates to recycle poly-Ub into mono-Ub, perhaps it necessitates the use of preformed chains for ubiquitination. Cardiac studies in Drosophila and mammalian models could work toward understanding any involvement of free chains in muscle function or dysfunction.

Finally, it will be necessary to tease out the details of unanchored poly-Ub inhibition of the proteasome. In vitro proteasomal activity assays in the presence of different free chain species – various lengths and linkage types – will help us understand which types of chains can inhibit the proteasome, but conventional in vitro studies cannot provide a full picture. Not all proteasomal degradation is Ub-dependent, and there are multiple binding sites for Ub at the proteasome [224]. Could some free poly-Ub species affect the turnover of only a subset of proteasomal substrates, based on where the chains bind the proteasome? Is there any effect on Ub-independent turnover, or do some chains simply outcompete degradation signals without affecting overall proteasomal
function? One potential avenue to explore these questions involves the establishment of stable, 
Ub⁶-expressing cell lines that can be used to assess proteasomal activity against a variety of 
substrates present in cells. Assessing the turnover of known proteasomal substrates, either 
gineered substrates in in vitro assays or endogenous proteins in the cells, would help determine 
specific proteasomal effects. Similar studies can be performed in transgenic flies, but with more 
difficulty due to the limited availability of quality fly antibodies.

In sum, ideas about untethered poly-Ub that have long been considered settled – that they 
are toxic and require rapid disassembly to prevent proteasomal inhibition – are far more 
complicated than previously understood. The complexities addressed throughout this dissertation 
make it clear that the unanchored ubiquitin field is rife with novel research opportunities.
## APPENDIX

<table>
<thead>
<tr>
<th>Linkage Type</th>
<th>Reported Cellular Processes</th>
<th>Abundance</th>
<th>Predominant E3/E2s Reported</th>
<th>Predominant DUBs Reported</th>
</tr>
</thead>
</table>
| **Lyssine-6** | Mitochondrial Homeostasis  
- Mitophagy delayed in cells with mutant Lys6Ub  
- Lys6Ub interacts with the fractionation machinery  
- Involved with mitochondrial membranes and proteins through ubiquitination  
- Increased in response to mitochondrial deprivation  
-表现 polyUb  
- Increases upon UV-induced genotoxic stress  
- Increases in response to mitochondrial deprivation | • Does not increase with proteasome inhibition  
• Increases upon UV-induced genotoxic stress  
• Increases in response to mitochondrial depletion | • USP50 (localization to mitochondria)  
• USP56  
• USP57 | • USP50 (localization to mitochondria)  
• USP56  
• USP57 |
| **Lyssine-11** | Cell Cycle Regulation  
- Triggers proteasomal degradation of cell cycle regulators during mitosis  
- Proapoptotic  
- Inhibits cell cycle proliferation  
- Leads to induction of apoptosis | • Increases in response to proteasome inhibition  
• Increases in response to mitochondrial depletion  
• Prototypical polyUb produced during mitosis and G1 in cancer cells | • Ubc20  
• Ubc25  
• Ubc26  
• Ubc27  
• Ubc29  
• Parkin  
• BAP31  
• UBB2  
• APEL  
• APEL  
• APEL  
• APEL  | • TRABID  
• APEL  
• APEL  |
| **Lyssine-27** | DNA Damage Response  
- The E2 UBE2D3 and E3 APCS/UBR5 form branched chains comprising Lys27 that are strong regulators of DNA damage response  
- Lack of Lys27-linked chains prevents activation of DNA damage response | • Lys27-linked chains are the major Ub chain type on chromatin following DNA damage  
• Lys27-linked polyUb of STING acts as a scaffold for the recruitment and activation of the kinase TANK-binding domain 1 (TBK1) that leads to the activation of transcription factor NF-κB and induction of type-I interferons and pro-inflammatory cytokines | • Lys27-linked chains are the major Ub chain type on chromatin following DNA damage  
• Lys27-linked polyUb of STING acts as a scaffold for the recruitment and activation of the kinase TANK-binding domain 1 (TBK1) that leads to the activation of transcription factor NF-κB and induction of type-I interferons and pro-inflammatory cytokines | • TRABID  
• APEL  
• APEL  |
| **Lyssine-29** | Proapoptotic Degradation  
- Associated with the 26S proteasome and contributes to substrates turnover in the Ub-fusion-degradation pathway | • Increases upon inhibition of the proteasome | • No specific Ub-Reported  
• UBA13UBC5CH5D3D2D9D8  
• UBB2  
• UBB2  
• UBB2  
• UBB2  | • TRABID |
| **Lyssine-33** | Post-Golgi Membrane Trafficking  
- Implicated in regulating traffic through the post-Golgi network | • Increases upon UV-based genotoxic stress | • No specific Ub-Reported  
• UBA13UBC5CH5D3D2D9D8  
• UBB2  
• UBB2  
• UBB2  
• UBB2  | • TRABID |
| **Lyssine-48** | Proapoptotic Degradation  
- Targets proteins to the 26S proteasome for degradation  
- Associated with Wnt signaling  
- Indicates reduced activity of β-catenin signaling  
- Increased in response to genotoxic stress | • Predominant linkage type in cells, often >90% of all linkages  
• Levenson upon proteasome inhibition  
• Increases in response to mitochondrial deprivation | • Ubc20  
• Ubc25  
• Ubc26  
• Ubc27  
• Ubc29  
• Parkin  
• Parkin  
• Parkin  
• Parkin  
• Parkin  | • USP56  
• USP57  
• USP57  
• USP57  |
| **Methionine-11** | Scaffolding to Facilitate Protein Interactions  
- Acts as a molecular scaffold for the formation and activation of various complexes and pathways:  
- Activation of NF-κB transcription factor  
- DNA repair  
- Inhibit immune responses  
- Mitophagy  
- Protein sorting  
- Assembly of protein complexes that drive mRNA splicing and translation  
- Propagation of Wnt signaling | • Second most abundant chain type  
• Increases in response to mitochondrial deprivation | • Ubc20  
• Ubc25  
• Ubc26  
• Ubc27  
• Ubc29  
• Parkin  
• Parkin  
• Parkin  
• Parkin  
• Parkin  | • CYLD  
• UBA13UBC5CH5D3D2D9D8  
• USP30  
• TRAUBIS  |
| **Methionine-1** | NF-κB signaling  
- Modifies the IκB kinase complex and NEMO in order to allosterically activate IκBα in the NF-κB pathway  
- Molecules mediate NF-κB activation  
- Reduces in response to activation of inflammatory signaling cascades  
- Rapidly synthesized in response to activation of inflammatory signaling cascades | • Rapidly synthesized in response to activation of inflammatory signaling cascades  
• Removes enzymes from specific cellular locations in response to DNA damage  
• Reduces in response to proteasome inhibition, most likely in favor of polyubiquitin chain formation | • Ubc20  
• Ubc25  
• Ubc26  
• Ubc27  
• Ubc29  
• Parkin  
• Parkin  
• Parkin  | • CYLD  
• UBA13UBC5CH5D3D2D9D8  
• USP57  
• USP30 |
| **Methionine-3** | Mediates Protein Interaction  
- Allows interaction with a number of other proteins:  
- Mitochondrial protein import  
- 20S proteasome  
- Ubiquitin conjugation  
- Functions in the mitophagy pathway  
- Cubilin  
- Sustains embryonic development  
- Ubiquitin conjugation  
- Proteasomal degradation signals, as well as heterotypic Lys11/Lys48-linked chains | • Reduced in response to proteasome inhibition, most likely in favor of polyubiquitin chain formation | • Ubc20  
• Ubc25  
• Ubc26  
• Ubc27  
• Ubc29  
• Parkin  
• Parkin  
• Parkin  | • USP10  
• USP22  
• USP56  
• USP57  |

Table S1. Outline of the known homotypic ubiquitin linkages along with their reported functions, abundance, and ubiquitin machinery associations. In addition to the primary literature cited in the table, the works of Komander and Rape, 2012 (Komander, D., & Rape, M. (2012). The ubiquitin code. Annu Rev Biochem, 81, 203-229.)
References for Table S1


57. Tsuchiya, H., Tanaka, K. & Saeki, Y. The parallel reaction monitoring method contributes to a highly
et al. 2011. Structure of the HECT:ubiquitin complex


Table S3.1. Effects of Ub⁶ expression throughout all tissues and stages, as previously published [79].

<table>
<thead>
<tr>
<th>sg-h-Gal4 driver (all tissues and stages)</th>
<th>Background Host Line</th>
<th>Ub⁴-Stop</th>
<th>Ub⁶-QG</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Expression</strong></td>
<td>None</td>
<td>Throughout development and throughout adulthood</td>
<td>Throughout development and throughout adulthood</td>
</tr>
<tr>
<td><strong>Protein detection</strong></td>
<td>No Ub⁶</td>
<td>Very robust signal by western blotting from simple lysates and from immunoprecipitations from whole flies and dissected tissues</td>
<td>Very robust signal by western blotting from simple lysates and from immunoprecipitations from whole flies and dissected tissues</td>
</tr>
<tr>
<td><strong>Unanchored Ub accumulation</strong></td>
<td>Not Applicable</td>
<td>Detected by western blotting</td>
<td>Detected by western blotting</td>
</tr>
<tr>
<td><strong>Development</strong></td>
<td>Normal at all stages</td>
<td>Normal at all stages</td>
<td>Normal at all stages</td>
</tr>
<tr>
<td><strong>Eclosion of adults</strong></td>
<td>Normal</td>
<td>Normal; larval, pupal and pharate adult deaths not different from controls</td>
<td>Normal; larval, pupal and pharate adult deaths not different from controls</td>
</tr>
<tr>
<td><strong>Adult longevity</strong></td>
<td>Normal</td>
<td>Normal (not statistically significant from controls that contain driver on the background of the Ub⁶ transgenes)</td>
<td>Normal (not statistically significant from controls that contain driver on the background of the Ub⁶ transgenes)</td>
</tr>
</tbody>
</table>

Table S3.2. Rationale for using DAVID analysis tool over IPA. Information was acquired through the databases themselves, as well as reviews written on the subject of functional analysis tools [159, 160, 162-164].

<table>
<thead>
<tr>
<th>DAVID</th>
<th>IPA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pros</strong></td>
<td><strong>Cons</strong></td>
</tr>
<tr>
<td>● Drosophila-specific gene analysis</td>
<td>● Running criteria must be fine tuned to avoid distortion</td>
</tr>
<tr>
<td>● Groups functionally related genes and terms into manageable number of biological modules, helping to reduce redundancy</td>
<td>● “Orphan” terms, i.e. term exclusion because of undefined fit into a specific function</td>
</tr>
<tr>
<td>● Gene-to-term and term-to-gene analysis</td>
<td>● Fewer designer tools</td>
</tr>
<tr>
<td>● Improves “functional group” definition</td>
<td>● Needs the most complete data set on file</td>
</tr>
<tr>
<td>● Fuzziness feature, that allows one gene to participate in more than one functional group</td>
<td>● Potential incongruence with GO terms</td>
</tr>
<tr>
<td>● Global view with “fuzzy heat map” visualization</td>
<td>● Many of our genes of interest lack human orthologs</td>
</tr>
<tr>
<td></td>
<td>● Ignores our Drosophila-specific genes of interest</td>
</tr>
</tbody>
</table>
### Gene Ontology: Biological Process

<table>
<thead>
<tr>
<th>Term</th>
<th>Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ub&lt;sup&gt;6&lt;/sup&gt;-GG vs. Control</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Upregulated</strong></td>
<td>proteolysis</td>
</tr>
<tr>
<td></td>
<td>CG18493, CG31265, CG31266, CG4653, CG7025,</td>
</tr>
<tr>
<td></td>
<td>CG8299, CG9673, ZetaTry</td>
</tr>
<tr>
<td></td>
<td>peptide catabolic process</td>
</tr>
<tr>
<td></td>
<td>CG31198, CG31343, CG42335, CG8773</td>
</tr>
<tr>
<td></td>
<td>mannose metabolic process</td>
</tr>
<tr>
<td></td>
<td>LManIII, LManV, LManVI</td>
</tr>
<tr>
<td></td>
<td>protein deglycosylation</td>
</tr>
<tr>
<td></td>
<td>LManIII, LManV, LManVI</td>
</tr>
<tr>
<td></td>
<td>melanin biosynthetic process from tyrosine</td>
</tr>
<tr>
<td></td>
<td>yellow-f2, Y</td>
</tr>
<tr>
<td><strong>Downregulated</strong></td>
<td>None detected</td>
</tr>
<tr>
<td><strong>Ub&lt;sup&gt;6&lt;/sup&gt;-Stop vs. Control</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Upregulated</strong></td>
<td>proteolysis</td>
</tr>
<tr>
<td></td>
<td>CG11911, CG11912, CG14820, CG31198,</td>
</tr>
<tr>
<td></td>
<td>CG31343, CG42335, CG6048, CG7025, CG7631,</td>
</tr>
<tr>
<td></td>
<td>CG9673, Jon6Sii, zetaTry</td>
</tr>
<tr>
<td></td>
<td>peptide catabolic process</td>
</tr>
<tr>
<td></td>
<td>CG31198, CG31343, CG42335</td>
</tr>
<tr>
<td></td>
<td>transmembrane transport</td>
</tr>
<tr>
<td></td>
<td>CG17751, CG32669, CG42825, CG4562, CG8785,</td>
</tr>
<tr>
<td></td>
<td>MFS1</td>
</tr>
<tr>
<td><strong>Downregulated</strong></td>
<td>carbohydrate metabolic process</td>
</tr>
<tr>
<td></td>
<td>Mal-A1, Mal-A6, Mal-A7, Nmdmc</td>
</tr>
<tr>
<td></td>
<td>folic acid-containing compound biosynthesis</td>
</tr>
<tr>
<td></td>
<td>process</td>
</tr>
<tr>
<td></td>
<td>Nmdmc, pug</td>
</tr>
<tr>
<td></td>
<td>tetrahydrofolate interconversion</td>
</tr>
<tr>
<td></td>
<td>CG3011, pug</td>
</tr>
<tr>
<td></td>
<td>de novo' IMP biosynthetic process</td>
</tr>
<tr>
<td></td>
<td>AdSL, ade3</td>
</tr>
<tr>
<td></td>
<td>one-carbon metabolic process</td>
</tr>
<tr>
<td></td>
<td>Nmdmc, pug</td>
</tr>
<tr>
<td></td>
<td>oxidation-reduction process</td>
</tr>
<tr>
<td></td>
<td>CG12766, Cyp4p2, Nmdmc, pug</td>
</tr>
<tr>
<td><strong>Ub&lt;sup&gt;6&lt;/sup&gt;-Stop vs. Ub&lt;sup&gt;6&lt;/sup&gt;-GG</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Upregulated</strong></td>
<td>None detected</td>
</tr>
<tr>
<td><strong>Downregulated</strong></td>
<td>carbohydrate metabolic process</td>
</tr>
<tr>
<td></td>
<td>Amy-d, Mal-A1, Mal-A4, Mal-A6, Mal-A7, Mal-</td>
</tr>
<tr>
<td></td>
<td>A8, tobi</td>
</tr>
<tr>
<td></td>
<td>oxidation-reduction process</td>
</tr>
<tr>
<td></td>
<td>CG12766, CG18003, CG1944, CG31075</td>
</tr>
</tbody>
</table>

Table S3.3. Expansion of Table 3.1. Symbols of differentially expressed genes organized by biological process. “CG” identifier denotes that the gene has not yet been named in *Drosophila.*
<table>
<thead>
<tr>
<th>Term</th>
<th>Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ub⁶-GG vs. Control</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Upregulated</strong></td>
<td></td>
</tr>
<tr>
<td>peptide binding</td>
<td>CG31198, CG31343, CG42335, CG8773</td>
</tr>
<tr>
<td>metalloaminopeptidase activity</td>
<td>CG31198, CG31343, CG42335, CG8773</td>
</tr>
<tr>
<td>alpha-mannosidase activity</td>
<td>LManIII, LManV, LManVI</td>
</tr>
<tr>
<td>metallopeptidase activity</td>
<td>CG31198, CG31343, CG42335, CG8773</td>
</tr>
<tr>
<td>serine-type endopeptidase activity</td>
<td>CG1304, CG31265, CG31266, CG4653, CG8299, CG9673, zetaTry</td>
</tr>
<tr>
<td>carbohydrate binding</td>
<td>CG14499, LManIII, LManV, LManVI</td>
</tr>
<tr>
<td>zinc ion binding</td>
<td>CG31198, CG31343, CG42335, CG7025, CG7631, CG8773, LManIII, LManV, LManVI</td>
</tr>
<tr>
<td>hydrolase activity</td>
<td>CG14120, CG31266, vanin-like, CG4653, CG9673</td>
</tr>
<tr>
<td>hydrolase activity, acting on C-N (not peptide) bonds, in linear amides</td>
<td>CG32751, vanin-like</td>
</tr>
<tr>
<td><strong>Downregulated</strong></td>
<td></td>
</tr>
<tr>
<td>catalytic activity</td>
<td>CG5955, Mal-A1, Mal-A6, Mal-A7</td>
</tr>
<tr>
<td>methenyltetrahydrofolate cyclohydrolase activity</td>
<td>Nmdmc, pug</td>
</tr>
<tr>
<td>methylenetetrahydrofolate dehydrogenase (NADP+) activity</td>
<td>Nmdmc, pug</td>
</tr>
<tr>
<td>formate-tetrahydrofolate ligase activity</td>
<td>Nmdmc, pug</td>
</tr>
<tr>
<td>maltose alpha-glucosidase activity</td>
<td>Mal-A1, Mal-A7</td>
</tr>
<tr>
<td>alpha-1,4-glucosidase activity</td>
<td>Mal-A1, Mal-A7</td>
</tr>
<tr>
<td><strong>Ub⁶-Stop vs. Ub⁶-GG</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Upregulated</strong></td>
<td></td>
</tr>
<tr>
<td>None detected</td>
<td></td>
</tr>
<tr>
<td><strong>Downregulated</strong></td>
<td></td>
</tr>
<tr>
<td>maltose alpha-glucosidase activity</td>
<td>Mal-A1, Mal-A4, Mal-A7, Mal-A8, tobi</td>
</tr>
<tr>
<td>alpha-1,4-glucosidase activity</td>
<td>Mal-A1, Mal-A4, Mal-A7, Mal-A8, tobi</td>
</tr>
<tr>
<td>catalytic activity</td>
<td>Amy-d, Mal-A1, Mal-A4, Mal-A6, Mal-A7, Mal-A8</td>
</tr>
</tbody>
</table>

Table S3.4. Expansion of Table 3.2. Symbols of differentially expressed genes organized by molecular function. “CG” identifier denotes that the gene has not yet been named in *Drosophila*. 
<table>
<thead>
<tr>
<th>Pathway</th>
<th>Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ub₆-GG vs. Control</strong></td>
<td></td>
</tr>
<tr>
<td>Upregulated</td>
<td>Lysosome: CG6903, LMANIII, LMANV, LMANVI, Vha100-4, Tsp29Fa</td>
</tr>
<tr>
<td></td>
<td>Other glycan degradation: LMANIII, LMANV, LMANVI</td>
</tr>
<tr>
<td>Downregulated</td>
<td>None detected</td>
</tr>
<tr>
<td><strong>Ub₆-Stop vs. Control</strong></td>
<td></td>
</tr>
<tr>
<td>Upregulated</td>
<td>None detected</td>
</tr>
<tr>
<td>Downregulated</td>
<td>One carbon pool by folate: ade3, CG3011, Nmdmc, pug</td>
</tr>
<tr>
<td></td>
<td>Metabolic pathways: ade, AdSL, CG11425, CG12766, CG13377, CG17224,</td>
</tr>
<tr>
<td></td>
<td>CG30016, CG3011, CG6903, Mal-A1, Mal-A7, Nmdmc, pug, Ugt35b, vanin-</td>
</tr>
<tr>
<td></td>
<td>like</td>
</tr>
<tr>
<td></td>
<td>Galactose metabolism: CG12766, Mal-A1, Mal-A7</td>
</tr>
<tr>
<td><strong>Ub₆-Stop vs. Ub₆-GG</strong></td>
<td></td>
</tr>
<tr>
<td>Upregulated</td>
<td>None detected</td>
</tr>
<tr>
<td>Downregulated</td>
<td>Starch and sucrose metabolism: Amy-d, Mal-A1, Mal-A4, Mal-A7, Mal-A8,</td>
</tr>
<tr>
<td></td>
<td>tobi</td>
</tr>
<tr>
<td></td>
<td>Metabolic pathways: Amy-d, CG11425, CG12766, CG18003, CG31075, Mal-A1,</td>
</tr>
<tr>
<td></td>
<td>Mal-A4, Mal-A7, Mal-A8, tobi</td>
</tr>
<tr>
<td></td>
<td>Glycerolipidmetabolism: CG11425, CG12766, CG31075</td>
</tr>
</tbody>
</table>

**Table S3.5.** Expansion of Table 3.3. Symbols of differentially expressed genes organized by KEGG pathway. “CG” identifier denotes that the gene has not yet been named in *Drosophila*. 
<table>
<thead>
<tr>
<th>Term</th>
<th># of genes</th>
<th>p-value</th>
<th>Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ub&lt;sup&gt;6&lt;/sup&gt;-GG vs. Control</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Upregulated</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>proteolysis *</td>
<td>9</td>
<td>2.30E-05</td>
<td>ANPEP, CPB1, CFD, HPN, KLK9, MEP1A, PRSS16, PRSS36, TPSD1</td>
</tr>
<tr>
<td>termination of signal transduction</td>
<td>2</td>
<td>7.30E-03</td>
<td>GBA, SMPD1</td>
</tr>
<tr>
<td>response to thyroid hormone</td>
<td>2</td>
<td>1.50E-02</td>
<td>GBA, HPN</td>
</tr>
<tr>
<td>response to pH</td>
<td>2</td>
<td>2.90E-02</td>
<td>GBA, TTPA</td>
</tr>
<tr>
<td>regulation of cell shape</td>
<td>3</td>
<td>4.60E-02</td>
<td>BAIAP2, FMNL1, HPN</td>
</tr>
<tr>
<td><strong>Downregulated</strong></td>
<td>None detected</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Ub&lt;sup&gt;6&lt;/sup&gt;-Stop vs. Control</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Upregulated</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>proteolysis *</td>
<td>7</td>
<td>7.20E-04</td>
<td>ANPEP, AZU1, CPA3, CPB1, HPN, MEP1A, TPSD1</td>
</tr>
<tr>
<td>pyrimidine nucleoside salvage</td>
<td>2</td>
<td>2.60E-02</td>
<td>CDA, UPP1</td>
</tr>
<tr>
<td>biotin metabolic process</td>
<td>2</td>
<td>3.00E-02</td>
<td>BTD, SLC5A6</td>
</tr>
<tr>
<td><strong>Downregulated</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>folic acid metabolic process</td>
<td>3</td>
<td>1.30E-04</td>
<td>MTHFD1, MTHFD2, SHMT1</td>
</tr>
<tr>
<td>purine nucleobase biosynthetic process</td>
<td>2</td>
<td>4.80E-03</td>
<td>GART, SHMT1</td>
</tr>
<tr>
<td>de novo IMP biosynthetic process</td>
<td>2</td>
<td>5.70E-03</td>
<td>AdSL, GART</td>
</tr>
<tr>
<td>folic acid-containing compound</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>biosynthesis process</td>
<td>2</td>
<td>5.70E-03</td>
<td>MTHFD2, MTHFD1</td>
</tr>
<tr>
<td>glycine metabolic process</td>
<td>2</td>
<td>8.50E-03</td>
<td>GART, SHMT1</td>
</tr>
<tr>
<td>tetrahydrofolate interconversion</td>
<td>2</td>
<td>9.50E-03</td>
<td>MTHFD1, SHMT1</td>
</tr>
<tr>
<td>tetrahydrofolate metabolic process</td>
<td>2</td>
<td>9.50E-03</td>
<td>MTHFD2, SHMT1</td>
</tr>
<tr>
<td>purine nucleotide biosynthetic process</td>
<td>2</td>
<td>1.10E-02</td>
<td>ADSL, MTHFD1</td>
</tr>
<tr>
<td>purine ribonucleoside monophosphate</td>
<td>2</td>
<td>1.20E-02</td>
<td>ADSL, GART, AKR1A1, CYP4B1, MTHFD1</td>
</tr>
<tr>
<td>biosynthetic process</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>oxidation-reduction process</td>
<td>4</td>
<td>1.70E-02</td>
<td>MTHFD2</td>
</tr>
<tr>
<td>one-carbon metabolic process</td>
<td>2</td>
<td>2.80E-02</td>
<td>MTHFD1, MTHFD2</td>
</tr>
<tr>
<td>amino acid transport</td>
<td>2</td>
<td>3.30E-02</td>
<td>SLC3A1, SLC36A1</td>
</tr>
<tr>
<td>protein tetramerization</td>
<td>2</td>
<td>3.70E-02</td>
<td>ADSL, SHMT1</td>
</tr>
<tr>
<td><strong>Ub&lt;sup&gt;6&lt;/sup&gt;-Stop vs. Ub&lt;sup&gt;6&lt;/sup&gt;-GG</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Upregulated</strong></td>
<td>None detected</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Downregulated</strong></td>
<td>carbohydrate metabolic process</td>
<td>3</td>
<td>2.20E-03</td>
</tr>
</tbody>
</table>

Table S3.6. Enriched Biological Process Gene Ontology terms as determined by DAVID analysis. Differentially expressed (Absolute Log2 Fold Change > 1, FDR < 0.05) RNA-Seq transcripts at each level of comparison (Ub6-GG vs. Control, Ub6-Stop vs. Control, Ub6-Stop vs. Ub6-GG) were assigned human orthologues by FlyBase.org and separated into lists of upregulated and downregulated genes for each condition. Each list was uploaded into DAVID’s Functional Annotation tool (https://david.ncifcrf.gov, v. 6.8) as a gene list and submitted using the official gene symbol as identifier and H. sapiens as background. The term BP_DIRECT was selected for chart creation within the Gene Ontology category, and terms were included as enriched if p-value < 0.05. * indicates overlap in terms between Ub6-GG vs. Control and Ub6-Stop vs. Control comparisons.
<table>
<thead>
<tr>
<th>Term</th>
<th># of genes</th>
<th>p-value</th>
<th>Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>upregulated serine-type peptidase activity</td>
<td>4</td>
<td>4.80E-04</td>
<td>CFD, HPN, PRSS16, TPSD1</td>
</tr>
<tr>
<td>upregulated serine-type endopeptidase activity *</td>
<td>5</td>
<td>3.30E-03</td>
<td>CFD, HPN, KLK9, PRSS36, TRAIP, ANPEP, CPB1, CDA, ENPEP, MAN2B1, MEP1A, SMPD1</td>
</tr>
<tr>
<td>upregulated zinc ion binding *</td>
<td>8</td>
<td>2.10E-02</td>
<td></td>
</tr>
<tr>
<td>upregulated apolipoprotein binding</td>
<td>2</td>
<td>3.30E-02</td>
<td>CANX, CSARB1</td>
</tr>
<tr>
<td>upregulated mannose binding</td>
<td>2</td>
<td>4.70E-02</td>
<td>CD207, MAN2B1</td>
</tr>
</tbody>
</table>

| downregulated                           | None detected |             |

| upregulated zinc ion binding *           | 8          | 1.20E-02    | TRAIP, ANPEP, BNC2, CPA3, CPB1, CDA, MEP1A |
| upregulated serine-type endopeptidase activity * | 4          | 1.80E-02    | AZU1, HPN, TFPI, TPSD1               |

| downregulated methylenetetrahydrofolate dehydrogenase (NAD+) activity | 2          | 2.70E-03    | MTHFD1, MTHFD2                      |
| downregulated methylenetetrahydrofolate dehydrogenase (NADP+) activity | 2          | 3.50E-03    | MTHFD1, MTHFD2                      |
| downregulated methenyltetrahydrofolate cyclohydrolase activity | 2          | 3.50E-03    | MTHFD1, MTHFD2                      |
| downregulated formate-tetrahydrofolate ligase activity | 2          | 3.50E-03    | MTHFD1, MTHFD2                      |
| downregulated catalytic activity          | 3          | 1.20E-02    | ADSL, GART, SLC3A1                  |
| downregulated amino acid transmembrane transporter activity | 2          | 4.10E-02    | SLC3A1, SLC36A1                     |

Table S3.7. Enriched Molecular Function Gene Ontology terms as determined by DAVID analysis. Differentially expressed (Absolute Log2 Fold Change > 1, FDR < 0.05) RNA-Seq transcripts at each level of comparison (Ub6-GG vs. Control, Ub6-Stop vs. Control, Ub6-Stop vs. Ub6-GG) were assigned human orthologues by FlyBase.org and separated into lists of upregulated and downregulated genes for each condition. Each list was uploaded into DAVID's Functional Annotation tool (https://david.ncifcrf.gov, v. 6.8) as a gene list and submitted using the official gene symbol as identifier and H. sapiens as background. The term MF_DIRECT was selected for chart creation within the Gene Ontology category, and terms were included as enriched if p-value < 0.05. * indicates overlap in terms between Ub6-GG vs. Control and Ub6-Stop vs. Control comparisons.
# Table S3.8. Enriched KEGG Pathway terms as determined by DAVID analysis.

Differentially expressed (Absolute Log2 Fold Change > 1, FDR < 0.05) RNA-Seq transcripts at each level of comparison (Ub6-GG vs. Control, Ub6-Stop vs. Control, Ub6-Stop vs. Ub6-GG) were assigned human orthologues by FlyBase.org and separated into lists of upregulated and downregulated genes for each condition. Each list was uploaded into DAVID's Functional Annotation tool (https://david.ncifcrf.gov, v. 6.8) as a gene list and submitted using the official gene symbol as identifier and *H. sapiens* as background. The term KEGG_PATHWAY was selected for chart creation within the Pathway category, and terms were included as enriched if p-value < 0.05. ^ indicates overlap in terms between Ub6-Stop vs. Control and Ub6-Stop vs. Ub6-GG comparisons, ^ indicates overlap in terms across all comparisons.

<table>
<thead>
<tr>
<th>KEGG Pathway</th>
<th>Pathway</th>
<th># of genes</th>
<th>p-value</th>
<th>Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ub6-GG vs. Control</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Upregulated</strong></td>
<td>Lysosome</td>
<td>5</td>
<td>1.00E-03</td>
<td>ATP6V0A4, CD63, GBA, MAN2B1, SMPD1, ALPPL2, ANPEP, ATP6V0A1, BTD, CDA, CHDH, CYP3A4, DPM3, GBA, SMPD1</td>
</tr>
<tr>
<td></td>
<td>Metabolic pathways ^</td>
<td>10</td>
<td>2.90E-02</td>
<td></td>
</tr>
<tr>
<td><strong>Downregulated</strong></td>
<td>None detected</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Ub6-Stop vs. Control</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Upregulated</strong></td>
<td>Drug metabolism- other enzymes</td>
<td>3</td>
<td>7.00E-03</td>
<td>CDA, UGT2A3, UPP1</td>
</tr>
<tr>
<td></td>
<td>Protein digestion and absorption</td>
<td>3</td>
<td>2.40E-02</td>
<td>CPA3, CPB1, MEP1A</td>
</tr>
<tr>
<td><strong>Downregulated</strong></td>
<td>One carbon pool by folate</td>
<td>4</td>
<td>4.50E-06</td>
<td>MTHFD2, MTHFD1, GART, SHMT1, ADSL, AKR1A1, CYP4F2, GART, HGSNAT, MTHFD1, MTHFD2, PLPP1, SHMT1</td>
</tr>
<tr>
<td></td>
<td>Metabolic pathways ^</td>
<td>9</td>
<td>2.40E-04</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Biosynthesis of antibiotics +</td>
<td>5</td>
<td>3.60E-04</td>
<td>TGDS, ADSL, AKR1A1, GART, SHMT1</td>
</tr>
<tr>
<td></td>
<td>Glycerolipid metabolism +</td>
<td>3</td>
<td>4.40E-03</td>
<td>AKR1A1, LPL, PLPP1</td>
</tr>
<tr>
<td><strong>Ub6-Stop vs. Ub6-GG</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Upregulated</strong></td>
<td>None detected</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Downregulated</strong></td>
<td>Metabolic pathways ^</td>
<td>6</td>
<td>8.90E-04</td>
<td>ALDH2, AKR1A1, AMY2B, CYP4F2, HAO2, PLPP1</td>
</tr>
<tr>
<td></td>
<td>Glycerolipid metabolism +</td>
<td>3</td>
<td>1.00E-03</td>
<td>ALDH2, AKR1A1, PLPP1</td>
</tr>
<tr>
<td></td>
<td>Biosynthesis of antibiotics +</td>
<td>3</td>
<td>1.30E-02</td>
<td>ALDH2, AKR1A1, HAO2</td>
</tr>
<tr>
<td>Gene Target</td>
<td>Forward primer 5’-3’</td>
<td>Reverse primer 5’-3’</td>
<td>Localization</td>
<td></td>
</tr>
<tr>
<td>-------------</td>
<td>---------------------</td>
<td>---------------------</td>
<td>--------------</td>
<td></td>
</tr>
<tr>
<td>Rp49</td>
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<td>CACCAGGAACCTCTTCTAGAATCCG</td>
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</tr>
<tr>
<td>CG11911</td>
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<td>GGGATTTGATCTGCTGCTATAAT</td>
<td>Midgut</td>
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</tr>
<tr>
<td>CG1304</td>
<td>TCCACGGAGTAATGGTACTA</td>
<td>GGTAGATCGATGAGGGCTGATAC</td>
<td>Midgut</td>
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</tr>
<tr>
<td>CG2650</td>
<td>GATGTGACGCAGAGATCAA</td>
<td>GAGAAATCTGCTGAGGGAATG</td>
<td>Rectal pad and eye</td>
<td></td>
</tr>
<tr>
<td>CG32751</td>
<td>CGATTTCTGCTCTGGAAA</td>
<td>CTGGCATATGTGACACTTAAT</td>
<td>Midgut and Spermatheca</td>
<td></td>
</tr>
<tr>
<td>CG4653</td>
<td>GGTGATCGTGAAGAAGCGCAC</td>
<td>CACCAGGAACTCTTTGAATCCGG</td>
<td>Global</td>
<td></td>
</tr>
<tr>
<td>Drs13</td>
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<td>GACCTCCGAAGTTCCAGATAG</td>
<td>Midgut</td>
<td></td>
</tr>
<tr>
<td>LysE</td>
<td>GCTGTTGGGCGCCTGTATT</td>
<td>GAAGATCCGGATCGGTCTGGAG</td>
<td>Midgut and accessory glands</td>
<td></td>
</tr>
<tr>
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<td>Midgut</td>
<td></td>
</tr>
<tr>
<td>qin</td>
<td>CTGATCGGCCAGACAGACTAAG</td>
<td>GACCGTAGACCGTCAAGTTAC</td>
<td>Testis, ovary, and eye</td>
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</tr>
<tr>
<td>Takl1</td>
<td>TCCCTCCCAACTACCTCTATG</td>
<td>AGGCCATTTGACCTTACC</td>
<td>Midgut, hindut, and Malpighian tubules</td>
<td></td>
</tr>
<tr>
<td>Ubi-p5E</td>
<td>GGACGTCCGGCAAGTAAAA</td>
<td>ATGGCTCAACCTCCAAAGTG</td>
<td>Global</td>
<td></td>
</tr>
</tbody>
</table>

Table S3.9. All primer sequences used for qRT-PCR and their corresponding genes’ expression patterns in *Drosophila melanogaster*. Localization according to FlyAtlas.org. “CG” identifier denotes that the gene has not yet been named in *Drosophila*.

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>RINe</th>
<th>28S/18S (Area)</th>
<th>Conc. (ng/µL)</th>
</tr>
</thead>
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<td>GG-1</td>
<td>9.3</td>
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</tr>
<tr>
<td>GG-2</td>
<td>7.6</td>
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</tr>
<tr>
<td>GG-3</td>
<td>8.6</td>
<td>0.1</td>
<td>111</td>
</tr>
<tr>
<td>GG-4</td>
<td>8.9</td>
<td>0.1</td>
<td>95.3</td>
</tr>
<tr>
<td>Stop-1</td>
<td>6.9</td>
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</tr>
<tr>
<td>Stop-2</td>
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<td>N/A</td>
<td>41.4</td>
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<tr>
<td>Stop-3</td>
<td>8.4</td>
<td>0.2</td>
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</tr>
<tr>
<td>Stop-4</td>
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<td>0.2</td>
<td>167</td>
</tr>
<tr>
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<td>0.2</td>
<td>76.3</td>
</tr>
<tr>
<td>Ctrl-2</td>
<td>7.5</td>
<td>0.3</td>
<td>199</td>
</tr>
<tr>
<td>Ctrl-3</td>
<td>8.0</td>
<td>0.1</td>
<td>65.1</td>
</tr>
<tr>
<td>Ctrl-4</td>
<td>7.8</td>
<td>0.2</td>
<td>101</td>
</tr>
</tbody>
</table>

Table S3.10. Overall RNA quality. Before RNA-Seq, an aliquot of the RNA was assessed by microfluidics using the ScreenTape for the Agilent 2200 TapeStation. The electrophoretogram (not pictured), RNA Integrity Number (RIN), and the ratio of the 28S:18S RNA bands are collectively examined to determine overall quality of the RNA.
### Drosophila gene names - FlyBase; "CG" denotes an unnamed gene

<table>
<thead>
<tr>
<th>Human Orthologue(s)</th>
<th>Log2 FC</th>
<th>Human Orthologue(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLC2A8, SLC2A10, SLC2A12, SLC2A13</td>
<td>1.89</td>
<td>undefined</td>
</tr>
</tbody>
</table>

### Human gene names – HGNC

#### Empty cells: no orthologues identified on FlyBase.org

All results up-to-date as of January 11, 2019

Genes with multiple hits: all potential orthologues had the same score on FlyBase.org
REFERENCES


ABSTRACT

VARIATIONS ON A THEME: INTRICACIES OF UNANCHORED POLY-UBIQUITIN SIGNALING AND TOXICITY

by

JESSICA BLOUNT-PACHECO

August 2020

Advisor: Dr. Sokol V. Todi
Major: Pharmacology
Degree: Doctor of Philosophy

Ubiquitin is an 8.5 kDa post-translational modifier involved in essentially all eukaryotic cellular processes. Through a process called ubiquitination, ubiquitinating enzymes chemically attach ubiquitin to substrate proteins to control their fates, resulting in anything from their recruitment into signaling pathways to their proteasomal degradation, with a plethora of possibilities in between. Ubiquitin molecules can also be attached to one another, resulting in poly-ubiquitin chains with various effects depending on the number of ubiquitin molecules and the specific amino acid residues used to link them together. While most poly-ubiquitin in the cell exists as conjugated species, there are also untethered poly-ubiquitin species that are not attached to substrates. These unanchored ubiquitin chains have been previously classified as toxic byproducts that interfere with proteasomal function in vitro and in yeast, and are thus believed to be disassembled rapidly to avoid toxicity. Conversely, several studies have indicated a signaling role for certain types of unanchored ubiquitin chains in innate immunity and stress responses. Recent work from our lab that places untethered poly-ubiquitin in an in vivo setting, the fruit fly Drosophila melanogaster, has established a more nuanced existence for these chains. By manipulating the chemical properties of one type of untethered ubiquitin chain – linear poly-ubiquitin – we have shown that these chains are not always toxic in vivo. Under normal
circumstances, they are innocuous, readily degraded by the proteasome, and used by endogenous ubiquitination machinery that transfers them to substrates \textit{in toto}. It is only under certain conditions that they become noxious and elicit a cellular response; for example, when unanchored, linear ubiquitin chains cannot be ubiquitinated, they induce aberrant NF-κB signaling, resulting in premature death in flies. Our work invites a re-evaluation of the credence that untethered poly-ubiquitin is always toxic, and more broadly points to untapped potential for unanchored chain involvement in additional pathways \textit{in vivo}.
AUTOBIOGRAPHICAL STATEMENT

EDUCATION

University of Michigan, Ann Arbor, MI
Bachelor of Science, Neuroscience – August, 2009

Wayne State University School of Medicine, Detroit, MI
Ph.D, Pharmacology – August, 2015-present; expected graduation August, 2020

PUBLICATIONS


